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The Role of CMV and EBV Reactivation in Immunocompromised Patients with Therapy-Refractory Chronic Skin Diseases

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Summary

Reactivation of latent cytomegalovirus (CMV) and Epstein-Barr virus (EBV) infection is a dreaded complication in the immunocompromised host, which is well studied in transplant settings. In contrast, evidence is sparse if the reactivation of viral infection may also be of clinical relevance in patients with chronic skin diseases. In fact, these patients fulfill two major prerequisites of CMV and EBV reactivation in the human organism, namely ongoing skin inflammation and therapy-induced immunosuppression. Therefore, they may be particularly at risk.

This study addressed the role of CMV and EBV reactivation in immunocompromised patients suffering from different treatment-refractory chronic skin diseases.

First, the rate of viral reactivation, defined as positivity for both virus-specific IgG and IgM, could be demonstrated to be increased in immunocompromised patients with therapy-refractory chronic skin diseases (n = 30) in comparison to nonimmunocompromised control patients (n = 90) (CMV: 16.7 % vs. 5.6 %; EBV: 6.7 % vs. 0.0 %). This is consistent with the more frequent detection of virus-specific DNA in lesional skin of immunocompromised patients with therapy-refractory chronic skin diseases (n = 14) as compared to nonlesional skin of nonimmunocompromised control patients (n = 14) (CMV: 21.4 % vs. 0.0 %; EBV: 30.8 % vs. 0.0 %). The results of the immunohistochemical stainings, T cell stimulation experiments, and the multiplex immunoassay suggest that the perpetuation of skin inflammation and the aggravation of the underlying skin disease may be related to the presence of lytic infection-associated viral antigens and the release of proinflammatory cytokines by virus-specific T cells in lesional skin. The absence of herpes simplex virus- and varicella-zoster virus-specific DNA in therapy-refractory skin lesions of immunocompromised patients substantiates the pathophysiological relevance of CMV and EBV reactivation. In addition, the presented case studies of immunocompromised patients with treatment-refractory courses of disease and reactivation of CMV infection who showed marked improvement of skin lesions upon antiviral therapeutic intervention strongly support a disease-modifying role of CMV reactivation. Finally, the epidemiological relevance of these findings was estimated. The analysis of two representative inpatient wards showed that 3.6 % of all patients did not respond adequately to current gold standard (immunosuppressive) therapy. These patients appear to be at risk for CMV reactivation and thus may benefit from early screening and antiviral treatment. The calculation of the number needed to screen revealed that (depending on the definition of a positive screening test) CMV-induced morbidity could be prevented in one patient by screening 9 to 30 patients at risk (i.e., patients showing persistence or progression of skin lesions despite intensive immunosuppressive/immunomodulating therapy) if the detection of CMV reactivation entailed antiviral treatment.

To summarize, the results of this study support the hypothesis that inflammation and immunosuppression predispose to reactivation of CMV and EBV infection in patients suffering from chronic skin diseases and that CMV (EBV) reactivation, in turn, hinders the efficacy of immunosuppressive/immunomodulating treatment and even worsens the skin pathology. Therefore, patients with chronic skin conditions who do not respond adequately to immunosuppressive/immunomodulating treatment regimens may be screened for CMV (EBV) reactivation. The initiation of antiviral treatment may improve the course of the skin disease and help to reduce medical resource utilization. This study raises vigilance against a so far widely neglected clinical condition in dermatology, which may become increasingly relevant in the future because more and more systemically acting drugs with immunosuppressive/immunomodulating properties (e.g., biologicals and small molecules) are administered.

List of Abbreviations

Abbreviation	Meaning
∆A	difference in absorbance
AIDS	acquired immunodeficiency syndrome
ALAT	alanine aminotransferase
ASAT	aspartate aminotransferase
BHLF	Bam H-fragment; lower frame
BSG	British Society of Gastroenterology
BZLF1	BamHI Z fragment leftward open reading frame 1
CD (e.g., CD34)	cluster of differentiation (e.g., cluster of differentiation 34)
CFSE	carboxyfluorescein succinimidyl ester
CG-PG	Control Group for Prospective Study Group
CG-PG(P)	Control Group for Prospective Study Group (PCR)
CG-RG	Control Group for Retrospective Study Group
CMV	cytomegalovirus
CNS	central nervous system
C_t	cycle threshold
CTCL	cutaneous T cell lymphoma
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EBER	Epstein-Barr virus-encoded RNA
EBNA (e.g., EBNA1)	Epstein-Barr virus nuclear antigen (e.g., Epstein-Barr virus nuclear antigen 1)
EBV	Epstein-Barr virus
EBV EA-D-p52/50	Epstein-Barr virus early antigen-diffuse-p52/50
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FFPE	formalin-fixed paraffin-embedded
GM-CSF	granulocyte-macrophage colony-stimulating factor
hCMV	human cytomegalovirus
HE	hematoxylin and eosin
HIV	human immunodeficiency virus
HLA-DR	human leukocyte antigen - DR isotype

Abbreviation	Meaning
HSV	herpes simplex virus
HUDC medium	human dendritic cell medium
IBD	inflammatory bowel disease
IE (e.g., IE-1)	immediate early protein (e.g., immediate early protein-1)
IFN-γ	interferon-γ
Ig (e.g., IgM)	immunoglobulin (e.g., immunoglobulin M)
IHC	immunohistochemistry
IL- (e.g., IL-4)	interleukin- (e.g., interleukin-4)
ILC (e.g., ILC1)	innate lymphoid cells (e.g., type 1 innate lymphoid cells)
IP-10	interferon-γ-induced protein 10
IQR	interquartile range
ISH	*in situ* hybridization
iTreg cells	induced regulatory T cells
IU	International Unit
IVIg	intravenous immunoglobulins
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MIEP	major immediate early promoter
mIU	milli-International Unit
MODCs	monocyte-derived dendritic cells
mRNA	messenger RNA
n	number (e.g., of patients or samples)
NAT	nucleic acid testing
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	natural killer cells
NKT cells	natural killer T cells
NNS	number needed to screen
NRS	numerical rating scale
n.s.	not significant
NU	Novagnost Unit
PASI	Psoriasis Area and Severity Index
PBLs	peripheral blood leukocytes
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDH	human pyruvate dehydrogenase

Abbreviation	Meaning
PG	Prospective Study Group
PG(P)	Prospective Study Group (PCR)
pp65	phosphoprotein 65
RG	Retrospective Study Group
RISH	RNA *in situ* hybridization
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SLE	systemic lupus erythematosus
Tc cells (e.g., Tc1 cells)	cytotoxic T cells (e.g., type 1 cytotoxic T cells)
TGF-β	transforming growth factor-β
Th cells (e.g., Th1 cells)	T helper cells (e.g., type 1 T helper cells)
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor-α
TNF receptor	tumor necrosis factor receptor
Tr1 cells	type 1 regulatory T cells
Treg cells	regulatory T cells
TT	tetanus toxoid
U	Unit
UC	ulcerative colitis
UNG	uracil-N-glycosylase
UVB	ultraviolet B
VCA	viral capsid antigen
VZV	varicella-zoster virus
WHO	World Health Organization
ZEBRA	BamHI Z Epstein-Barr virus replication activator

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1 Introduction

1.1 Cytomegalovirus

Cytomegalovirus (CMV), also known as human herpesvirus 5, is a member of the beta-herpesvirus subfamily of the *Herpesviridae* family. It is a double-stranded DNA virus with a linear genome and an icosahedral shape. CMV consists of four structural elements (i.e., outer lipid envelope, tegument, nucleocapsid, and internal nucleoprotein core). (Dioverti and Razonable, 2016)

1.1.1 CMV infection/disease

Since 1993, the Cytomegalovirus Drug Development Forum has provided definitions of CMV disease and CMV infection to enable consistent reporting of outcomes in clinical trials. The latest update was released in 2017. The definitions were initially meant for application to transplant recipients, in whom CMV infection and disease are important reasons for morbidity and mortality, but can also be applied to other immunocompromised patients. (Ljungman et al., 2017)

“CMV infection is defined as virus isolation or detection of viral proteins (antigens) or nucleic acid in any body fluid or tissue specimen” (Ljungman et al., 2017).

The mucosal and glandular epithelial cells likely represent the sites of entry and exit for CMV. The virus is able to replicate lytically to produce infectious virus in many different cell types in all major organs during the active phase of infection. In some cell types (e.g., myeloid progenitor cells), latent CMV infection is established. The virus is disseminated in the human organism by latently infected monocytes. If the conditions are appropriate, CMV is reactivated from latency, and the production of infectious virus is restarted. (Forte et al., 2020)

1.1.1.1 Primary CMV infection

“Primary CMV infection is defined as the first detection of CMV infection in an individual who has no evidence of CMV exposure before […]” (Ljungman et al., 2017).

Most CMV infections are acquired during childhood and early adulthood (Dioverti and Razonable, 2016). Transmission of CMV infection is possible through contact with infectious body fluids (e.g., blood, breast milk, saliva, urine, and genital secretions) (Forte et al., 2020). Furthermore, CMV infection can be transmitted intrauterine, through organ/tissue transplantation, and blood transfusion (Dioverti and Razonable, 2016). In the immunocompetent host, primary CMV infection is typically subclinical (Forte et al., 2020). The host’s immune response is able to clear cells that are lytically infected and produce virus (Forte et al., 2020). However, the development of a nonspecific febrile illness or an infectious mononucleosis-like syndrome is possible (Dioverti and

Razonable, 2016). The CMV seroprevalence rate differs greatly depending on geographic location, age, and socioeconomic status (Dioverti and Razonable, 2016). In the United States, the overall CMV seroprevalence was reported to range at 50.4 % (1988–2004), whereas it approaches 100 % among older people in developing countries (Dioverti and Razonable, 2016).

1.1.1.2 Latent CMV infection

“Latency is characterized by the maintenance of the viral genome in the absence of virus production and the ability to reactivate” (Heald-Sargent et al., 2020).

During lytic infection, replication of CMV has been described in a variety of permissive cell types (Heald-Sargent et al., 2020). In contrast, latent CMV infection is maintained in a limited subset of cells, including CD34-positive hematopoietic progenitor cells in the bone marrow and monocytes in peripheral blood (Heald-Sargent et al., 2020). There might be additional, nonhematopoietic sites of CMV latency in solid organs (e.g., tissue-resident macrophages or dendritic cells, endothelial cells), but this is still a matter of research (Forte et al., 2020). The ability to reactivate from latent CMV infection allows the virus to escape from a host organism whose survival is threatened (e.g., by cellular damage or infections with other pathogens) (Forte et al., 2020).

1.1.1.3 Mechanisms of CMV reactivation

“‘Recurrent infection’ is defined as new CMV infection in a patient with previous evidence of CMV infection who has not had virus detected for an interval of at least 4 weeks during active surveillance. Recurrent infection may result from reactivation of latent virus (endogenous) or reinfection (exogenous).” (Ljungman et al., 2017)

Reactivation of latent CMV infection occurs intermittently throughout life. In immunocompetent individuals, the CMV-specific immunologic memory permits effective control of viral replication. In contrast, the loss of CMV-specific T cell immunity in immunocompromised patients may result in uncontrolled viral replication and subsequent clinical disease. (Dioverti and Razonable, 2016)

For many years, the mechanism by which CMV is reactivated from latency has been poorly understood, and different models have been discussed (Hummel and Abecassis, 2002). However, since then, the understanding of latent CMV infection has considerably evolved, and the stimuli leading to the reactivation of CMV infection have been elucidated. Forte et al. (2020) outlined that the temporal pattern of gene regulation was the same in all herpesviruses. First, a small number of immediate early genes are expressed (Forte et al., 2020). Then, the expression of early and late genes follows (Forte et al., 2020). The major immediate early proteins IE-1 and IE-2 are essential for the activation of early gene expression and for the subsequent phases of lytic replication (Heald-Sargent et al., 2020). The expression of IE-1 and IE-2 is regulated by the major immediate early promoter (MIEP), which contains binding motifs for different transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and is

considered to be a major control point in latency and reactivation of CMV (Heald-Sargent et al., 2020).

Heald-Sargent et al. (2020) delineated that there was growing evidence for a two-hit model of CMV reactivation following the transplantation of a latently infected solid organ (Figure 1):

The reactivation of CMV infection is initiated by molecular events (activation of transcription factors that bind to the MIEP, remodeling of viral chromatin) that activate CMV gene expression and stimulate local virus production (hit 1). This initial activation of viral gene expression can be mediated by ischemia/reperfusion injury, oxidative stress, DNA damage, or inflammatory mediators. These factors may act synergistically.

The loss of immune control permits amplification of viral replication and facilitates dissemination of reactivated virus throughout the host (hit 2). The cause of immunosuppression may be intrinsic (disease-related immune compromise) or extrinsic (administration of immunosuppressive drugs). (Heald-Sargent et al., 2020)

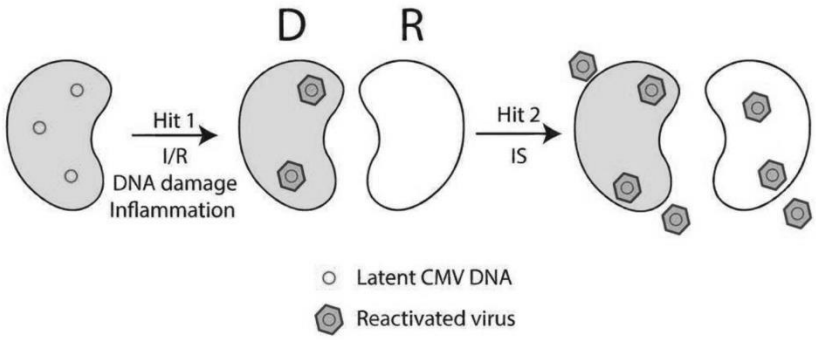


Figure 1: Two-hit model of cytomegalovirus reactivation after solid organ transplantation.
D = donor; I/R = ischemia/reperfusion; IS = immunosuppression; R = recipient.
Figure modified from Heald-Sargent et al. (2020).

This model might also be applicable outside the transplant setting.

1.1.1.4 CMV disease

In healthy and immunocompetent individuals, both primary CMV infection and reactivation are usually relatively benign (Hummel and Abecassis, 2002). In contrast, the immunocompromised host may suffer from severe and even fatal disease of different organ systems (Dioverti and Razonable, 2016).

The generic term “CMV disease” comprises “CMV end-organ diseases” and “CMV syndrome”. The disease definition “CMV syndrome” should only be used in the context of solid organ transplantation. For the diagnosis of probable CMV syndrome, detection of CMV in blood (by virus isolation, rapid culture, antigenemia, or PCR) is obligatory together with at least two other criteria (fever, malaise/fatigue, leukopenia/neutropenia, ≥ 5 % atypical lymphocytes, thrombocytopenia, elevation of ALAT/ASAT). The group of “CMV end-organ diseases” includes, amongst others,

CMV pneumonia, CMV gastrointestinal disease, CMV encephalitis and ventriculitis, and CMV retinitis. Three categories (proven, probable, possible) have to be distinguished. Each category requires the presence of appropriate clinical signs and/or symptoms. The classification as "proven CMV end-organ disease" always demands the detection of CMV in tissue of the respective organ by histopathology, immunohistochemistry (IHC), virus isolation, rapid culture, DNA hybridization, or (in the case of CMV encephalitis and ventriculitis) quantitative PCR from CNS tissue. For the diagnosis of proven CMV retinitis, typical ophthalmological signs are sufficient. In some end-organ disease categories (CMV pneumonia, CMV gastrointestinal disease), detection of high CMV DNA levels in the relevant tissue via quantitative nucleic acid testing (e.g., PCR) is newly accepted as "possible CMV end-organ disease", especially when CMV DNA is not detectable in blood obtained at the same time. However, defining viral load cutoff levels has not yet been possible. (Ljungman et al., 2017)

It must be noted that CMV infection is not synonymous with CMV disease in the immunocompromised host (Fietze et al., 1994). The development of CMV disease requires both exposure to (reactivated) replicating virus and failure of the immune system to limit virus spreading and to destroy CMV-infected cells (i.e., insufficient antiviral immune response) (Fietze et al., 1994). The populations at risk include patients with AIDS, solid-organ transplant patients, hematopoietic stem-cell transplant recipients, and newborns (Dioverti and Razonable, 2016). Other immunocompromised individuals (e.g., patients with leukemia, lymphoma, or other malignancies and patients treated with immunosuppressants) may also develop CMV disease (Dioverti and Razonable, 2016). Interestingly, even subclinical active CMV infection and, more importantly, the immune responses it triggers can have tremendous pathophysiological consequences. Indeed, symptomless CMV infection has been associated with renal allograft injury (Reinke et al., 1994).

1.1.2 Diagnostics and treatment

1.1.2.1 Diagnostic tools

For the diagnosis of CMV infection, nonmolecular and molecular laboratory methods are available. Nonmolecular techniques include serology, antigenemia assays, viral culture, and histopathology. Molecular methods comprise PCR (most common) and non-PCR amplification and detection of CMV DNA. The resources available in the respective center guide the choice of which methods to use. (Dioverti and Razonable, 2016)

Serology

Primary CMV infection results in the production of CMV-specific IgM and IgG antibodies, the latter persisting lifelong. Detection of CMV-specific immunoglobulins can be helpful in the diagnosis of acute or previous CMV infection. The principle of this test is described in section 2.2.6.1. Detection of CMV IgG implies past CMV infection, whereas the presence of CMV IgM or a ratio

of ≥ 4 in paired serum CMV IgG titers indicates acute or recent CMV infection. However, it has to be considered that CMV IgM may persist for months in some individuals, that CMV IgM may be produced during CMV reactivation, and that some conditions may cause false-positive results of CMV IgM. In contrast, the immunocompromised host may have delayed or attenuated antibody production, and some individuals may even fail to mount a serologic response upon CMV infection, which leads to false-negative results. (Dioverti and Razonable, 2016)

Antigenemia assays

"Antigenemia is defined as the detection of CMV pp65 antigen in PBLs [peripheral blood leukocytes, author's note]" (Ljungman et al., 2017).

Detecting CMV-specific antigens in neutrophils is useful to diagnose acute CMV infection in the immunocompromised host, assess its severity, and monitor treatment response. The principle of this method is that cytospin preparations are fixed and permeabilized. The presence of CMV-specific matrix phosphoprotein 65 (pp65) is revealed by immunofluorescence, immunoperoxidase, or other antigen detection methods. The number of antigen-positive cells is counted and related to the total number of cells evaluated. The sensitivity of the pp65-antigenemia assay is much higher compared to viral culture, and in some studies, its performance was comparable to molecular tests. However, there are considerable limitations, which make it a less useful diagnostic tool. First, it is a subjective method lacking standardization across laboratories. Secondly, neutrophil granulocytes are needed in adequate numbers to perform the test. Thirdly, the samples must be processed within 6–8 hours as the *ex vivo* lifespan of neutrophils is short. Last but not least, this method cannot detect free viruses in biological fluids, which may be more indicative of active viral replication. (Dioverti and Razonable, 2016)

Nucleic acid testing

"DNAemia is defined as the detection of CMV DNA in samples of plasma, serum, whole blood, or isolated PBLs, or in buffy-coat [sic!] specimens" (Ljungman et al., 2017).

In contemporary clinical practice, CMV nucleic acid testing (NAT) is preferentially used to rapidly diagnose CMV infection in immunocompromised patients. This molecular method is based on the detection and/or amplification of CMV-specific nucleic acids in clinical specimens. The sensitivity is higher compared to nonmolecular methods, and detection of CMV infection is possible at early stages, even before serologic conversion. The most commonly used molecular method is CMV PCR. The target of CMV NAT usually is DNA, though detection of RNA through reverse transcription (RT)-PCR is possible. CMV NAT assays detecting DNA are highly sensitive but have a higher risk of detecting inactive latent CMV infection than RNA testing, which is more specific and indicative of active CMV infection. However, DNA is stable in clinical specimens over time. In contrast, RNA is readily degraded, leading to false-negative results and a lower sensitivity. (Dioverti and Razonable, 2016)

Qualitative CMV DNA tests, which report a sample as negative or positive, have a high sensitivity in the diagnosis of CMV infection. However, the specificity is only modest, and the positive predictive value is low compared to quantitative tests. Importantly, qualitative assays do not reliably discriminate latent from active CMV infection. Quantitative CMV NATs report viral loads (amount of virus, typically normalized to the volume of input specimen), which can be correlated to disease/infection severity and enable real-time monitoring of antiviral treatment response. High viral loads or a rising trend indicate active CMV disease, whereas low viral loads point to latent or subclinical infection. However, there is no widely accepted threshold for predicting CMV disease. (Dioverti and Razonable, 2016)

Viral culture

Detection of CMV using labor-intensive culture techniques is highly predictive of CMV disease and relatively specific. The principle of the conventional tube cell culture is that clinical specimens (e.g., blood) are inoculated into human fibroblast cultures and incubated. Once cytopathic effects are observed, the identity of the viral isolate is confirmed by immunofluorescence using CMV-specific antisera. The clinical utility is limited by the slow turn-around time (2–4 weeks) and the poor sensitivity. The shell-vial assay accelerates the viral culture process (turn-around time of 16–48 hours) using low-speed centrifugation and monoclonal antibodies directed against early antigens of replicating CMV. However, its sensitivity is still relatively low compared to antigenemia/molecular assays, and CMV activity in clinical specimens decreases rapidly. Therefore, viral culture is no longer the first-line method for the laboratory diagnosis of CMV infection. (Dioverti and Razonable, 2016)

Histopathology

The histologic detection of CMV-infected cells is highly specific and indicates the presence of active tissue-invasive CMV disease. Typically, giant cells with a dense intranuclear inclusion and surrounding halo (classic “owl’s eye”) can be seen in hematoxylin and eosin (HE) stain. This histological feature is formed when the mass of viral particles shrinks away from the nuclear membrane during fixation. The appearance of herpes simplex virus (HSV) intranuclear inclusions may be similar. However, CMV is the only member of the Herpesviridae family that shows both intranuclear and intracytoplasmic inclusions. The major drawback of histopathology is the need to invasively obtain a tissue sample. Immunohistochemical stainings of tissue specimens and *in situ* hybridization (ISH) can be carried out to demonstrate the presence of CMV antigens (e.g., viral matrix protein pp65) and CMV DNA, respectively. (Dioverti and Razonable, 2016)

Detection of CMV-specific T cells

It has been shown that CMV infection induces a distinct virus-specific CD4- and CD8-positive T cell response. There are different ways to measure the cell-mediated immune response to CMV infection, including MHC multimer (for identification and enumeration of CMV-specific T cells)

and functional assays (e.g., enzyme-linked immunospot, flowcytometric intracellular cytokine staining), which detect IFN-γ release or other cytokine-secreting cells upon *in vitro* stimulation with antigen. (Dioverti and Razonable, 2016)

1.1.2.2 Treatment strategies

Treatment of CMV disease consists of administration of antiviral drugs and (if arguable) reduction of immunosuppressive agents. The antiviral agents of choice are intravenous ganciclovir and oral valganciclovir. The duration of antiviral treatment is guided by the clinical and virologic response, as indicated by quantitative NAT and/or pp65 antigenemia tests on a weekly basis. It is recommended to maintain viral suppression for at least 2 weeks before antiviral treatment is discontinued. Rising or non-declining viral loads over at least 2 weeks suggest drug-resistant CMV infection. Foscarnet and cidofovir are used as second-line agents. The role of intravenous immunoglobulins (IVIg) and CMV immunoglobulin as adjuncts to antiviral therapy is unclear. Novel antiviral drugs are currently evaluated and/or have already been approved for special indications. (Dioverti and Razonable, 2016)

1.1.3 How does CMV infection (potentially) affect the skin?

The following chapter will describe the known cutaneous effects of CMV infection. In addition, three major principles in the pathogenesis and treatment of chronic skin diseases and their relation to CMV infection will be outlined. Finally, the hypothesis underlying this research project will be developed.

1.1.3.1 Cutaneous CMV involvement

The rarity of reports of the cutaneous effects of CMV infection in literature is attributed to the fact that clinical and pathological features are not pathognomonic and may be difficult to identify, leading to misdiagnosis. The actual incidence of dermatological CMV manifestations may be significantly higher. (Drozd et al., 2019)

The involvement of the skin during CMV infection mainly affects the immunocompromised host. The skin lesions may be nonspecific or specific. The nonspecific skin eruptions, which are mostly attributable to immunological abnormalities following viral infection or hypersensitivity reactions in the context of antibiotic treatment, include maculopapular rashes and urticarial and scarlatiniform eruptions. The different specific lesions with histological evidence of CMV infection in endothelial cells of dermal vessels that have been reported can be grouped into localized (cutaneous and oral ulcerations, crusted papules, nodules) and widespread (erythematous and purpuric morbilliform eruptions, verrucous lesions, perifollicular papulopustules, urticaria, and vesiculobullous eruptions) lesions. In contrast, the development of cutaneous CMV involvement in the immunocompetent host is very rare. (Drago et al., 2000)

In their more recent comprehensive review, Drozd et al. (2019) outlined that the possible dermatological manifestations associated with CMV infection were numerous, including erythema, papules, nodules, plaques, vesicles and bullae, pustules, petechiae and purpura, and erosions. There are reports of CMV-related morbilliform rash, vasculitis, pruritus, and edema. However, the by far most common CMV-associated cutaneous lesions are ulcers. These ulcers are predominantly located in the genital, gluteal, and perianal region and the oral cavity, but there are also reports of dispersed ulcers and lower extremity ulcers. The preferential location in the perianal area has been related to fecal shedding during periods of reactivation. The character of CMV-associated cutaneous ulcers is not uniform. Indeed, both painful and tender or non-tender lesions have been described. (Drozd et al., 2019)

The diagnosis of cutaneous CMV-associated lesions is not easily made by the clinical picture. Indeed, the first diagnosis is often not correct. The histopathological examination of skin biopsies is the most commonly utilized method. Other common diagnostic tests include immunohistochemical staining of skin samples, PCR from skin biopsies and/or blood samples, serological tests, and CMV pp65 blood antigen assay. (Drozd et al., 2019)
The histopathological evidence of cutaneous CMV infection can primarily be found in endothelial cells and less frequently in fibrocytes and inflammatory cells. In contrast, epithelial cells (epidermal keratinocytes, eccrine ductal cells) are rarely affected. (Resnik et al., 2000)

The early recognition of cutaneous CMV manifestations, which may indicate systemic disease and an unfavorable prognosis, is imperative (Drozd et al., 2019). Though untreated CMV infection in the immunocompromised host may be associated with mortality, the outcomes with adequate treatment are often favorable (Drozd et al., 2019).

1.1.3.2 Three major principles in the pathogenesis and treatment of chronic skin diseases

The major principles in the pathogenesis of chronic skin diseases include autoimmunity and inflammation. Their treatment usually involves immunosuppressive and/or immunomodulating agents to control autoimmune reactions and inflammation. The following subsections will outline the main features of autoimmunity, skin inflammation, and immunosuppression and the mechanisms underlying their association with CMV infection.

Autoimmunity

The term “autoimmunity” refers to the failure of immunologic tolerance leading to an immune response against host antigens, which potentially results in tissue damage and the development of “autoimmune diseases”. To initiate autoimmune reactions, a combination of genetic predisposition, immune dysregulation, and environmental factors is needed. The environmental factors include viral infections, which usually trigger a potent immune response to contain the infection.

However, in some cases, this inflammatory reaction overwhelms immune regulatory mechanisms and potentially leads to the onset or exacerbation of autoimmune diseases. (Hussein and Rahal, 2019)

Hussein and Rahal (2019) outlined infection-associated mechanisms underlying autoimmunity:

1) The presentation of microbial antigens that molecularly resemble self-epitopes to cross-reactive T cells, which recognize both the microbial antigen and the respective self-antigen, may induce an autoimmune reaction (= molecular mimicry).
2) The infection may result in inflammatory signals that inadvertently activate autoreactive T cells (= bystander activation).
3) The release of self-antigens from host tissue, damaged directly by a persisting infectious agent or indirectly by the immune response to infection, may induce an autoimmune reaction against these self-antigens. This means that the immune reaction spreads from microbial antigens to self-antigens that are not cross-reactive (= epitope spreading).
4) The inflammatory microenvironment resulting from the host immune response to infection may enhance and alter the processing of self-antigens by antigen-presenting cells. This may lead to the exposure of cryptic epitopes, which are then presented to self-reactive T cells and initiate autoimmune processes (= presentation of cryptic antigens). (Hussein and Rahal, 2019)

Pan et al. (2019) delineated additional mechanisms that underlie the involvement of infectious agents in the pathogenesis of systemic lupus erythematosus (SLE) and, in general, autoimmune reactions. These mechanisms included, amongst others, superantigen production (resulting in the activation of numerous T cells with different antigenic specificities), persistence of viral infections (providing constant stimulation to the immune system), and viral lymphotropism (prompting the development of polyclonal lymphocytes) (Pan et al., 2019).

The wide range of target cells and tissues, its immunomodulating abilities, and its persistence in the host organism with alternating phases of latency and lytic replication may enable CMV to contribute to the development of autoimmunity. The autoimmune diseases that have been associated with CMV infection include systemic sclerosis and SLE. (Hussein and Rahal, 2019)

Skin inflammation

Eyerich and Eyerich (2018) summarized that the pathogenesis of most non-communicable inflammatory skin diseases was based on the interaction of lymphocytes and keratinocytes. The dominating lymphocyte subset and the secreted cytokines induce six distinct cutaneous immune response patterns:

1) The lichenoid pattern is characterized by a cytotoxic immune response against keratinocytes of the basal layer (“interface dermatitis”). The skin lesions are dominated by type 1 immune cells (Tc1 cells, Th1 cells, ILC1, NKT cells, and NK cells), which are secreting IFN-γ and TNF-α. The major physiologic function of this pattern is the elimination of keratinocytes that are (pre-)carcinogenic or potentially infected with intracellular microbes.

2a) The eczematous pattern with impaired epidermal barrier, reduced antimicrobial peptides, and recruitment of eosinophils is mediated by type 2 immune cells (Th2 cells, ILC2), which are secreting IL-4, IL-5, IL-13, and IL-31. The major physiologic role of this immune pattern is defense against extracellular parasites.

2b) The bullous pattern, which is characterized by antibody deposits resulting in blistering and loss of epithelial integrity, is elicited by IgE, IgG, and IgA autoantibodies. The formation of these antibody subclasses directed against structural proteins of the skin is induced by type 2 lymphocytes via secretion of IL-4 and IL-5, respectively. The physiologic role of this immune response pattern is the neutralization of extracellular microbes.

3) The psoriatic pattern is mediated by type 3 immune cells (Th17 cells, Tc17 cells, ILC3, and Th22 cells), which physiologically warrant homeostasis at barrier organs and wound healing. The secretion of IL-17A, IL-17F, IL-21, IL-22, and TNF-α leads to acanthosis, high metabolic activity, and infiltration of neutrophils.

4a) The fibrogenic pattern with rarefication of cells and deposits of extracellular matrix results from prolonged lymphocyte anti-inflammatory activity, usually related to counter-regulation of a preceding inflammatory response. This immune response pattern is elicited by regulatory T cells such as iTreg cells, Th3 cells, and Tr1 cells (type 4 lymphocytes), and the driving cytokines are IL-10 and TGF-β.

4b) The granulomatous pattern involves both proinflammatory and regulatory T cells with TNF-α (non-Treg) and IL-10 as the leading cytokines. The physiological role of granuloma formation is the limitation of potentially harmful molecules that cannot be eliminated.

There is usually one predominant pattern, but a mixture of patterns may also be observed, particularly in chronic skin conditions. (Eyerich and Eyerich, 2018)

To conclude, the distinct cutaneous immune response patterns that have been described by Eyerich and Eyerich (2018) are elicited by the production of different cytokines with a major role for TNF-α in the development of the lichenoid and psoriatic pattern. The release of inflammatory cytokines (particularly TNF-α) is also known to activate CMV gene expression and stimulate local virus production (Heald-Sargent et al., 2020).

Immunosuppression/immunomodulation

The administration of topical agents is insufficient in certain phases/stages of many dermatological conditions. Instead, the use of systemically acting drugs, including biologicals, immunosuppressants, and immunomodulators, becomes necessary. The systemic treatment of skin diseases in general and the administration of specific antibody therapies in particular have considerably increased in the last decades and in recent years, respectively. (Schreml and Gollnick, 2018)

Immunosuppressive agents that act by different mechanisms have different effects on the course of CMV infection. For instance, cyclosporine interferes significantly with the host's ability to control CMV infection but only has minimal impact on the reactivation of latent virus. In contrast, pan-T cell antibodies inducing cytokine release are extremely potent in reactivating the virus from latency. The resulting T cell depletion prevents sufficient antiviral immune response, although the effect on the course of active infection is much smaller. It is important to consider both the effects of the individual components of an immunosuppressive regimen and their combined impact on CMV infection. (Rubin, 1990; Fietze et al., 1994)

1.1.3.3 Hypothesis of this research project

The clinical experience has shown that some patients with chronic skin diseases do not respond adequately to immunosuppressive/immunomodulating standard treatment but instead show persistence or progression of skin lesions. The insufficient control of disease activity represents a significant challenge for clinicians and patients.

Latent CMV infection and, thus, the potential of CMV reactivation is widespread in Germany. In a nationally representative sample of German adults (aged 18–79 years, samples from 1998), the overall seroprevalence of CMV-specific antibodies (IgG, IgM, and IgA) ranged at 56.7 % (Lachmann et al., 2018). Reactivation of CMV infection might be particularly relevant in patients with chronic skin diseases. These patients fulfill two major prerequisites of CMV reactivation, namely ongoing skin inflammation and therapy-induced immunosuppression; and, not least, CMV infection has already been associated with the pathogenesis of autoimmune skin conditions. The inherent altered immune function might additionally increase the risk of infections, as described for SLE patients (Doaty et al., 2016). The inflammatory cytokines released from CMV-infected cells and the host's antiviral immune response might, in turn, aggravate ongoing skin inflammation, starting a vicious circle of viral reactivation and inflammation.

To summarize, the hypothesis of this research project is that inflammation and immunosuppression might predispose to reactivation of CMV infection in patients suffering from chronic skin diseases and that CMV reactivation, in turn, might hinder the efficacy of immunosuppressive/immunomodulating treatment and even worsen the skin pathology.

1.2 Epstein-Barr virus

Besides CMV, there are other viruses that have a high seroprevalence in the general population, persist in the human organism following primary infection, may be reactivated during states of immunosuppression, and have been linked to the development of autoimmune skin diseases. There is no doubt that the Epstein-Barr virus (EBV) is one of the most prominent examples, as outlined in the following chapter. Interestingly, the incidence of EBV reactivation has been

demonstrated to be enhanced in hematopoietic stem cell transplant recipients who have also reactivated CMV (Al Hamed et al., 2020). Similarly, Hatayama et al. (2020) observed frequent co-reactivation of EBV in patients with CMV DNAemia during immunosuppressive treatment and/or chemotherapy. Though this research project focuses on CMV reactivation, the parallels between these two viruses prompted us to additionally investigate EBV reactivation.

1.2.1 EBV infection/disease

EBV, also known as human herpesvirus 4, belongs to the *Herpesviridae* family and its *Gammaherpesvirinae* subfamily (Grywalska and Rolinski, 2015). EBV is a double-stranded DNA virus (Marques-Piubelli et al., 2020), which is most commonly transmitted through respiratory secretions (Nowalk and Green, 2016). The seroprevalence among adults worldwide ranges at over 90 % (Nowalk and Green, 2016). The life cycle of EBV infection consists of two distinct phases, i.e., lytic replication in epithelial cells and lifelong latency with periodical reactivation in memory B cells and possibly also in epithelial cells (Andrei et al., 2019).

Most primary EBV infections in younger children are benign and often subclinical, whereas young adults are more likely to suffer from pathognomonic infectious mononucleosis (Nowalk and Green, 2016). In the immunocompetent host, EBV reactivation is controlled by the immune system (Andrei et al., 2019). In contrast, immunosuppression may result in the loss of EBV replication control and the development of EBV-related malignancies (primarily B cell lymphomas and nasopharyngeal carcinoma) (Andrei et al., 2019). The association between EBV infection and specific malignancies can also be observed in immunocompetent individuals, but the neoplastic potential of EBV infection is higher in immunocompromised hosts (Nowalk and Green, 2016). Besides, there are different other manifestations of EBV-driven disease, e.g., encephalitis, pneumonitis, and hepatitis (Nowalk and Green, 2016). Nowalk and Green (2016) outlined that the outcome of EBV infection in immunocompromised individuals depended on several key factors, including the degree of immunosuppression and the level of EBV-specific T cell activity. Finally, EBV infection has been associated with the pathogenesis of autoimmune diseases, including SLE (Hussein and Rahal, 2019; Pan et al., 2019).

1.2.2 Diagnostics and treatment

In the immunocompetent host, the development of EBV-specific antibodies – initially IgM and IgG to viral capsid antigen (VCA), months after infection IgG to EBV nuclear antigen (EBNA) – reliably hallmarks acute and chronic infection. Determining EBV loads in peripheral blood (e.g., by PCR) is widely recommended to diagnose and monitor EBV infection and disease in immunocompromised patients. To definitively diagnose EBV disease, the examination of a biopsy from the affected tissue is required. (Nowalk and Green, 2016)

Treatment with antiviral agents plays a limited role in EBV infection. The first-line therapeutic approach is stimulating the host immune response (by reduction or discontinuation of immunosuppressants, if possible). Second-line treatment consists of destruction of EBV-infected lymphocytes (e.g., by rituximab), chemotherapy, and adoptive immunotherapy. (Nowalk and Green, 2016)

1.3 Aims of the thesis

The reactivation of CMV and/or EBV infection is a serious complication in immunocompromised patients, which is able to dramatically impair clinical outcomes. The susceptibility to CMV and EBV reactivation may be particularly high in patients with chronic skin diseases, which are known to be associated with increased local and systemic levels of inflammatory cytokines and reduced immune status due to inherent altered immune function and/or iatrogenic immunosuppression. However, this patient population has not yet been in the focus of clinical and scientific interest. Thus, little is known about the potential role of CMV and EBV reactivation. In particular, the awareness of viral reactivation and possible implications falls far below par compared to other immunocompromised patient groups (e.g., transplant recipients).

This doctoral thesis aims to elucidate the role of CMV and EBV reactivation in patients who suffer from chronic skin conditions and do not respond (adequately) to immunosuppressive/immunomodulating treatment. Indeed, both CMV and EBV are highly prevalent, and at least for CMV infection, efficient antiviral therapies are available. Therefore, detection of viral reactivation at an early stage may be clinically relevant to initiate antiviral treatment, to break the vicious circle of skin inflammation and viral reactivation, and to eventually control skin diseases.

The objectives of this research project are as follows:

1) To screen the presence of CMV and EBV in lesional skin of immunocompromised patients with chronic skin diseases.
2) To comprehensively examine the role of CMV and EBV reactivation in patients with chronic skin diseases who show persistence or progression of skin lesions despite intensive immunosuppressive/immunomodulating standard treatment. This includes…
   a) determining the frequency of CMV and EBV reactivation.
   b) investigating the presence of CMV and EBV (DNA, RNA and/or proteins) in skin lesions.
   c) deciphering the frequency, phenotype, and functional role of CMV- and EBV-specific T cells in lesional skin.
   d) exploring the effects of antiviral treatment in patients showing CMV reactivation.
3) To estimate the proportion of dermatological patients at risk for CMV reactivation.
4) To evaluate the benefit of screening for CMV reactivation and subsequent antiviral treatment.

If this proof-of-concept design shows a clinical relevance of CMV and/or EBV reactivation in immunocompromised patients with therapy-refractory chronic skin diseases, it could induce a paradigm shift towards monitoring and antiviral treatment of special patient groups.

2 Material and Methods

2.1 Material

2.1.1 Sample acquisition

Table 1: Sample acquisition – Consumables

Consumable	**Company (head office)**
Biopsy Punch 6.0 mm	Stiefel a GSK Company (Brentford, UK)
Cap for 26 ml Cup (#76.569)	Sarstedt (Nümbrecht)
CELLSTAR Test Tube, 15 ml (#188271)	Greiner Bio-One (Frickenhausen)
Cellulose Swabs off the roll (#13356)	Lohmann & Rauscher (Rengsdorf)
Dafilon 3/0 (#0936243)	B. Braun Melsungen (Melsungen)
Disposable Cup, 26 ml, PP (#75.568)	Sarstedt (Nümbrecht)
Durapore Surgical Tape (#1538-1)	3M (Saint Paul, USA)
Feather Disposable Scalpel, No.11 (#02.001.30.011)	Feather Safety Razor (Osaka, Japan)
Fixomull stretch 15 x 20 cm (#72592-02)	BSN medical (Hamburg)
Injekt 2 ml Luer Solo (#4606027V)	B. Braun Melsungen (Melsungen)
Octeniderm colorless (#118211)	Schülke & Mayr (Norderstedt)
Octenisept colorless (#28031)	Schülke & Mayr (Norderstedt)
Raucodrape 50 x 60 cm (#33041)	Lohmann & Rauscher (Rengsdorf)
Sterican Gr. 20, 27 G x ¾″ (#4657705)	B. Braun Melsungen (Melsungen)
Sterile surgical set (scissors, forceps, etc.)	Diverse manufacturers
SafeSeal Micro Tube, 1.5 ml (#72.706.400)	Sarstedt (Nümbrecht)
Safety-Multifly Needle 21 G x ¾″ (#85.1638.235)	Sarstedt (Nümbrecht)
Screw Cap Micro Tube, 2 ml (#72.694.106)	Sarstedt (Nümbrecht)
S-Monovette, 9 ml: K3E (#02.1066.001), Z (#02.1063)	Sarstedt (Nümbrecht)
Sempermed supreme (surgical gloves, sterile)	Semperit Technische Produkte (Vienna, Austria)
Single-Use Tourniquet (#95.1006)	Sarstedt (Nümbrecht)

Table 2: Sample acquisition – Reagents

Reagent	**Company (head office)**
DPBS w/o $Ca^{2+}Mg^{2+}$ (#14190-169)	Thermo Fisher Scientific (Waltham, USA)
Formaldehyde Solution 3.5–3.7 %	Otto Fischer GmbH & Co. KG (Saarbrücken)
RNAlater RNA Stabilization Reagent (#76104)	Qiagen (Hilden)
Xylonest 1 %, 50 ml	AstraZeneca (London, UK)

2.1.2 Molecular biology

Table 3: Molecular biology – Consumables
(Table continues on the next page.)

Consumable	**Company (head office)**
CELLSTAR Serological Pipette, 25 ml (#760180)	Greiner Bio-One (Frickenhausen)
CELLSTAR Test Tube, 15 ml (#188271)	Greiner Bio-One (Frickenhausen)
Deparaffinization Solution (#19093)	Qiagen (Hilden)
FrameStar 384 (384-well PCR plate) (#4ti-0384/C)	4titude (Wotton, UK)
Microtome Blades N35	Feather Safety Razor (Osaka, Japan)

Table 3: Molecular biology – Consumables
(Table continued from the previous page.)

Consumable	**Company (head office)**
Pipet Tips SurPhob SafeSeal: 10 µl (extra-long) (#VT0200), 100 µl (#VT0230), 1250 µl (#VT0270)	Biozym Scientific (Hessisch Oldendorf)
Precision Wipes (#05511/#7552)	Kimberly-Clark (Dallas, USA)
qPCR Seal (#4ti-0560)	4titude (Wotton, UK)
SafeSeal Micro Tube: 1.5 ml (#72.706.400), 2.0 ml (#72.695.400)	Sarstedt (Nümbrecht)
Stainless Steel Beads, 5 mm (#69989)	Qiagen (Hilden)

Table 4: Molecular biology – Devices

Device	**Type**	**Company (head office)**
Centrifuge	Megafuge 1.0R	Heraeus (Hanau)
Freezer (-20 °C)	GS 3183 Comfort	Liebherr (Bulle, Switzerland)
Heating block	Thermomixer 5437	Eppendorf (Hamburg)
Microcentrifuge	PerfectSpin 24R	PEQLAB Biotechnologie (Erlangen)
Microtome	RM 2255	Leica Biosystems (Nußloch)
Microtome accessories	–	Diverse manufacturers
Pipet controller	Falcon Pipet Controller	Corning (Corning, USA)
Pipet	Eppendorf Reference Eppendorf Research (Plus)	Eppendorf (Hamburg)
Refrigerator	KUw 1740	Liebherr (Bulle, Switzerland)
Real-time PCR machine	LightCycler 480 Instrument II ViiA 7 Real-Time PCR System	Roche Diagnostics (Rotkreuz, Switzerland) Applied Biosystems (Foster City, USA)
Spectrophotometer	NanoDrop ND-1000	PEQLAB Biotechnologie (Erlangen)
Sterile scissors	–	Diverse manufacturers
Thermoblock	PCR 384	Eppendorf (Hamburg)
TissueLyser	TissueLyser, Adapter Set (2 x 24)	Qiagen (Hilden)
Vortexer	Vortex-Genie 2	Bender & Hobein (Zurich, Switzerland)

Table 5: Molecular biology – Kits

Kit	**Company (head office)**
QIAamp DNA FFPE Tissue Kit (#56404)	Qiagen (Hilden)
QIAamp DNA Micro Kit (#56304)	Qiagen (Hilden)
QIAamp DNA Mini Kit (#51304)	Qiagen (Hilden)
RealStar CMV PCR Kit 1.0 (#021013)	altona Diagnostics (Hamburg)
RealStar EBV PCR Kit 1.0 (#131013)	altona Diagnostics (Hamburg)
RealStar HSV PCR Kit 1.0 (#061013)	altona Diagnostics (Hamburg)
RealStar VZV PCR Kit 1.0 (#071013)	altona Diagnostics (Hamburg)

Table 6: Molecular biology – Primers/probes

Target (human)	**Direction**	**Sequence (5'–3')**	**Company (head office)**
PDH-primer	forward	TCG ATC GGG ACT GCT TTC C	Metabion (Planegg)
PDH-primer	reverse	CCC ACA ACC TAG CAC CAA AAG A	Metabion (Planegg)
PDH-probe	–	6-FAM CAT CTC CTT TTG CTT GGC AAA TCT GAT CC-TAMRA	Metabion (Planegg)

Table 7: Molecular biology – Reagents

Reagent	Company (head office)
DPBS w/o $Ca^{2+}Mg^{2+}$ (#14190-169)	Thermo Fisher Scientific (Waltham, USA)
Ethanol (C_2H_5OH) (#1.00983.1000)	Merck (Darmstadt)
RNase-free Water (#129112)	Qiagen (Hilden)
TaqMan Fast Advanced Master Mix (#4444557)	Thermo Fisher Scientific (Waltham, USA)
UltraPure DEPC-treated Water (#R0601)	Thermo Fisher Scientific (Waltham, USA)

Table 8: Molecular biology – Software

Software	Company (head office)
NanoDrop ND-1000 V3.8.1	PEQLAB Biotechnologie (Erlangen)
ViiA 7 Software v1.0	Applied Biosystems (Foster City, USA)

2.1.3 Immunohistochemistry and *in situ* hybridization

Table 9: IHC and ISH – Antibodies

Target	Clone	Host	Company (head office)
Cytomegalovirus (#M0854) [immediate early antigen, early antigen]	CCH2 + DDG9	Mouse	Dako Denmark A/S (Glostrup, Denmark)
Epstein-Barr virus (#MAB8186) [early antigen-diffuse (EA-D)-p52/50]	R3	Mouse	Merck (Darmstadt)

Table 10: IHC and ISH – Consumables

Consumable	Company (head office)
Microtome Blades N35	Feather Safety Razor (Osaka, Japan)
SuperFrost Plus Adhesion Microscope Slides (#J1800AMNZ)	Thermo Fisher Scientific (Waltham, USA)

Table 11: IHC and ISH – Devices

Device	Type	Company (head office)
Automated slide stainer	BenchMark ULTRA BOND-MAX	Roche Diagnostics (Rotkreuz, Switzerland) Leica Biosystems (Nußloch)
Incubation shaker	Ecotron ET25-TA-00	Infors (Bottmingen, Switzerland)
Microtome	RM 2255	Leica Biosystems (Nußloch)
Microtome accessories	–	Diverse manufacturers

Table 12: IHC and ISH – Reagents

Reagent	Company (head office)
BOND Epitope Retrieval Solution 1 (#AR9961)	Leica Biosystems (Nußloch)
BOND Polymer Refine Red Detection (#DS9390)	Leica Biosystems (Nußloch)
BOND Primary Antibody Diluent (#AR9352)	Leica Biosystems (Nußloch)
INFORM EBER (Epstein-Barr Virus Early RNA) Probe (#05278660001)	Roche Diagnostics (Rotkreuz, Switzerland)
ISH iVIEW Blue Detection Kit (#05278511001)	Roche Diagnostics (Rotkreuz, Switzerland)
ISH Protease 2 (#05273323001)	Roche Diagnostics (Rotkreuz, Switzerland)
OptiView DAB IHC Detection Kit (#06396500001)	Roche Diagnostics (Rotkreuz, Switzerland)
Red Counterstain II (#05272017001)	Roche Diagnostics (Rotkreuz, Switzerland)
ULTRA Cell Conditioning (ULTRA CC2) (#05424542001)	Roche Diagnostics (Rotkreuz, Switzerland)

2.1.4 ELISA and multiplex immunoassay

Table 13: ELISA and multiplex immunoassay – Consumables

Consumable	Company (head office)
Nunc Sealing Tape (#236269)	Thermo Fisher Scientific (Waltham, USA)
Pipette Tips (2–200 µl) (#732028)	BRAND (Wertheim)
Pipette Tips (300 µl) (#70.765.100)	Sarstedt (Nümbrecht)
Pipet Tips SurPhob: 10 µl (extra-long) (#VT0103), 200 µl (#VT0143), 1250 µl (#VT0173)	Biozym Scientific (Hessisch Oldendorf)
TC Plate 96 Well, Suspension, R (#83.3925.500)	Sarstedt (Nümbrecht)

Table 14: ELISA and multiplex immunoassay – Devices

Device	Type	Company (head office)
ELISA reader	Epoch	BioTek (Winooski, USA)
Graduated cylinder	250 ml, 500 ml	VITLAB (Großostheim)
Graduated pipet	BLAUBRAND (10 ml, 20 ml)	BRAND (Wertheim)
Incubator	HERAcell	Heraeus (Hanau)
Microplate wash station	Bio-Plex Pro II Wash Station	Bio-Rad Laboratories (Hercules, USA)
Microtiter plate shaker	MTS 2/4 digital microtiter shaker	IKA-Werke (Staufen)
Multi-channel pipet	Transferpette-8: 2.5–25 µl, 20–200 µl, 30–300 µl	BRAND (Wertheim)
Multiplex array system	Bio-Plex 200 System	Bio-Rad Laboratories (Hercules, USA)
Pipet controller	Eppendorf Easypet	Eppendorf (Hamburg)
Single-channel pipet	Eppendorf Research (Plus)	Eppendorf (Hamburg)
Vortexer	Vortex-Genie 2	Scientific Industries (Bohemia, USA)

Table 15: ELISA and multiplex immunoassay – Kits

Kit	Company (head office)
Enzygnost Anti-CMV/IgG (#OWBA155/#10446580)	Siemens Healthcare Diagnostics Products (Marburg)/ DiaSorin (Saluggia, Italy)
Enzygnost Anti-CMV/IgM (#OWBK155/#10446583)	
Novagnost EBV-EBNA IgG (#EBVG0580DB/#10445800)	
Novagnost EBV-VCA IgM (#EBVM0150DB/#10445801)	
Supplementary Reagents for Enzygnost/TMB (#OUVP17)	
Bio-Plex Pro Human Cytokine 27-plex Assay (#M500KCAF0Y)	Bio-Rad Laboratories (Hercules, USA)

Table 16: ELISA and multiplex immunoassay – Reagents

Reagent	Company (head office)
Aqua ad iniectabilia	Berlin-Chemie (Berlin)

Table 17: ELISA and multiplex immunoassay – Software

Software	Company (head office)
Gen5 2.0 All-In-One Microplate Reader Software	BioTek (Winooski, USA)
Bio-Plex Manager 6.0 Software	Bio-Rad Laboratories (Hercules, USA)

2.1.5 Cell culture and flow cytometry

Table 18: Cell culture and flow cytometry – Buffers and supplemented media

Buffer/supplemented medium	Composition
FACS buffer	94.98 % DPBS w/o $Ca^{2+}Mg^{2+}$, 5 % FBS, 0.02 % Sodium azide
HUDC medium	88.9 % RPMI, 9.9 % FBS, 1 % L-Glutamine, 0.2 % Gentamicin
Monocyte freezing medium	90 % FBS, 10 % DMSO
T cell proliferation medium	91 % RPMI, 5 % Human serum, 1 % L-Glutamine, 1 % NEAA, 1 % Penicillin-Streptomycin, 1 % Sodium Pyruvate
T cell freezing medium	50 % RPMI, 40 % FBS, 10 % DMSO

Table 19: Cell culture and flow cytometry – Consumables

Consumable	Company (head office)
CELLSTAR Serological Pipette: 1 ml (#604181), 2 ml (#710180), 5 ml (#606180), 10 ml (#607180), 25 ml (#760180)	Greiner Bio-One (Frickenhausen)
CELLSTAR Test Tube: 15 ml (#188271), 50 ml (#227261)	Greiner Bio-One (Frickenhausen)
CryoPure Tube, 1.8 ml (#72.379)	Sarstedt (Nümbrecht)
Falcon Non-Tissue Culture-Treated Plate, Flat Bottom: 6-well (#351146), 24-well (#351147), 48-well (#351178)	Corning (Corning, USA)
Falcon Non-Tissue Culture-Treated Plate, 96-well: Flat Bottom (#351172), Round Bottom (#351177)	Corning (Corning, USA)
Falcon 5 ml Polystyrene Round-Bottom Tube (#352052)	Corning (Corning, USA)
Filtropur V50, 500 ml (0.2 µm) (#83.3941.001)	Sarstedt (Nümbrecht)
Micro Test Plate 96 Well, R (#82.1582.001)	Sarstedt (Nümbrecht)
Pipet Tips ep T.I.P.S. Standard/Bulk: 0.5–20 µl (#0030 000.854), 2–200 µl (#0030 000.870)	Eppendorf (Hamburg)
Pipet Tips ep T.I.P.S. Standard, 50–1000 µl (#0030 000.919)	Eppendorf (Hamburg)
Quali - 96 well Tube - System - Single Tubes 1.2 ml (#G057-T)	Kisker Biotech (Steinfurt)

Table 20: Cell culture and flow cytometry – Devices

Device	Type	Company (head office)
Biological safety cabinet	HERAsafe	Heraeus (Hanau)
Centrifuge	Heraeus Megafuge 40R Multifuge 1L-R	Thermo Fisher Scientific (Waltham, USA) Heraeus (Hanau)
Chemistry pump unit	Vacuubrand PC 2004 VARIO	Vacuubrand (Wertheim)
Flow cytometer	BD LSRFortessa	BD Biosciences (San Jose, USA)
Freezer (-86 °C)	HERAfreeze, HFU 686 Basic	Thermo Fisher Scientific (Waltham, USA)
Freezing container	CoolCell 5ml LX	BioCision (San Rafael, USA)
Hemocytometer	Neubauer improved	Marienfeld Superior (Lauda-Königshofen)
Incubator	HERAcell	Heraeus (Hanau)
Liquid nitrogen container	Locator 6 Plus LS6000	Thermo Fisher Scientific (Waltham, USA) tec-lab (Taunusstein)
MACS	autoMACS Pro Separator	Miltenyi Biotec (Bergisch Gladbach)
Microscope	Axiovert 25	Zeiss (Oberkochen)
Pipet controller	Falcon Pipet Controller	Corning (Corning, USA)
Pipet	Eppendorf Reference Eppendorf Research (Plus)	Eppendorf (Hamburg)
Refrigerator	VKS 2101 KS (02)	Riedel Cooling (Kulmbach)

Table 21: Cell culture and flow cytometry – Reagents

Reagent	**Company (head office)**
autoMACS Pro Washing Solution (#130-092-987)	Miltenyi Biotec (Bergisch Gladbach)
autoMACS Running Buffer – MACS Separation Buffer (#130-091-221)	Miltenyi Biotec (Bergisch Gladbach)
CD14 MicroBeads, human (#130-050-201)	Miltenyi Biotec (Bergisch Gladbach)
CellTrace CFSE Cell Proliferation Kit, for flow cytometry (#C34554)	Thermo Fisher Scientific (Waltham, USA)
DPBS w/o $Ca^{2+}Mg^{2+}$ (#14190169)	Thermo Fisher Scientific (Waltham, USA)
Ethanol (C_2H_5OH) (#1.00983.1000)	Merck (Darmstadt)
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation (#L34965)	Thermo Fisher Scientific (Waltham, USA)
Lymphoprep (#1114547)	Axis-Shield PoC AS (Oslo, Norway)
Sodium azide	Sigma-Aldrich (Saint Louis, USA)
Trypan Blue Stain (0.4 %) (#15250-061)	Thermo Fisher Scientific (Waltham, USA)
UltraPure 0.5 mM EDTA, pH 8.0 (#15575-038)	Thermo Fisher Scientific (Waltham, USA)

Table 22: Cell culture – Media/supplements

Medium/supplement	**Company (head office)**
Anti-CD3 mAb, murine (#555329)	BD Biosciences (San Jose, USA)
Anti-CD28 mAb, murine (#555725)	BD Biosciences (San Jose, USA)
DMSO (#A3672.0250)	AppliChem (Darmstadt)
Gentamicin (10 mg/ml) (#15710049)	Thermo Fisher Scientific (Waltham, USA)
GM-CSF, human, recombinant (#130-093-864)	Miltenyi Biotec (Bergisch Gladbach)
hCMV Cell Lysate Antigen (#CMV-CL-100)	The Native Antigen Company (Kidlington, UK)
Human Serum (#H4522)	Sigma-Aldrich (Saint Louis, USA)
HyClone Fetal Bovine Serum (#SV30160.03)	GE Healthcare (Chicago, USA)
IL-2, human, recombinant (#02238131)	Novartis Pharma (Basel, Switzerland)
IL-4, human, recombinant (#130-093-920)	Miltenyi Biotec (Bergisch Gladbach)
L-Glutamine (200 mM) (100x) (#25030024)	Thermo Fisher Scientific (Waltham, USA)
MEM NEAA (100X) (#11140035)	Thermo Fisher Scientific (Waltham, USA)
Penicillin-Streptomycin (10,000 U/ml) (#15140122)	Thermo Fisher Scientific (Waltham, USA)
RPMI 1640 Medium (#21875091)	Thermo Fisher Scientific (Waltham, USA)
Sodium Pyruvate 100 mM (#11360039)	Thermo Fisher Scientific (Waltham, USA)
T-activated BZLF1, recombinant (#12312001)	Lophius Biosciences (Regensburg)
T-activated EBNA3A, recombinant (#12312002)	Lophius Biosciences (Regensburg)
Tetanus toxoid (#ALX-630-108-C100)	Enzo Life Sciences (Farmingdale, USA)

Table 23: Flow cytometry – Antibodies

Target	**Fluorochrome**	**Clone**	**Host**	**Dilution [1:X]**	**Company (head office)**
CD3 (#317327)	BV711	OKT3	Mouse	100	BioLegend (San Diego, USA)
CD4 (#300526)	AF700	RPA-T4	Mouse	500	
HLA-DR (#307630)	PerCp-Cy5.5	L243	Mouse	50	
CD8 (#561423)	APC-H7	SK1	Mouse	50	BD Biosciences (San Jose, USA)
CD14 (#557923)	AF700	M5E2	Mouse	50	
CD80 (#561134)	APC-H7 (APC-Cy7)	L307.4	Mouse	100	
CD83 (#551073)	APC	HB15e	Mouse	50	
CD86 (#562390)	PECF594 (PE-Texas Red)	2331 (FUN-1)	Mouse	100	
CD209 (#551264)	FITC	DCN46	Mouse	20	

Table 24: Flow cytometry – Software

Software	Company (head office)
BD FACSDiva Software v6.2	BD Biosciences (San Jose, USA)
FlowJo v10	FlowJo LLC (Ashland, USA)

2.2 Methods

2.2.1 Patient cohorts

All patients were admitted to the Department of Dermatology and Allergology of the Technical University of Munich. The study followed the Declaration of Helsinki and was approved by the local ethics committee (Klinikum rechts der Isar, 514/17 S). The demographic and clinical data were extracted from the patients' charts and electronic records. Biomaterial was obtained from the Biobank Biederstein, which is approved by the local ethics committee (Klinikum rechts der Isar, 5590/12) and follows data protection rules. Patients gave their written informed consent.

2.2.1.1 Overview and inclusion criteria

To investigate the role of CMV and EBV reactivation in immunocompromised patients suffering from (therapy-refractory) chronic skin diseases, biomaterial of a total number of 68 patients was analyzed. To be enrolled in this study, patients had to be diagnosed with an autoimmune or inflammatory skin disease, chronic ulcer, or skin lymphoma. In addition, patients had to be treated with immunosuppressive/immunomodulating agents in or outside the context of the underlying skin disease at the time of biomaterial acquisition. The patients were recruited both retrospectively and prospectively (see below). The results were compared to 147 age- and sex-matched control patients, who suffered from skin conditions within the same disease categories but had not been under systemic immunosuppressive/immunomodulating treatment for at least 6 months before biomaterial acquisition. The controls were recruited retrospectively.

In the retrospective analysis, lesional skin of 43 patients who had been treated with biologicals, chemotherapy and/or immunosuppressants for at least 2 weeks prior to the acquisition of the skin biopsy **[= Retrospective Study Group, RG]** was screened for the presence of CMV and EBV. The results were compared to nonlesional skin of 43 control patients (1:1 matching) **[= Control Group for Retrospective Study Group, CG-RG]**.

The prospective study included patients who had not shown adequate treatment response (i.e., persistence or progression of skin lesions) during a period of at least 8 weeks of intensive therapy with biologicals and/or immunosuppressants, which was evaluated by at least two experienced board-certified dermatologists. The seroprevalences of CMV- and EBV-specific antibodies could be determined in 30 patients **[= Prospective Study Group, PG]** and were compared to 90 con-

trol patients (1:3 matching) **[= Control Group for Prospective Study Group, CG-PG]**. In addition, lesional skin of 14 patients **[= Prospective Study Group (PCR), PG(P)] [= subgroup of PG]** was analyzed for the presence of CMV and EBV. The results were compared to nonlesional skin of 14 control patients (1:1 matching) **[= Control Group for Prospective Study Group (PCR), CG-PG(P)]**.

2.2.1.2 Demographic data and clinical characteristics

The following subsection gives a detailed overview of the demographic data (Table 25) and clinical characteristics (diagnosis distribution (Table 26), use of immunosuppressive/immunomodulating agents (Table 27)) of the different study groups and the respective control groups.

Table 25: Demographic data

Group	**Female** n (%)	**Age (years)** median (interquartile range, range)	**Age difference** (control(s) vs. patient)
CG-RG (n = 43)	23 (53.5)	71.0 (53.0–77.0, 24.0–89.0)	± 3 years
RG (n = 43)	23 (53.5)	72.0 (52.0–78.0, 23.0–90.0)	
CG-PG (n = 90)	66 (73.3)	60.5 (47.0–70.0, 40.0–99.0)	± 0 years (in one case: ± 4 years)
PG (n = 30)	22 (73.3)	60.5 (46.5–70.3, 40.0–97.0)	
CG-PG(P) (n = 14)	10 (71.4)	55.5 (45.8–67.0, 40.0–88.0)	± 3 years
PG(P) (n = 14)	10 (71.4)	56.5 (43.8–67.0, 40.0–88.0)	

Table 26: Diagnosis distribution.
EBA = epidermolysis bullosa acquisita; PAPASH = pyoderma gangrenosum, acne, psoriasis, arthritis, and suppurative hidradenitis; PRP = pityriasis rubra pilaris.

Diagnosis n (%)	**CG-RG (n = 43)**	**RG (n = 43)**	**CG-PG (n = 90)**	**PG (n = 30)**	**CG-PG(P) (n = 14)**	**PG(P) (n = 14)**
Autoimmune skin diseases	**0 (0.0)**	**20 (46.5)**	**2 (2.2)**	**18 (60.0)**	**0 (0.0)**	**6 (42.9)**
Bullous pemphigoid	–	12 (27.9)	1 (1.1)	2 (6.7)	–	1 (7.1)
Dermatomyositis	–	1 (2.3)	–	2 (6.7)	–	1 (7.1)
EBA	–	1 (2.3)	–	1 (3.3)	–	–
Linear IgA bullous dermatosis	–	1 (2.3)	–	1 (3.3)	–	–
Pemphigus vulgaris	–	4 (9.3)	1 (1.1)	5 (16.7)	–	1 (7.1)
SLE	–	–	–	5 (16.7)	–	3 (21.4)
Systemic sclerosis	–	1 (2.3)	–	2 (6.7)	–	–
Chronic ulcers	**0 (0.0)**	**15 (34.9)**	**2 (2.2)**	**4 (13.3)**	**0 (0.0)**	**3 (21.4)**
Pyoderma gangrenosum	–	8 (18.6)	–	4 (13.3)	–	3 (21.4)
Ulcus cruris	–	7 (16.3)	2 (2.2)	–	–	–
Inflammatory skin diseases	**41 (95.3)**	**1 (2.3)**	**86 (95.6)**	**8 (26.7)**	**14 (100)**	**5 (35.7)**
Autoinflammatory conditions (PRP, Still's disease)	2 (4.7)	–	–	–	–	–
Dermatitis (e.g., atopic/ nummular/prurigoform)	13 (30.2)	–	54 (60.0)	–	7 (50.0)	–
Drug-induced exanthema	–	–	6 (6.7)	2 (6.7)	–	1 (7.1)
Erythroderma	–	–	–	1 (3.3)	–	1 (7.1)
Lichen ruber, lichen sclerosus	1 (2.3)	–	1 (1.1)	1 (3.3)	1 (7.1)	–
PAPASH syndrome	–	1 (2.3)	–	1 (3.3)	–	–
Prurigo simplex subacuta	1 (2.3)	–	5 (5.6)	–	1 (7.1)	–
Psoriasis (e.g., inversa/ vulgaris (and arthropathica))	22 (51.2)	–	8 (8.9)	3 (10.0)	3 (21.4)	3 (21.4)
Urticaria, urticarial vasculitis	1 (2.3)	–	10 (11.1)	–	–	–
Other inflammatory conditions	1 (2.3)	–	2 (2.2)	–	2 (14.3)	–
Skin lymphomas	**2 (4.7)**	**7 (16.3)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**
Mycosis fungoides	1 (2.3)	3 (7.0)	–	–	–	–
Sézary syndrome	1 (2.3)	2 (4.7)	–	–	–	–
Other skin lymphomas	–	2 (4.7)	–	–	–	–

Table 27: Use of immunosuppressive/immunomodulating agents

Medication n (%)	**RG (n = 43)**	**PG (n = 30)**	**PG(P) (n = 14)**
Biologicals	**3 (7.0)**	**12 (40.0)**	**5 (35.7)**
Adalimumab	–	1 (3.3)	1 (7.1)
Belimumab	–	3 (10.0)	1 (7.1)
Infliximab	2 (4.7)	4 (13.3)	2 (14.3)
Ixekizumab	–	1 (3.3)	1 (7.1)
Rituximab	1 (2.3)	3 (10.0)	–
Chemotherapy	**3 (7.0)**	**0 (0.0)**	**0 (0.0)**
Immunosuppressants	**49 (114.0)**	**35 (116.7)**	**15 (107.1)**
Azathioprine	5 (11.6)	4 (13.3)	2 (14.3)
Cyclophosphamide	1 (2.3)	–	–
Cyclosporine	2 (4.7)	2 (6.7)	1 (7.1)
Glucocorticoids	36 (83.7)	23 (76.7)	11 (78.6)
Methotrexate	2 (4.7)	3 (10.0)	1 (7.1)
Mycophenolic acid	3 (7.0)	3 (10.0)	–
Combination therapy (≥ 2 agents)	**12 (27.9)**	**15 (50.0)**	**6 (42.9)**

2.2.2 Medical records research

The medical records of all patients and control patients were searched for the clinical and/or mycological diagnosis of oral thrush and determination of lymphocyte counts to be able to draw conclusions on the degree of immunosuppression.

2.2.3 Sample acquisition

The sample acquisition included blood withdrawal and/or obtaining a skin biopsy.

Blood was usually drawn from a forearm vein after local disinfection. To draw blood for serology, blood tubes that had been prepared with clotting activator were used. The tubes were centrifuged at 2,000 rpm for 10 min (at room temperature). Then, the serum fraction could be aspirated. The serum samples were stored at -80 °C until experiments were started. To isolate peripheral blood mononuclear cells (PBMCs), blood withdrawal was performed with tubes that had been prepared with EDTA. In addition, EDTA-anticoagulated venous blood was sent to the Institute of Virology of the Technical University of Munich and the Helmholtz Center Munich for CMV- and EBV-specific PCR following interim storage at -80 °C. In a very small number of cases, the presence of CMV and EBV DNA was investigated in the serum sample (instead of EDTA blood).

To obtain the skin biopsy, the surgical area of interest was disinfected and locally anesthetized with Xylonest 1 %. Then, a 6 mm punch biopsy was taken from a representative skin lesion under sterile conditions and divided into three equal parts with a scalpel. The first part was preserved in RNAlater RNA stabilization reagent at -80 °C until the isolation of total DNA. The second part of the biopsy was stored in DPBS w/o $Ca^{2+}Mg^{2+}$ and used for T cell isolation. The third part was sent for routine histologic evaluation to confirm the diagnosis. The formalin-fixed paraffin-embedded (FFPE) tissue samples were further used for IHC, ISH, and (if RNAlater preserved tissue was not available) total DNA isolation.

2.2.4 Molecular biology

2.2.4.1 Isolation of DNA

Isolation of DNA from FFPE tissue sections, RNAlater preserved tissue, and PBMCs was carried out with Qiagen Kits, which utilize spin column-based sample preparation procedures. The concentration and purity of the eluates were determined by spectrophotometric measurement using NanoDrop ND-1000. DNA was stored at -20 °C until PCR experiments were started.

Isolation of DNA from FFPE tissue sections

To isolate DNA from FFPE tissue sections, QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution were used. To avoid cross-contamination, the blade holder and the brush were cleaned with 70 % ethanol, and the microtome blade was changed before cutting the next sample.

The manufacturer's protocol was modified as follows: DNA was isolated from a total number of 12 FFPE tissue sections with a thickness of 10 µm each. The volumes of Deparaffinization Solution (480 µl) and proteinase K (30 µl) were adjusted proportionally. The samples were centrifuged at full speed (i.e., 17,200 x g) for 10 min prior to elution to eliminate any traces of ethanol (which is a strong inhibitor in the following real-time PCR). To increase DNA yields, the column loaded with 20 µl Buffer ATE was incubated for 5 min at room temperature before elution.

Isolation of DNA from RNAlater preserved tissue

Isolation of DNA from RNAlater preserved tissue was carried out with QIAamp DNA Micro Kit and Qiagen TissueLyser. The frozen tissue sample was carefully dissected with a pair of sterile scissors. Then, it was placed in a 2 ml microcentrifuge tube containing one stainless steel bead (5 mm mean diameter), and 180 µl Buffer ATL was added. The TissueLyser was operated at 23 Hz for 5 min. Next, 20 µl proteinase K was added to the disrupted and homogenized sample, mixed by pulse-vortexing, and incubated at 56 °C and 700 rpm overnight. The following steps were performed according to the manufacturer's instructions. Prior to elution, the sample was centrifuged at full speed (i.e., 17,200 x g) for 10 min to avoid ethanol carryover. The column loaded with 20 µl Buffer AE was incubated for 5 min at room temperature to enhance DNA yields.

Isolation of DNA from PBMCs

For the isolation of DNA from PBMCs (total of 5 x 10^6 PBMCs in 200 µl DPBS w/o $Ca^{2+}Mg^{2+}$), QIAamp DNA Mini Kit was used as instructed in the manufacturer's protocol. To increase DNA yields, the column loaded with 200 µl Buffer AE was incubated for 5 min at room temperature.

2.2.4.2 Virus-specific real-time PCR

CMV- and EBV-specific PCR

Detection of CMV and EBV DNA in skin biopsies was carried out with RealStar PCR Kits, which use polymerase chain reaction for the amplification of specific target sequences and target-specific fluorescent dye-labeled probes for the detection of PCR amplicons, on a ViiA 7 Real-Time PCR System. The heterologous Internal Control was used as a PCR inhibition control. Reagents Master A and Master B contain all components (buffers, enzymes, primers, and probes) needed to amplify and detect virus-specific DNA and Internal Control. To ascertain the detection of all relevant viral genotypes, the manufacturer had checked the selected oligonucleotides against publicly available sequences using sequence comparison analysis. For cost reasons, the volumes of Master A and Master B reagents per reaction had to be reduced by 50 %. Thus, the number of reactions per kit could be doubled. The volumes of the other reagents were adjusted proportionally, resulting in 10.0 µl Master Mix and 5.0 µl DNA template per reaction. The rest of the reaction setup was performed as described in the manufacturer's protocol. Nuclease-free water and Quantification Standards were used as negative and positive controls, respectively.

PCR analysis of positive controls (CMV-infected placenta, EBV-infected tonsils), which had been obtained from the Institute of Pathology of the Technical University of Munich, ensured that the described modification to the protocol did not substantially interfere with PCR performance. The appropriate amount of DNA template for this PCR assay was evaluated with a serial dilution of DNA isolated from CMV-infected placenta. Depending on DNA quality and yield obtained from the respective specimen, DNA amounts of 30 ng (FFPE tissue with low DNA yield, RNAlater preserved tissue) and 50 ng (FFPE tissue with high DNA yield) were used. The reactions were carried out in duplicates. The experiment was run using the following conditions:

Table 28: Virus-specific real-time PCR – Fluorescence detectors

Target	**Reporter**	**Quencher**
Virus-specific DNA	FAM	None
Internal Control	VIC (instead of JOE)	None

Table 29: Virus-specific real-time PCR – Temperature profile and dye acquisition

	Stage	**Cycle Repeats**	**Acquisition**	**Temperature**	**Time**
Denaturation	Hold	1	–	95 °C	10:00 min
Amplification	Cycling	45	–	95 °C	0:15 min
			√	58 °C	1:00 min

For a qualitative diagnostic test run to be valid, the positive control had to show a positive signal in the FAM detection channel, and both negative and positive controls had to yield a positive signal in the VIC detection channel. The PCR results of the samples were considered positive, provided that at least one subsample showed a positive signal in the FAM detection channel. The C_t values needed to be ≤ 40 to rule out nonspecific amplification.

HSV- and VZV-specific PCR

Detection of HSV and varicella-zoster virus (VZV) DNA in skin biopsies was carried out using RealStar PCR Kits. The modifications to the protocol were the same as described in the previous section. To be able to detect HSV-1 and HSV-2 DNA simultaneously, an additional detection channel (Cy5) had to be used. This experiment was run on a LightCycler 480 Instrument II according to the manufacturer's instructions.

2.2.4.3 PDH PCR

To confirm the presence of amplifiable DNA and to correct for the variable amount of DNA in tissue samples, real-time PCR targeting the housekeeping gene "human pyruvate dehydrogenase" (PDH) was performed. The sequences of PDH primers and probe had been published previously (Knöll et al., 2003). TaqMan Fast Advanced Master Mix was used. The manufacturer's protocol ("RT-PCR for TaqMan and Custom TaqMan Gene Expression Assays") was modified using 300 nM of each primer and 300 nM of the probe instead of TaqMan Assay. The volumes of all reagents were adjusted proportionally to a total amount of 5 µl DNA template. Nuclease-free water was used as a negative control. DNA isolated from PBMCs served as a positive control

and to generate a standard curve. The reactions were carried out in duplicates (samples) and triplicates (controls), respectively. TaqMan Fast Advanced Master Mix contains uracil-N-glycosylase (UNG) to degrade carryover amplicons, which could result in false-positive amplification during PCR. The experiment was run using the following protocol:

Table 30: PDH PCR – Temperature profile and dye acquisition

	Stage	Cycle Repeats	Acquisition	Temperature	Time
UNG incubation	Hold	1	–	50 °C	2:00 min
Polymerase activation	Hold	1	–	95 °C	0:20 min
Amplification	Cycling	40	–	95 °C	0:01 min
			√	60 °C	0:20 min

To evaluate the number of cells in each sample, a serial dilution of DNA extracted from PBMCs was used (Table 31). The eluates from three healthy blood donors were mixed to minimize random errors. The average DNA yield from 5 x 10^6 PBMCs was 16,920 ng (i.e., 3.384 pg DNA/cell). Then, DNA was repeatedly diluted 1:5 with nuclease-free water. The dilution series was subjected to PDH PCR (using three replicates per dilution), and a standard curve was generated in each PCR run based on the mean threshold cycle per dilution.

Table 31: PDH PCR – Standard curve

ID	Dilution	Concentration (ng/µl)	Volume (µl)	DNA quantity (pg)	Cell count
V0	–	84.600	5	423,000	125,000
V1	1:5	16.920	5	84,600	25,000
V2	1:25	3.384	5	16,920	5,000
V3	1:125	0.677	5	3,384	1,000
V4	1:625	0.135	5	677	200
V5	1:3,125	0.027	5	135	40
V6	1:15,625	0.005	5	27	8

2.2.4.4 Viral DNA load determination

RealStar PCR Kits include Quantification Standards (QS1–QS4), which have been calibrated against the respective WHO International Standards for Nucleic Acid Amplification Techniques and contain standardized concentrations of virus-specific DNA. The Quantification Standards could be used to generate a standard curve and to quantify the amount of virus-specific DNA in the samples (Table 32). Depending on the qualitative PCR results, the mean value of the amount of virus-specific DNA was calculated (in the case of two positive subsamples), or the respective single value was used (in the case of one positive subsample).

Table 32: Virus-specific real-time PCR – Standard Curve

ID	Concentration (IU/µl)	Volume (µl)	Virus-specific DNA quantity (IU)
QS1	1.00E+04	5	50,000
QS2	1.00E+03	5	5,000
QS3	1.00E+02	5	500
QS4	1.00E+01	5	50

For the validity of a quantitative diagnostic test run, the generated standard curves were required to achieve defined control parameter values. The presence of virus-specific DNA in tissue specimens was quantified based on the number of cells in the sample. To determine the viral DNA load (in mIU/cell), the absolute amount of virus-specific DNA (in IU) was divided by the cell number, which had been evaluated separately using PDH PCR.

2.2.5 Immunohistochemistry and *in situ* hybridization

To detect viral gene products in the skin samples, IHC and ISH were performed. First, 2 µm sections were obtained from FFPE tissue blocks, and 2–3 sections per specimen were mounted on a SuperFrost Plus adhesion microscope slide. Then, the samples dried overnight at 37 °C.

CMV IHC and EBV-encoded RNA (EBER) ISH were carried out by the Institute of Pathology of the Technical University of Munich. Tissue sections were stained with the fully automated BenchMark ULTRA instrument according to the guidelines from the supplier.
Immunohistochemical staining for CMV was performed using a mixture of two monoclonal antibodies that react with CMV immediate early antigen and early antigen (clone CCH2 + DDG9). Tissue sections were pretreated with citrate-based buffer (ULTRA CC2). Then, incubation with anti-CMV antibodies (at a dilution of 1:30) for 24 min was carried out. Finally, the target antigen was visualized with OptiView DAB IHC Detection Kit, which causes a brown-colored precipitate. To identify cells expressing EBER, INFORM EBER Probe was used in conjunction with ISH iVIEW Blue Detection Kit, ISH Protease 2, and Red Counterstain II. The hybridization of the INFORM EBER Probe to EBER transcripts results in the formation of a blue precipitate.

Immunohistochemistry for EBV was performed by PD Dr. med. Natalie Garzorz-Stark, PhD. Tissue sections were stained with the fully automated BOND-MAX system according to the manufacturer's instructions. For the heat-induced epitope retrieval of FFPE tissue, citrate-based pH 6 BOND Epitope Retrieval Solution 1 was used (100 °C, 20 min). Then, tissue sections were incubated with the anti-EBV early antigen-diffuse (EA-D)-p52/50 antibody (clone R3) (at a dilution of 1:2,000) for 15 min. Finally, the target antigen was visualized with BOND Polymer Refine Red Detection, which contains Fast Red chromogen.

2.2.6 ELISA and multiplex immunoassay

2.2.6.1 Detection of virus-specific antibodies

Detection of virus-specific antibodies in serum was based on the ELISA technique. The principle of this method is that virus-specific antibodies in the sample bind to immobilized, recombinant antigens on the microplate. Peroxidase-labeled anti-human IgG/IgM binds to these antigen-antibody complexes. The enzyme part of the conjugate converts TMB substrate solution, resulting in a blue reaction product. The color changes to yellow when stop solution is added to terminate

the reaction. The intensity of the yellow color can be measured by an ELISA microplate reader at 450 nm using a reference wavelength of 650 nm. The absorbance value is proportional to the quantity of virus-specific antibodies in the sample. The experiments were carried out according to the manufacturers' instructions.

Enzygnost Kits were applied for the detection of CMV-specific antibodies. The left row of each strip of the microplate contained antigen from human fibroblasts that had been infected with CMV (Towne strain). The right row was coated with antigen derived from uninfected cells. The difference in color intensities was used as a measure of the immunochemical reactivity of the CMV-specific antibodies in the sample. To achieve an optimal reproducibility of results, the differences in absorbance (ΔA) had to be multiplied by a correction factor. Based on the corrected ΔA values, samples were classified as negative [$\Delta A < 0.100$ (= cutoff)], equivocal [$0.100 \leq \Delta A \leq 0.200$], or positive [$\Delta A > 0.200$]. Test samples with an equivocal result were re-tested in duplicate. If the result was confirmed, the samples were classified as equivocal, otherwise as positive or negative. The lot-dependent constants α and β were used to calculate IgG titers with the α-method according to the protocol. The result of the IgM test was quantified by calculating the quotient from the cutoff value (= CMV IgM ratio).

Novagnost Kits were applied for the detection of EBV-specific antibodies. The manufacturer had pre-coated microtiter wells with appropriate antigens (recombinant EBNA1 antigen, synthetic p18 peptide). To facilitate the interpretation of results, Novagnost Units (NU) were calculated from the absorbance value of the serum sample and the cutoff controls. The sample was considered positive if the absorbance value exceeded the cutoff by more than 15 % (> 11.5 NU). Samples with an absorbance value of up to 15 % above or below the cutoff (8.5–11.5 NU) were classified as "grey zone" (i.e., not clearly positive or negative). If the absorbance value was lower than 15 % below the cutoff (< 8.5 NU), samples were regarded as negative.

Hereinafter, test results that had been classified as "equivocal" (CMV-specific antibodies) or "grey zone" (EBV-specific antibodies) will consistently be referred to as "borderline".

2.2.6.2 Multiplex immunoassay

The concentrations of 27 cytokines, chemokines, and growth factors in cell-free supernatants derived from the co-culture of lesional T cells and activated monocyte-derived dendritic cells (MODCs) (see section 2.2.7.4) were measured using the Bio-Plex Pro Human Cytokine 27-plex Assay. The principle of this method is similar to that of a sandwich ELISA. The capture antibodies, which are directed against the biomarker of interest, are covalently bound to fluorescently dyed magnetic microspheres (so-called beads), which have a distinct color code. This allows simultaneous detection of different molecules in a multiplex assay. To create a sandwich complex, a biotinylated detection antibody is added. The addition of streptavidin-phycoerythrin con-

jugate results in the formation of the final detection complex. The plate reader is a flow cytometry-based instrument, which uses two lasers operating at different wavelengths to illuminate the fluorescent dyes within each bead (for assay identification) and to excite phycoerythrin to generate a reporter signal (for quantification of the analyte). The experiment was performed according to the manufacturer's recommendations.

2.2.7 Cell culture and flow cytometry

All cell culture experiments were performed in a Biosafety Level 1 laboratory under sterile conditions.

2.2.7.1 Isolation of PBMCs from human blood

PBMCs were isolated from 50 ml EDTA-anticoagulated whole blood, which had to be diluted 1:2 with DPBS w/o $Ca^{2+}Mg^{2+}$. Thereof, 25 ml was carefully layered onto 15 ml Lymphoprep without mixing both solutions. Then, the tubes were centrifuged (2,200 rpm) at room temperature for 15 min without brake. Lymphoprep is a density gradient medium. Erythrocytes and granulocytes have a higher density than mononuclear cells and, therefore, sediment through the Lymphoprep layer during centrifugation. The resulting PBMC bands were collected and washed two times by filling up with DPBS w/o $Ca^{2+}Mg^{2+}$ + 5 mM EDTA and centrifugation (first 1,600 rpm, second 1,200 rpm) at room temperature for 10 min. Finally, the PBMC pellet was resuspended in 10 ml DPBS w/o $Ca^{2+}Mg^{2+}$ + 5 mM EDTA, and the cells were counted.

2.2.7.2 Isolation of monocytes via magnetic-activated cell sorting

To isolate monocytes, magnetic-activated cell sorting (MACS) was used. First, the PBMC suspension was centrifuged (300 x g) at 4 °C for 10 min, and the supernatant was aspirated. Then, the cell pellet was resuspended in 120 µl autoMACS Running Buffer per 10^8 cells, and 30 µl CD14 MicroBeads per 10^8 cells was added. The cell suspension was mixed well and incubated in the refrigerator (at 2–8 °C) for 15 min. The cells were washed by adding 2 ml autoMACS Running Buffer per 10^7 cells and centrifugation (300 x g) at 4 °C for 10 min. Then, the supernatant was removed, and up to 10^8 cells were resuspended in 500 µl autoMACS Running Buffer. Magnetic-activated cell sorting was carried out using autoMACS Pro Separator. The separated cell fractions were counted and centrifuged (1,200 rpm) at 4 °C for 10 min. The liquid was discarded, and the CD14-positive cell fraction was resuspended in monocyte freezing medium (at 4–6 x 10^6 cells/ml). Finally, the cells were incubated in a freezing container (which cools -1 °C/min) at -80 °C for at least 80 min and transferred to liquid nitrogen.

2.2.7.3 Isolation of T cells from lesional skin biopsies

To isolate T cells from lesional skin biopsies, the T cell proliferation medium had to be supplemented with IL-2 to a working concentration of 60 U/ml. The biopsy was carefully inserted into a non-tissue culture-treated 24-well with a flat bottom that had been filled with 2 ml of T cell proliferation medium (supplemented with IL-2). The first cells that emigrate from a skin biopsy are not T cells but erythrocytes. For this reason, the biopsy was transferred into a new 24-well with 2 ml of fresh T cell proliferation medium (supplemented with IL-2) after an incubation time of 24 h (37 °C, 5 % CO_2, 100 % humidity). Thereafter, medium exchange was done every second day (Monday, Wednesday, and Friday), i.e., 1 ml medium was discarded, and 1 ml of fresh T cell proliferation medium (supplemented with 2 x IL-2 [120 U/ml]) was added. By reaching 30–50 % confluency or, at the latest, 14 days after biopsy insertion, T cells were restimulated with monoclonal anti-CD3 antibodies and the costimulatory anti-CD28 antibodies to activate their T cell receptors. First, 1 ml medium was carefully aspirated, and the skin biopsy was removed. Then, T cells were resuspended and transferred to a 24-well that had been coated with 0.75 µg/ml anti-CD3 mAb (in 1 ml DPBS w/o $Ca^{2+}Mg^{2+}$ at 37 °C for 2 h). Next, 1 ml of fresh T cell proliferation medium (supplemented with 1.5 µg/ml anti-CD28 mAb, i.e., final concentration of 0.75 µg/ml) was added and incubated for 48 h (37 °C, 5 % CO_2, 100 % humidity). T cell stimulation was usually performed without IL-2. If there were only few T cells, IL-2 was added to a final concentration of 20 U/ml. After 48 h, 1 ml medium was discarded. T cells were resuspended and transferred into a new non-coated 24-well. Next, 1 ml of fresh T cell proliferation medium (supplemented with 2 x IL-2 [120 U/ml]) was added. After reaching 80–100 % confluency, T cells were split for further expansion, i.e., T cells were resuspended, 1 ml cell suspension was transferred into a new 24-well, and both wells were filled up with fresh T cell proliferation medium (supplemented with 2 x IL-2 [120 U/ml]). Medium exchange was continued as described until T cells ceased from proliferation and did not show proliferation clusters anymore. Then, but not earlier than 2 weeks after stimulation, T cells were harvested, centrifuged (1,200 rpm) at 4 °C for 10 min, and frozen in T cell freezing medium (2 wells/vial and ml) using a freezing container as described.

2.2.7.4 T cell proliferation assay

To test CMV and EBV reactivity of lesional skin-derived T cells, a T cell proliferation assay was established. T cells isolated from lesional skin were co-cultured with MODCs that had been stimulated with human CMV (hCMV) cell lysate antigen and recombinant EBV proteins, respectively. All experiments were carried out by Dr. rer. nat. Manja Jargosch.

Thawing and cultivation of T cells (days 1–17)

Following removal from liquid nitrogen, the vials that contained frozen T cells were immediately placed into warm water and thawed until a small rest of ice was still visible. Next, T cells were washed with 20 ml DPBS w/o $Ca^{2+}Mg^{2+}$ and centrifuged (1,200 rpm) at 4 °C for 10 min, and the

supernatant was aspirated. To be restimulated, T cells were resuspended in 2 ml T cell proliferation medium (supplemented with 60 U/ml IL-2 and 0.75 µg/ml anti-CD28 mAb) and transferred into a 24-well that had been coated with 0.75 µg/ml anti-CD3 mAb (in 1 ml DPBS w/o $Ca^{2+}Mg^{2+}$ at 37 °C for 2 h). After 48 h, 1 ml medium was discarded. T cells were resuspended and transferred into a new non-coated 24-well. Then, 1 ml fresh T cell proliferation medium (supplemented with 2 x IL-2 [120 U/ml]) was added, and T cells were cultivated at 37 °C and 5 % CO_2 for 14 more days. Both medium exchange and splitting of T cells were performed as described (see section 2.2.7.3). Finally, T cells were harvested and centrifuged (1,200 rpm) at 4 °C for 10 min. The supernatant was aspirated. T cells were resuspended in DPBS w/o $Ca^{2+}Mg^{2+}$ and counted.

Generation of monocyte-derived dendritic cells (days 9–16)

Thawing of frozen monocytes followed the same procedure as described for T cells. Instead of T cell proliferation medium, the cells were resuspended in 1 ml human dendritic cell (HUDC) medium (supplemented with 150 U/ml IL-4 and 150 U/ml GM-CSF). The cell concentration was adjusted to 1.0 x 10^6 cells/ml. Next, 3.0 x 10^6 monocytes were seeded into a non-tissue culture-treated 6-well and cultivated at 37 °C and 5 % CO_2. After 4 days, 3.0 ml of fresh HUDC medium (supplemented with IL-4 and GM-CSF) was carefully added. To obtain immature dendritic cells, differentiation was continued for 3 days. MODCs do not grow adherently but are free-floating in the culture medium. Thus, cells could be harvested by pipetting up and down the cell suspension two times carefully. Additionally, the well was washed with DPBS w/o $Ca^{2+}Mg^{2+}$ once. The suspension was centrifuged (1,200 rpm) at 4 °C for 10 min, and the supernatant was discarded. The cells were resuspended in 0.5 ml HUDC medium, and the cell concentration was adjusted to 1.0 x 10^6 cells/ml. To control the differentiation of monocytes into MODCs, flow cytometric analysis of 100,000 to 300,000 cells was performed (see section “Flow cytometry (day 27)”). The downregulation of CD14 and the upregulation of CD80, CD83, CD86, CD209, and HLA-DR (exemplarily shown for patient PG4 in Figure 2) confirmed that monocytes had differentiated into MODCs.

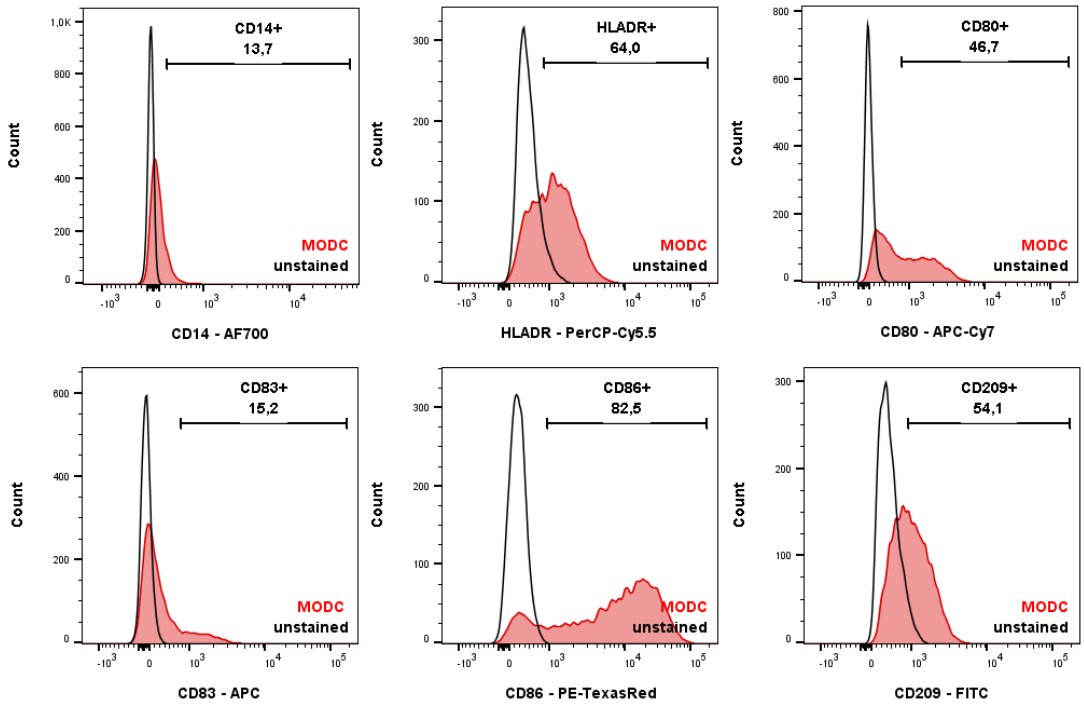


Figure 2: Differentiation control of monocytes into MODCs.
Exemplarily shown for patient PG4.

Activation of MODCs with antigen (day 16)

To obtain mature dendritic cells, 100,000 MODCs (in 200 µl HUDC medium) were incubated with antigens in non-tissue culture-treated 96-wells with a flat bottom for 24 h (at 37 °C and 5 % CO_2). To stimulate MODCs, HUDC medium was supplemented with hCMV cell lysate antigen (10 µg/ml), recombinant EBV proteins (5 µl T-activated BZLF1 and 5 µl T-activated EBNA3A/ 10 µl T-activated BZLF1), and tetanus toxoid (TT) (10 µg/ml) as a presentation control, respectively. Furthermore, HUDC medium without supplemented antigen was used as a reference measurement.

CFSE staining of lesional T cells (day 17)

Lesional T cells were labeled with CellTrace CFSE Cell Proliferation Kit for flow cytometry. CFSE is a fluorescent dye that is retained on and within cells. It allows to monitor cell proliferation by flow cytometry as its fluorescence intensity is divided equally between daughter cells. First, CellTrace CFSE was reconstituted in 18 µl DMSO and diluted 1:5,000 in DPBS w/o $Ca^{2+}Mg^{2+}$ to a final concentration of 1 µM. Next, T cells were washed with 2 ml DPBS w/o $Ca^{2+}Mg^{2+}$ and centrifuged (1,200 rpm, 4 °C, 10 min). The cell pellet was resuspended in 1 µM CFSE to a final cell concentration of 1.0 x 10^7 cells/ml. Then, T cells were stained with gentle agitation at room temperature. The reaction was stopped after 4 min, and cells were washed with 5 ml of cold DPBS w/o $Ca^{2+}Mg^{2+}$ and 10 % FBS. The cell suspension was centrifuged (1,200 rpm) at 4 °C for 10 min,

and washing was repeated with 2 ml of cold DPBS w/o $Ca^{2+}Mg^{2+}$ and 10 % FBS. Finally, T cells were resuspended in T cell proliferation medium to a final cell concentration of 1.0 x 10^6 cells/ml.

Co-culture of lesional T cells and activated MODCs (days 17–27)

500,000 CFSE-labeled T cells were incubated with 25,000 antigen-stimulated MODCs each (20:1) for 10 days at 37 °C (5 % CO_2) in T cell proliferation medium (+ 5 % FBS) using non-tissue culture-treated 48-wells with a flat bottom. After 6 days, 20 U/ml IL-2 was added to the co-culture. The cell-free supernatant was aspirated and stored frozen (-80 °C) until the multiplex immunoassay (see section 2.2.6.2) was started. In contrast to the first experiment (MODC, MODC+CMV, MODC+TT, MODC+BZLF1+EBNA3A), 10 U/ml IL-2 was added to the co-culture at the beginning of the second experiment (MODC, MODC+BZLF1, MODC+BZLF1+EBNA3A) because T cells were exhausted as a result of repeated freezing and thawing.

Flow cytometry (day 27)

Finally, flow cytometric analysis was performed to determine the frequency and phenotype of CMV- and EBV-specific lesional T cells. The principle of this method is that cells are illuminated with light of different wavelengths when passing a flow cell. Scattered light and the fluorescence of fluorochrome-labeled antibodies are used to characterize cells by size, granularity, and surface proteins.

First, the cells were harvested, transferred into non-tissue culture-treated 96-wells with a round bottom, and centrifuged. All centrifugation steps were carried out at room temperature and 2,000 rpm for 1 min. Then, the supernatant was discarded, and the cells were stained.
For the live/dead cell staining, the cell pellet was washed with 2 x 200 µl DPBS w/o $Ca^{2+}Mg^{2+}$, resuspended in 100 µl Aqua solution (diluted 1:1,000 in DPBS w/o $Ca^{2+}Mg^{2+}$), and incubated at 4 °C for 30 min (protected from light). Finally, the cell pellet was washed with 2 x 200 µl FACS buffer. The principle of this stain is that aqua solution reacts with free amines. In dead cells, which have a compromised membrane, the dye's reactivity is not limited to the amines on the cell surface. Instead, it is able to react with free amines in the cell interior, resulting in a more than 50-fold higher fluorescence as compared to viable cells.
For the staining of surface proteins, the cell pellet was resuspended in 20 µl FACS buffer plus fluorochrome-conjugated antibodies (CD3, CD4, and CD8) and incubated at 4 °C for 20 min. To control the differentiation of monocytes into MODCs (see section "Generation of monocyte-derived dendritic cells (days 9–16)"), antibodies directed against CD14, CD80, CD83, CD86, CD209, and HLA-DR were used. Finally, the cell pellet was washed with 1 x 200 µl FACS buffer, resuspended in 200 µl FACS buffer, and transferred into small FACS tubes.
The stained samples were acquired with the BD LSRFortessa flow cytometer. The results were analyzed using FlowJo v10 software.

The following gating strategy (illustrated in Figure 3) was used:

Lymphocytes → single cells → living cells → CD3+ cells → CD3+ proliferated T cells

→ CD4+ T cells

→ CD8+ T cells

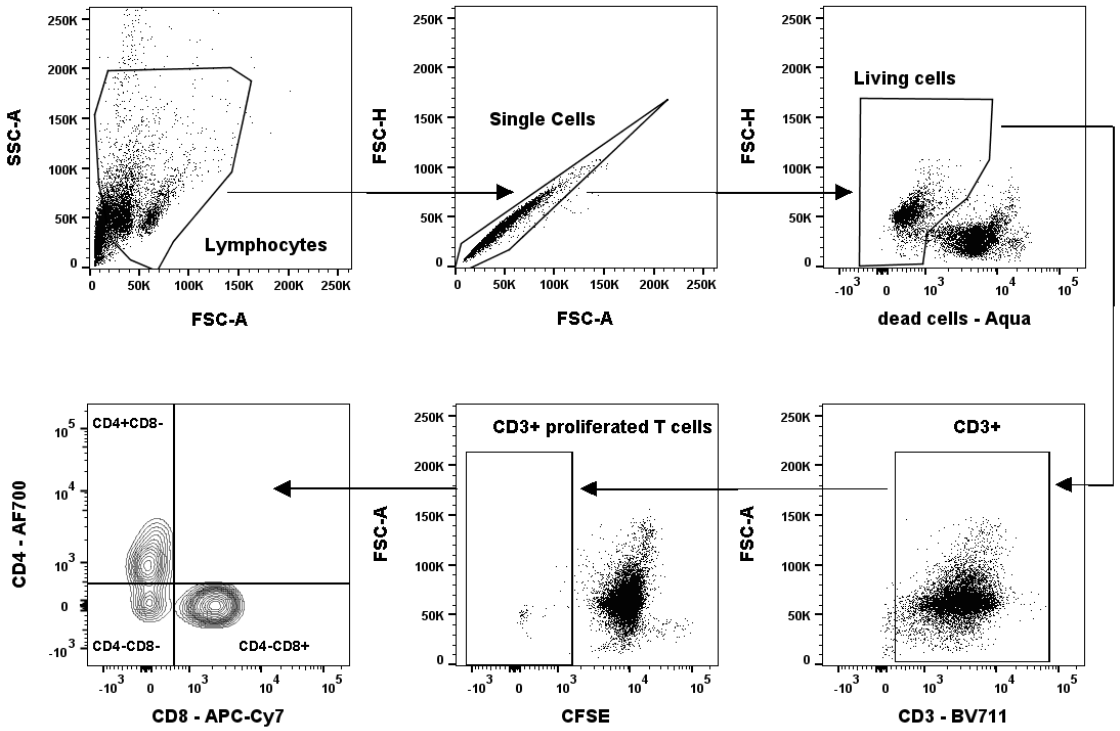


Figure 3: Gating strategy.
FSC-A = forward scatter area; FSC-H = forward scatter height; SSC-A = side scatter area.

2.2.8 Number needed to screen

To evaluate the benefit of screening for CMV reactivation and subsequent antiviral treatment, the number needed to screen (NNS) was computed.

The NNS is a statistic for disease screening, which has been developed by Rembold (1998). It is based on the number needed to treat concept and allows to compare strategies for disease screening. The NNS is calculated as the reciprocal of the absolute risk reduction. The ideal NNS is 1, indicating that all patients who are screened will benefit if detection is followed by treatment. Positive numbers imply that screening prevents a death/adverse event (with higher values in less effective screening programs). In contrast, negative numbers indicate that the patient is harmed by screening and following treatment. (Rembold, 1998)

2.2.9 Statistical analysis

Statistical analysis was carried out using SPSS Statistics 26 for Windows. Categorical variables were summarized by absolute frequencies and percentages. Quantitative variables are presented as median with interquartile range (IQR) and range. To compare the control groups with

the study groups, the chi-square test was calculated for nominal dependent variables, provided that the following conditions were met: number of degrees of freedom $n > 1$, sample size $n > 50$, and all expected counts $n > 5$. If the number of degrees of freedom was $n \leq 1$ and/or the sample size was $20 \leq n \leq 50$, Yates's corrected chi-square was computed (continuity correction). Fisher's exact test was used if the sample size was $n < 20$ and/or any expected count ranged at $n \leq 5$. The linear-by-linear association test was applied to ordinally scaled response variables. To compare quantitative variables, the Mann-Whitney U test was calculated. Two-tailed p values less than 0.05 were considered statistically significant. The software GraphPad Prism 6.01 was used to create graphs. The p values were labeled as follows: $p \geq 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

3 Results

3.1 Immunocompromised patients with chronic skin diseases

First, the presence of CMV and EBV was screened in lesional skin of immunocompromised patients with chronic skin diseases **["Retrospective Study Group, RG"]**. The results were compared to nonlesional skin of nonimmunocompromised control patients with chronic skin diseases **["Control Group for Retrospective Study Group, CG-RG"]**.

The medical records research revealed that both relative and absolute lymphocyte counts were significantly decreased in RG compared to CG-RG (Figure 4, A+B). In contrast, the frequency of oral thrush did not differ significantly between both groups (Figure 4C).

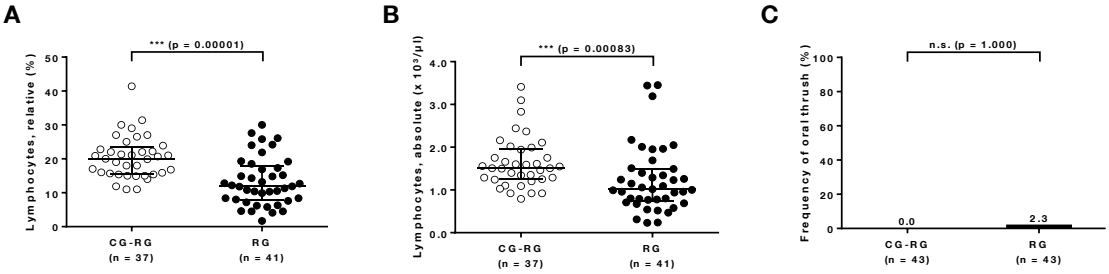


Figure 4: Lymphocyte counts and oral thrush [CG-RG vs. RG]

3.1.1 Detection of EBV DNA (but not CMV DNA) in lesional skin

FFPE tissue and RNAlater preserved tissue (preferred option) had been used to isolate DNA, depending on the availability of biomaterial (Table 33).

Table 33: Biomaterial used for DNA isolation [CG-RG vs. RG]

Group	**FFPE tissue** n (%)	**RNAlater preserved tissue** n (%)
CG-RG (n = 43)	13 (30.2)	30 (69.8)
RG (n = 43)	42 (97.7)	1 (2.3)

The detection of CMV DNA by real-time PCR was neither possible in nonlesional skin samples from CG-RG (n = 43) nor in lesional skin samples from RG (n = 43) (Figure 5, A+B).
Similarly, EBV DNA was not detectable in nonlesional skin of patients from CG-RG (n = 43). In contrast, the presence of EBV DNA could be demonstrated in lesional skin of 6 out of 43 patients (14.0 %) from RG. This difference was found to be statistically significant (Figure 5C). The median EBV DNA load (n = 6) ranged at 0.20 mIU/cell with a minimum of 0.06 mIU/cell and a maximum of 4.64 mIU/cell (IQR = 0.10 to 3.97 mIU/cell) (Figure 5D).

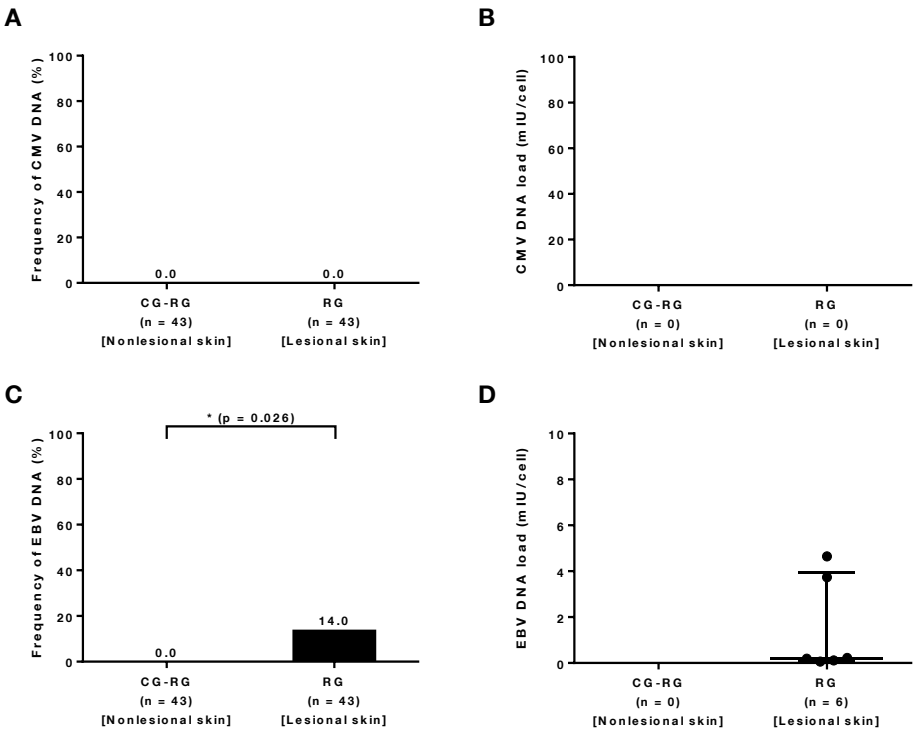


Figure 5: Detection of CMV- and EBV-specific DNA in skin biopsies [CG-RG vs. RG]

3.1.2 Demonstration of EBER and EBV EA-D in lesional skin

To gain further insights into EBV gene expression in the tissue, EBER ISH and EBV IHC were performed. In RG, EBV DNA had been detected by PCR in 6 out of 43 lesional skin biopsies (all of them FFPE samples). One FFPE sample had been completely depleted for DNA isolation. The remaining five FFPE specimens were analyzed for the presence of EBER by ISH. Immunoreactivity could be observed in 2 out of 5 samples (patients RG1 and RG2). Patient RG1, who suffered from known large cell T cell lymphoma suspicious for collision B cell tumor, showed EBER positivity in blasts and small lymphocytes (Figure 6A). Patient RG2, who had been diagnosed with pyoderma gangrenosum, showed a discrete EBER-positive signal within the lymphocytic infiltrate (Figure 6B). Both patient RG1 and RG2 harbored comparatively high EBV DNA loads in lesional skin (4.64 mIU/cell and 3.74 mIU/cell).

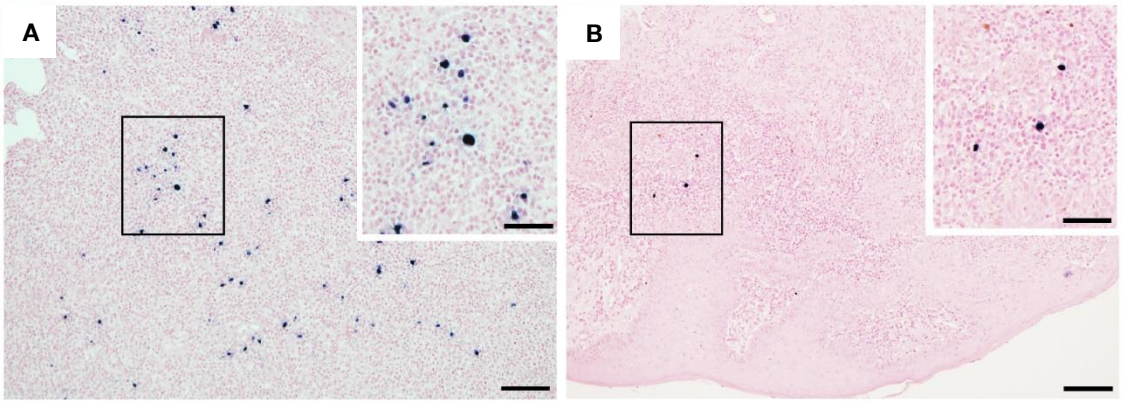


Figure 6: EBER *in situ* hybridization [RG].
(A) Patient RG1 with known large cell T cell lymphoma suspicious for collision B cell tumor.
(B) Patient RG2 with pyoderma gangrenosum. Figure modified from Speth et al. (2022).
Scale bars (in A and B) = 100 µm (overview) and 50 µm (inset).

In addition, both patients (RG1 and RG2) showed positivity for EBV EA-D-p52/50 on protein level as detected by IHC (Figure 7, A+B).

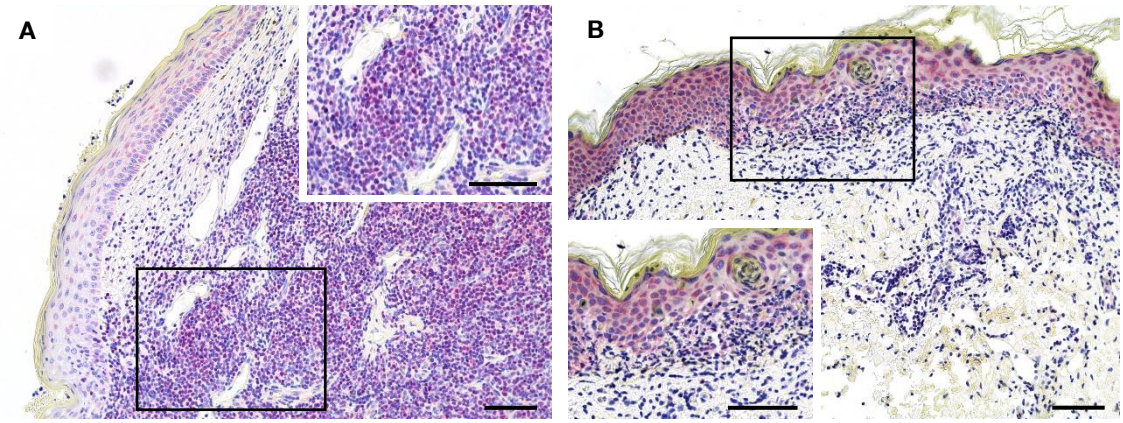


Figure 7: Immunohistochemical staining for EBV [RG].
(A) Patient RG1 with known large cell T cell lymphoma suspicious for collision B cell tumor.
(B) Patient RG2 with pyoderma gangrenosum.
Scale bars (in A and B) = 50 µm (overview and inset).

3.2 Immunocompromised patients with treatment-refractory chronic skin diseases

Next, we sought to comprehensively examine the role of CMV and EBV reactivation in patients with chronic skin diseases who show persistence or progression of skin lesions despite intensive immunosuppressive/immunomodulating standard treatment.

3.2.1 Detection of virus-specific antibodies in serum

The seroprevalences of CMV- and EBV-specific antibodies among immunocompromised patients with therapy-refractory chronic skin diseases were determined **["Prospective Study**

Group, PG"]. Table A1 (see Appendix) gives a detailed overview of all patients' demographic data, clinical characteristics, and diagnostic results. The findings were compared to nonimmunocompromised control patients with chronic skin diseases **["Control Group for Prospective Study Group, CG-PG"]**.

The medical records research showed that relative and absolute lymphocyte counts did not differ significantly between CG-PG and PG (Figure 8, A+B). However, the frequency of oral thrush was significantly higher in PG than in CG-PG (Figure 8C).

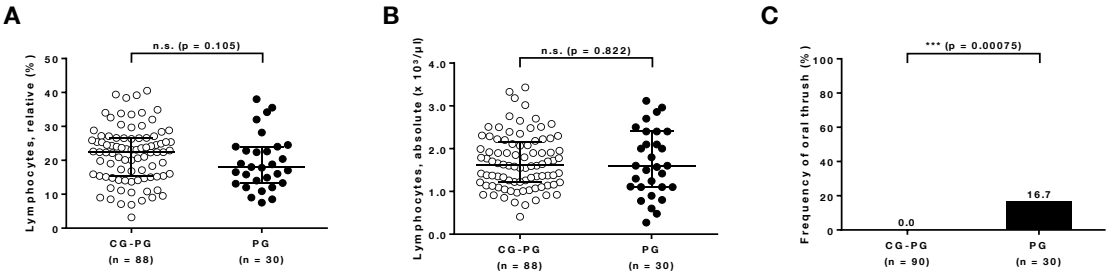


Figure 8: Lymphocyte counts and oral thrush [CG-PG vs. PG]

Detection of CMV (EBNA1) IgG indicated latent CMV (EBV) infection. Demonstration of CMV IgM (EBV-VCA IgM) in conjunction with CMV (EBNA1) IgG positivity pointed to reactivation of latent CMV (EBV) infection. In addition to the seroprevalences, the virus-specific antibody titers of the samples that had been tested positive were compared. Two samples from PG had been analyzed by an external laboratory, which did not report antibody titers. Treatment with IVIg might potentially have impacted the serological results of 14 out of 30 patients (46.7 %) from PG.

3.2.1.1 Frequencies and titers of CMV-specific antibodies

In CG-PG, 56 out of 90 patients (62.2 %) were positive for CMV IgG as compared to 21 out of 30 patients (70.0 %) in PG (Figure 9A). The CMV IgG titers in CG-PG (n = 56) ranged from 1,080 to 89,568 with a median of 19,980 (IQR = 12,414 to 29,922). The median CMV IgG titer in PG (n = 19) was 26,405 with a minimum of 1,043 and a maximum of 127,178 (IQR = 16,832 to 34,383) (Figure 9C).

CMV IgM could be detected in 5 out of 90 patients (5.6 %) in CG-PG, with an additional number of 11 patients (12.2 %) showing borderline values. The median CMV IgM ratio (n = 5) was 4.2, with a minimum of 2.2 and a maximum of 5.4 (IQR = 2.4 to 4.9). In PG, CMV IgM could be found in 5 out of 30 patients (16.7 %), plus borderline values in 2 patients (6.7 %). The CMV IgM ratio (n = 4) ranged from 2.8 to 8.0 with a median of 4.5 (IQR = 2.8 to 7.5) (Figure 9, B+D). All individuals who showed a positive CMV IgM result also had detectable CMV IgG, suggesting reactivation of latent CMV infection in 5.6 % of controls (CG-PG) and 16.7 %of patients (PG).

The differences in frequencies and titers of CMV-specific antibodies between PG and CG-PG were not statistically significant.

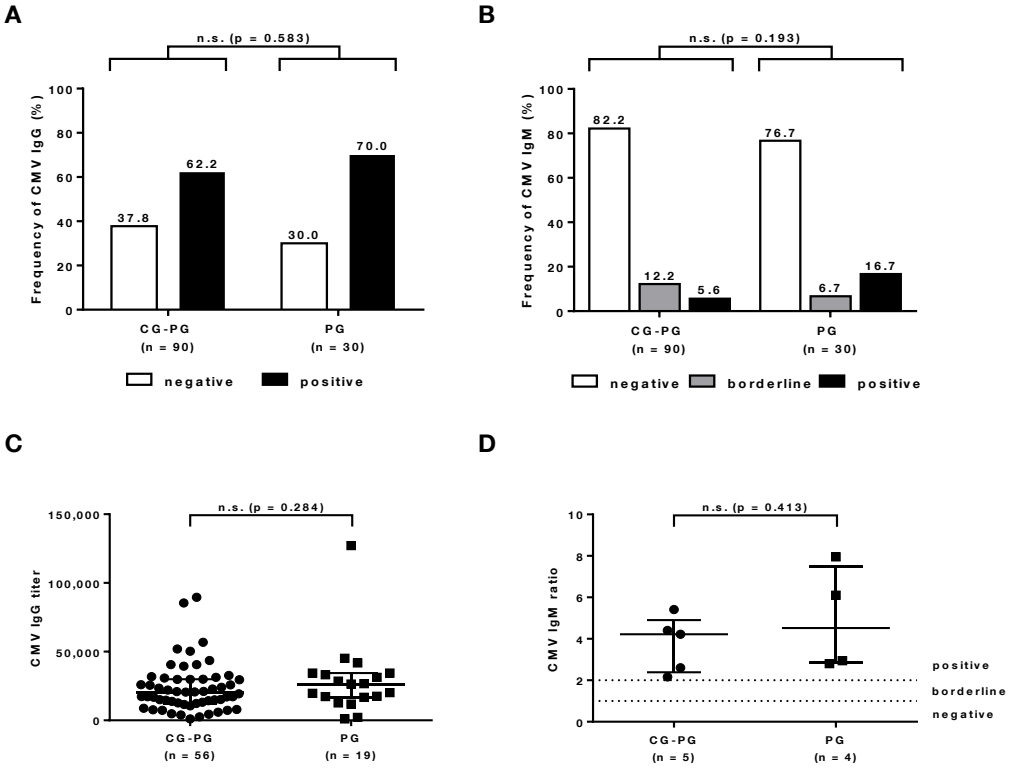


Figure 9: Frequencies and titers of CMV IgG and CMV IgM [CG-PG vs. PG]

3.2.1.2 Frequencies and titers of EBV-specific antibodies

The seroprevalence of EBNA1 IgG in CG-PG was 87.8 % (79 out of 90 patients), with borderline results in an additional number of 2 patients (2.2 %). The median EBNA1 IgG titer (n = 79) ranged at 56.6 NU with a minimum of 13.6 NU and a maximum of 71.7 NU (IQR = 44.2 to 65.9 NU). In PG, 26 out of 30 patients (86.7 %) were positive for EBNA1 IgG with titers (n = 24) ranging from 11.9 NU to 99.0 NU and a median of 72.7 NU (IQR = 55.2 to 88.9 NU). The statistical analysis revealed that the titers of EBNA1 IgG were significantly increased in PG compared to CG-PG (Figure 10, A+C).

In contrast to the complete absence of EBV-VCA IgM in CG-PG (n = 90), EBV-VCA IgM could be detected in 2 out of 30 patients (6.7 %) in PG (with titers of 12.8 NU and 13.6 NU). The results were evaluated to be borderline in 2 out of 90 patients (2.2 %) and 1 out of 30 patients (3.3 %), respectively. The difference between PG and CG-PG regarding the qualitative EBV-VCA IgM results was statistically significant (Figure 10, B+D). All individuals who showed a positive EBV-VCA IgM result also had detectable EBNA1 IgG, hinting at reactivation of latent EBV infection in 0.0 % of controls (CG-PG) and 6.7 % of patients (PG).

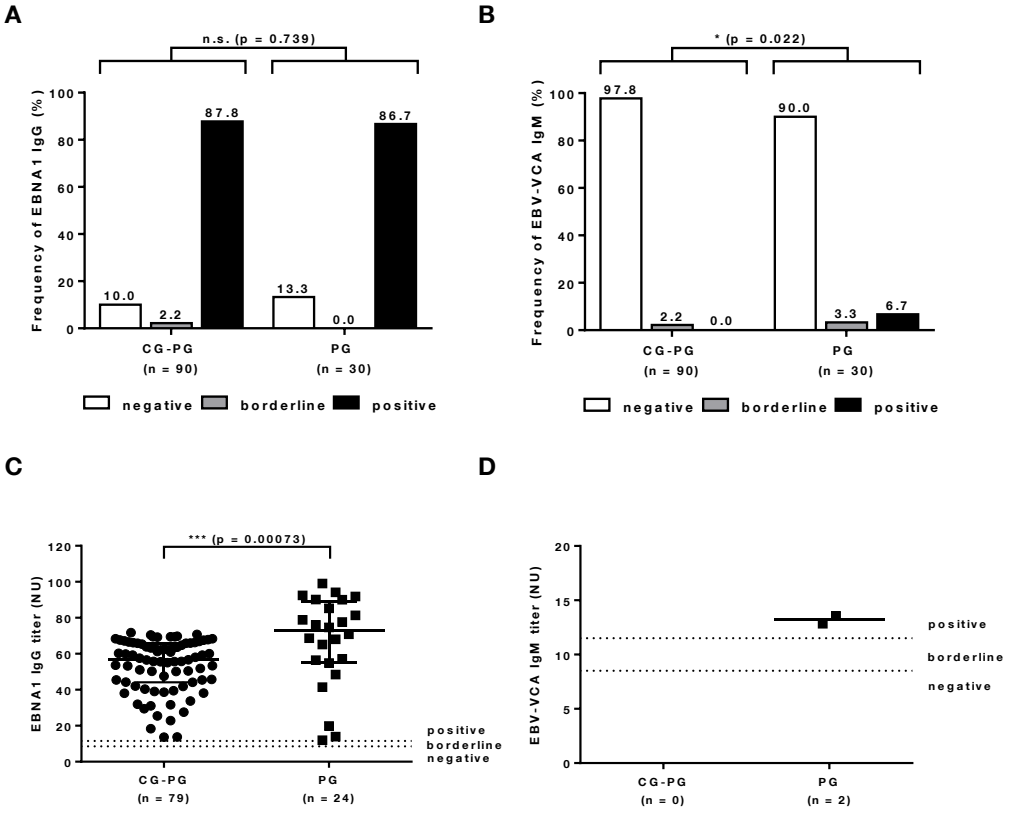


Figure 10: Frequencies and titers of EBNA1 IgG and EBV-VCA IgM [CG-PG vs. PG]

3.2.2 Presence of CMV- and EBV-specific DNA in blood

The peripheral blood of the patients from the **"Prospective Study Group, PG"** was subsequently analyzed by CMV- and EBV-specific PCR to corroborate the serological diagnosis of CMV and EBV reactivation. Indeed, the blood samples of 2 out of 30 patients (6.7 %) could be demonstrated to harbor CMV DNA. These patients had shown positive and borderline CMV IgM results, respectively. In addition, EBV-specific DNA could be detected in the blood samples of 3 out of 29 patients (10.3 %). Thereof, one patient had a borderline EBV-VCA IgM result, and two patients had tested negative for EBV-VCA IgM. The viral DNA loads are indicated in Table A1 (see Appendix).

3.2.3 Detection of virus-specific DNA, RNA and/or proteins in skin lesions

To shed light on the potential impact of CMV and EBV reactivation on skin pathology in immunocompromised patients with therapy-refractory chronic skin diseases, the presence of virus-specific DNA, RNA and/or proteins was investigated in lesional skin **["Prospective Study Group (PCR), PG(P)"] [= subgroup of PG]**. Table A2 (see Appendix) gives a detailed overview of all patients' demographic data, clinical characteristics, and diagnostic results. The findings were

compared to nonlesional skin of nonimmunocompromised control patients with chronic skin diseases **["Control Group for Prospective Study Group (PCR), CG-PG(P)"]**.

There was no statistically significant difference between CG-PG(P) and PG(P) in terms of relative and absolute lymphocyte counts (Figure 11, A+B). Though not significantly different from the control group, a high rate of oral thrush (21.4 %) could be identified in PG(P) (Figure 11C).

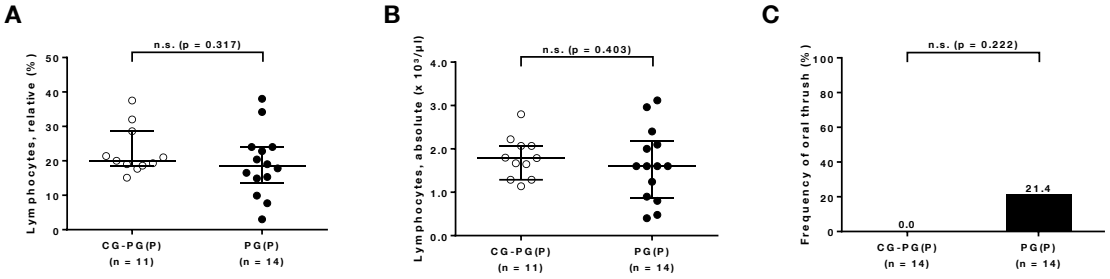


Figure 11: Lymphocyte counts and oral thrush [CG-PG(P) vs. PG(P)]

To isolate DNA, FFPE tissue and RNAlater preserved tissue (preferred option) had been used, depending on the availability of biomaterial (Table 34).

Table 34: Biomaterial used for DNA isolation [CG-PG(P) vs. PG(P)]

Group	**FFPE tissue** n (%)	**RNAlater preserved tissue** n (%)
CG-PG(P) (n = 14)	2 (14.3)	12 (85.7)
PG(P) (n = 14)	4 (28.6)	10 (71.4)

3.2.3.1 Demonstration of CMV DNA and CMV proteins in lesional skin

The detection of CMV DNA by real-time PCR was not possible in nonlesional skin samples from CG-PG(P) (n = 14). In contrast, the presence of CMV DNA could be demonstrated in lesional skin specimens of 3 out of 14 patients (21.4 %) from PG(P). However, the difference between PG(P) and CG-PG(P) was not statistically significant (Figure 12A). The CMV DNA loads (n = 2) ranged at 11.95 mIU/cell and 58.43 mIU/cell, respectively (Figure 12B). The third positive sample had been analyzed by an external laboratory, which did not provide data regarding the CMV DNA load.

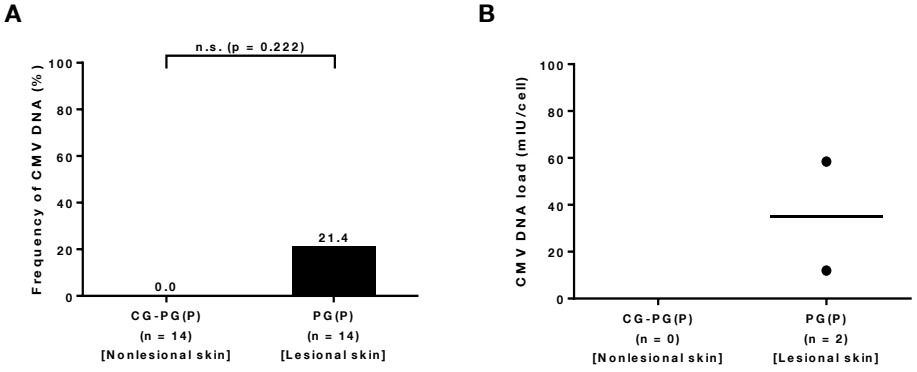


Figure 12: Detection of CMV-specific DNA in skin biopsies [CG-PG(P) vs. PG(P)]

To study the expression of CMV proteins in the tissue, CMV IHC was carried out. As described above, CMV-specific DNA had been detected in lesional skin biopsies of 3 out of 14 patients from PG(P), including one FFPE and two RNAlater preserved tissue samples. The FFPE sample had been investigated for the presence of CMV DNA by an external laboratory and had not been returned. Thus, it was not available for CMV IHC. In the case of the RNAlater preserved tissue specimens, the corresponding FFPE samples (representing a fraction from the same skin biopsy or another lesional skin biopsy obtained on the same day) were examined. In patient PG3, who had been diagnosed with pyoderma gangrenosum, the immunohistochemical analysis for CMV immediate early antigen and early antigen showed a weak positive nuclear signal (Figure 13). In patient PG5, who suffered from bullous pemphigoid, the immunohistochemical staining for CMV antigens did not label any of the respective tissue sections.

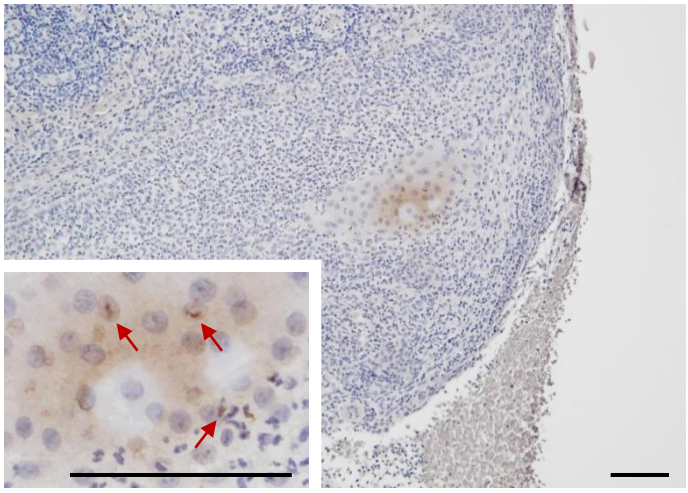


Figure 13: Immunohistochemical staining for CMV [PG(P)].
Patient PG3 with pyoderma gangrenosum.
Scale bars = 100 µm (overview and inset).
Figure modified from Speth et al. (2022).

3.2.3.2 Demonstration of EBV DNA, EBER, and EBV EA-D in lesional skin

EBV DNA could not be detected by real-time PCR in nonlesional skin of patients from CG-PG(P) (n = 14), whereas 4 out of 13 patients (30.8 %) from PG(P) harbored EBV-specific DNA in lesional skin (Figure 14A). Here, the median EBV DNA load (n = 4) was 0.33 mIU/cell with a range from 0.02 mIU/cell to 6.74 mIU/cell (IQR = 0.05 to 5.19 mIU/cell) (Figure 14B). The statistical analysis showed that the presence of EBV DNA was significantly more frequent in lesional skin from PG(P) than in nonlesional skin from CG-PG(P).

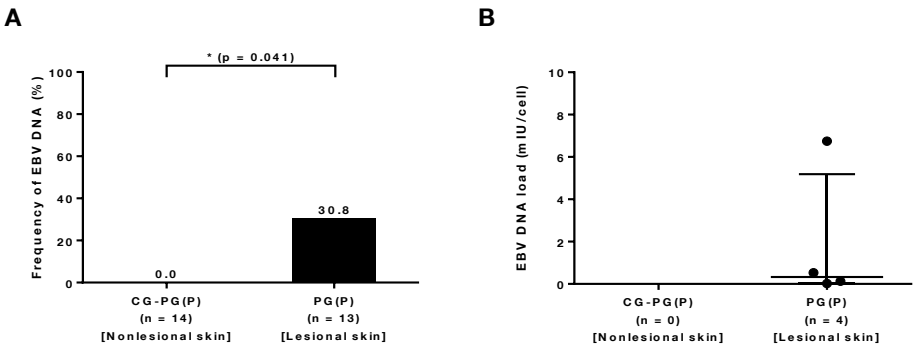


Figure 14: Detection of EBV-specific DNA in skin biopsies [CG-PG(P) vs. PG(P)]

Finally, EBER ISH and EBV IHC were performed to gain further insights into EBV gene expression in the tissue. In PG(P), as mentioned above, the presence of EBV DNA had been demonstrated in 4 out of 13 lesional skin biopsies (all of them RNAlater preserved samples):
patient PG5 with bullous pemphigoid, patient PG8 with SLE, patient PG11 with pyoderma gangrenosum, and patient PG12 with psoriasis vulgaris (and arthropathica).
The corresponding FFPE specimens (representing a fraction from the same skin biopsy or another lesional skin biopsy obtained on the same day) were analyzed by EBER ISH and EBV IHC. The FFPE sample of one patient had been obtained some days prior to the RNAlater preserved specimen. To sum up, EBER ISH did not label any of the respective tissue sections. In contrast, three of the four patients harboring EBV DNA in lesional skin (PG5, PG8, and PG12, but not PG11) also showed positivity for EBV EA-D-p52/50 on protein level as detected by IHC (Figure 15, A–C).

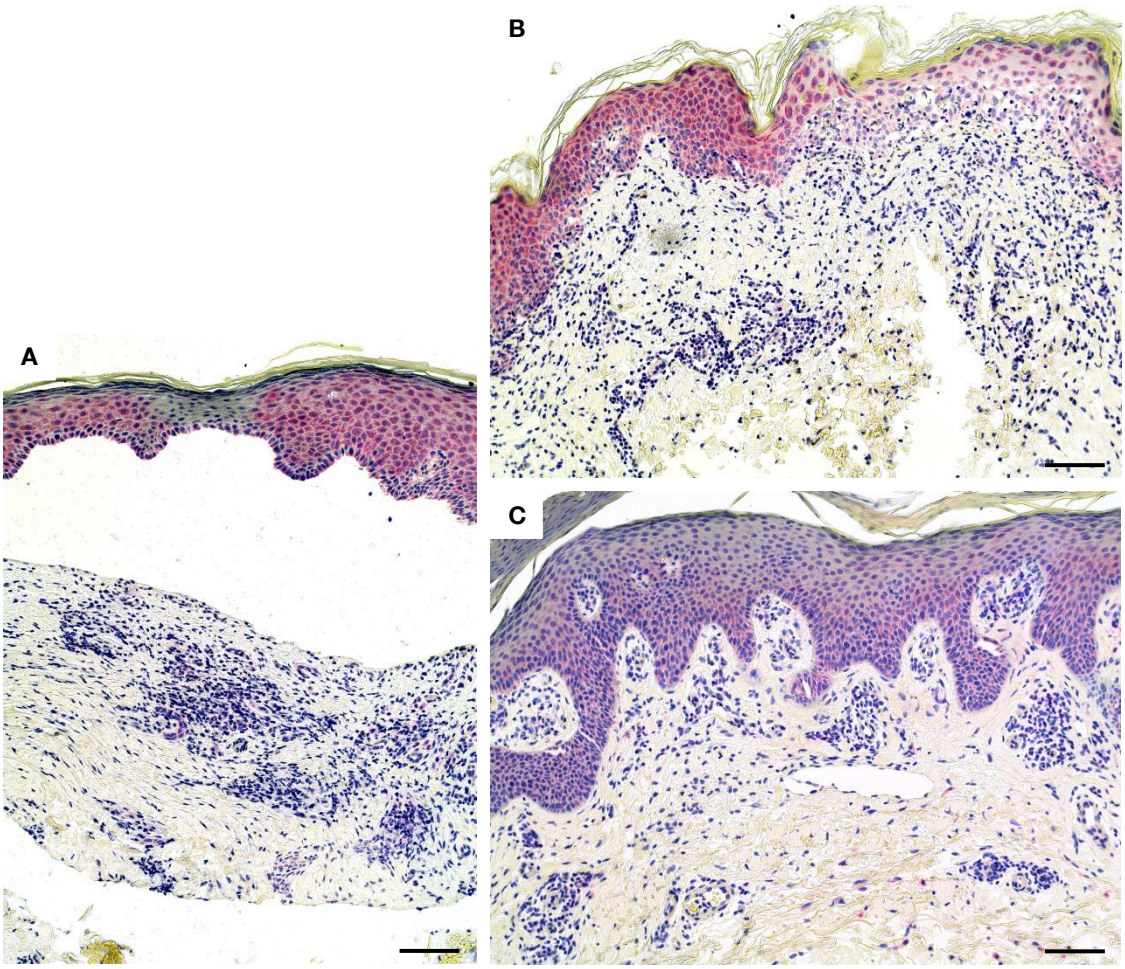


Figure 15: Immunohistochemical staining for EBV [PG(P)].
(A) Patient PG5 with bullous pemphigoid.
(B) Patient PG8 with SLE.
(C) Patient PG12 with psoriasis vulgaris (and arthropathica).
Scale bars (in A, B, and C) = 50 µm.

3.2.3.3 No detection of HSV DNA and VZV DNA in lesional skin

In addition, HSV- and VZV-specific PCR was carried out to disclose potential involvement of these herpesviruses, which might be reactivated simultaneously with CMV and/or EBV. However, as far as examined, neither HSV-1/2 DNA nor VZV DNA could be detected in the (non)lesional skin specimens from CG-PG(P) (n = 12) and PG(P) (n = 13), respectively.

3.2.4 Presence of CMV- and EBV-specific T cells in skin lesions

To elucidate whether CMV and EBV antigens in the skin are able to trigger immune responses and thus aggravate ongoing cutaneous inflammation, T cells had been isolated from lesional skin biopsies of two patients from the **"Prospective Study Group, PG"**:
patient PG4 with psoriasis inversa and capitis (and arthropathica) and patient PG8 with SLE.
To complement their serological profiles, EBV-VCA IgG was determined at the Institute of Virology of the Technical University of Munich and the Helmholtz Center Munich.

In brief, the production of CMV- and EBV-specific IgG could be demonstrated in both patients. In addition, patient PG4 had detectable CMV IgM hinting at CMV reactivation, while patient PG8 showed a borderline CMV IgM result. Patient PG8 harbored EBV DNA in lesional skin and showed positivity for EBV EA-D-p52/50, suggesting EBV reactivation (Figure 15B) (Figure 16A).

3.2.4.1 Frequency and phenotype

First, the frequency and phenotype of skin-derived T cells that had proliferated upon presentation of hCMV cell lysate antigen and recombinant EBV proteins (BZLF1+EBNA3A) by autologous human MODCs were investigated (Figure 16, B+C).

The *in vitro* proliferation rates of lesional T cells that had been co-cultured with unstimulated MODCs ranged at 0.15 % (patient PG4) and 0.31 % (patient PG8), respectively. These results could be used as a reference for the *in vitro* proliferation rates of lesional T cells upon presentation of tetanus toxoid and viral antigens by autologous MODCs.
The co-culture with MODCs that had been activated with tetanus toxoid (used as presentation control) prompted 3.23 % of lesional T cells from patient PG4 [21.5-fold proliferation rate] and 1.61 % of lesional T cells from patient PG8 [5.2-fold proliferation rate] to proliferate.

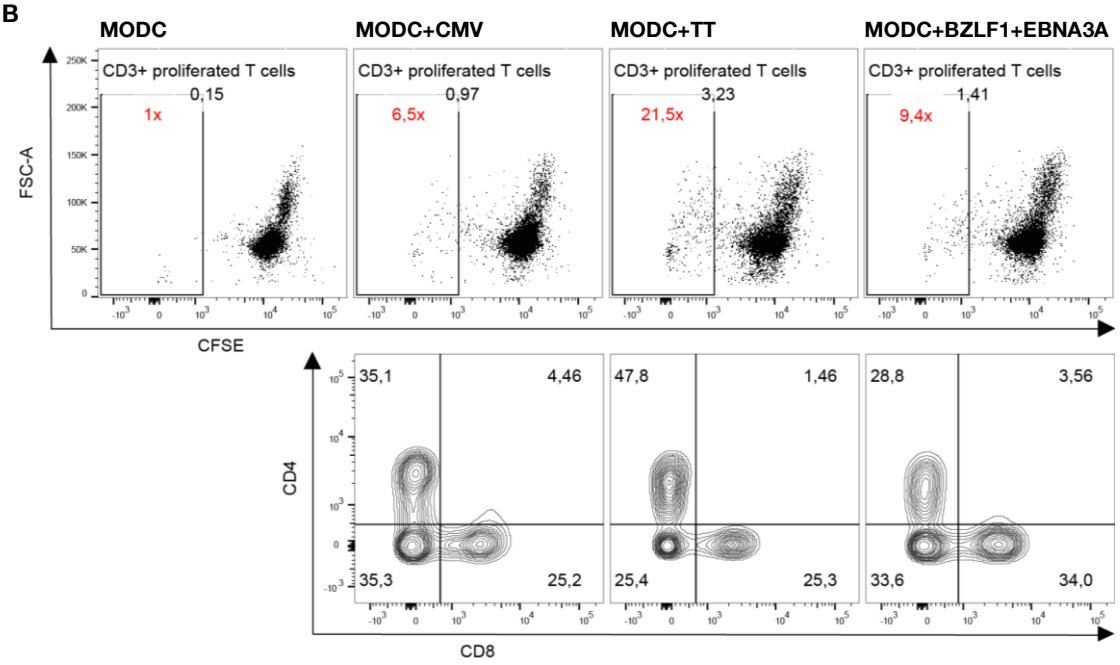
In the case of patient PG4, 0.97 % of lesional T cells proliferated [6.5-fold proliferation rate] upon presentation of hCMV cell lysate antigen by autologous MODCs. Following co-culture with MODCs that had been stimulated with recombinant EBV proteins (BZLF1+EBNA3A), 1.41 % of lesional T cells proliferated [9.4-fold proliferation rate].
In the case of patient PG8, 4.34 % of lesional T cells proliferated [14-fold proliferation rate] upon presentation of hCMV cell lysate antigen by autologous MODCs. Following co-culture with MODCs that had been stimulated with recombinant EBV proteins (BZLF1+EBNA3A), 1.05 % of lesional T cells proliferated [3.4-fold proliferation rate].
To summarize, the flow cytometric analysis revealed the presence of a distinct population of CMV- and EBV-specific T cells in lesional skin of both patient PG4 and patient PG8.

The frequency of CD4-positive T cells within the fraction of proliferating CD3-positive T cells was higher as compared to CD8-positive T cells, exclusive of T cell proliferation upon co-culture with MODCs stimulated with recombinant EBV proteins (BZLF1+EBNA3A) in patient PG4.
The proportion of CD4-/CD8-negative T cells within the fraction of proliferating CD3-positive T cells ranged from 25.4 % to 35.3 % (patient PG4) and from 27.0 % to 30.9 % (patient PG8).
The frequency of CD4-/CD8-positive T cells within the fraction of proliferating CD3-positive T cells ranged from 1.46 % to 4.46 % (patient PG4) and from 6.78 % to 7.51 % (patient PG8).

A

	PG4	PG8		PG4	PG8
CMV IgG	+	+	**EBNA1 IgG/EBV-VCA IgG**	+/+	-/+
CMV IgM	+	±	**EBV-VCA IgM**	-	-
CMV PCR [EDTA blood]	-	-	**EBV PCR [EDTA blood]**	- (*)	-
CMV PCR [lesional skin]	-	-	**EBV PCR [lesional skin]**	-	+

B



C

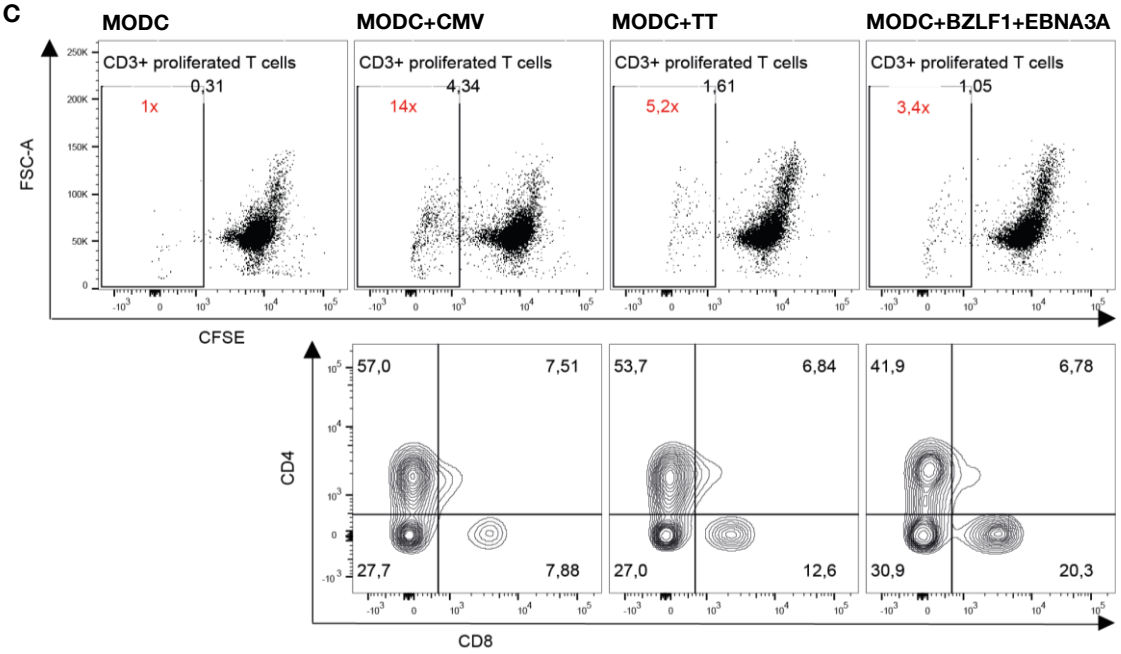


Figure 16: Flow cytometric analysis of lesional T cells (l) that had been co-cultured with unstimulated MODCs (MODC),
MODCs activated with hCMV cell lysate antigen (MODC+CMV),
MODCs activated with tetanus toxoid (MODC+TT) and
MODCs activated with recombinant EBV proteins (MODC+BZLF1+EBNA3A), respectively.
(Figure legend continues on the next page.)

(Figure legend continued from the previous page.)
(A) Overview of diagnostic results of patients PG4 and PG8:
*+ = positive; ± = borderline; - = negative; * = PCR analysis of serum (instead of EDTA blood).*
(B) Flow cytometry of patient PG4 with psoriasis inversa and capitis (and arthropathica).
(C) Flow cytometry of patient PG8 with SLE.
FSC-A = forward scatter area.

In the case of patient PG4, an additional stimulation experiment with BZLF1 (a lytic infection-related immediate early antigen) and EBNA3A (a latent infection-associated nuclear antigen) was carried out (Figure 17). It could be demonstrated that not only stimulation with BZLF1 in combination with EBNA3A (125 %) but also stimulation with BZLF1 alone (117 %) resulted in higher *in vitro* proliferation rates of lesional T cells as compared to the control (100 %). This finding corroborated the presence of EBV-specific T cells in lesional skin. It is worth mentioning that the addition of IL-2 to the co-culture at the beginning of this experiment also prompted T cells that are not EBV-specific to proliferate. For this reason, the frequency of proliferating T cells could be observed to be markedly elevated compared to the other experiment (Figure 16B). Accordingly, the relative increase of the *in vitro* proliferation rate upon presentation of recombinant EBV proteins (compared to unstimulated MODCs) was less pronounced. In comparison to the other experiment, the frequency of CD4-positive T cells within the fraction of proliferating CD3-positive T cells was profoundly elevated, whereas the proportions of both CD4-/CD8-negative T cells and CD4-/CD8-positive T cells were reduced.

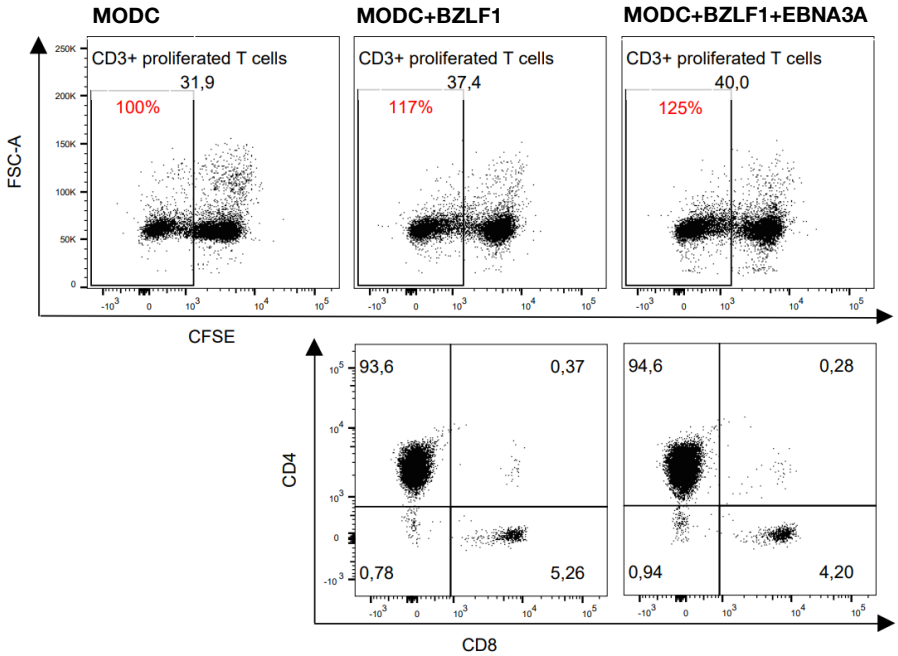
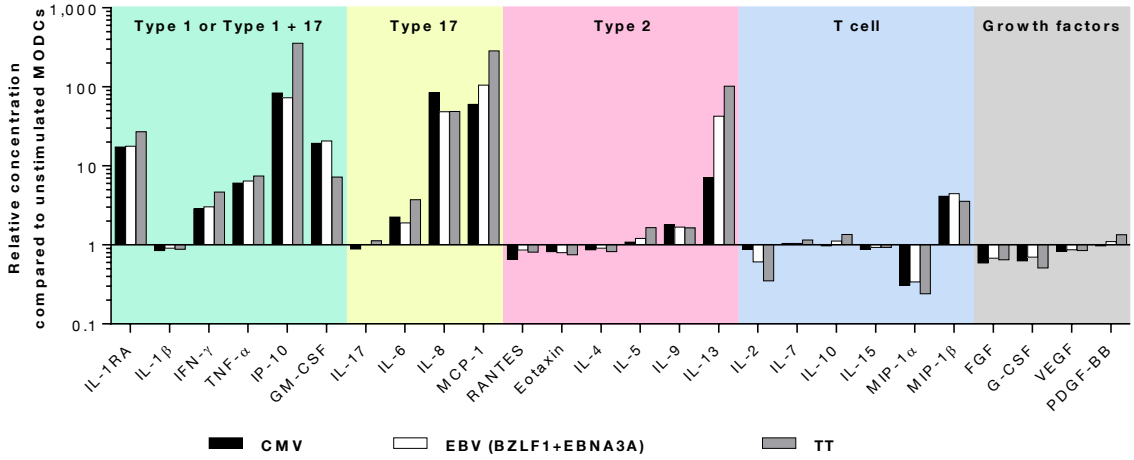


Figure 17: Flow cytometric analysis of lesional T cells (II) that had been co-cultured with unstimulated MODCs (MODC),
MODCs activated with BZLF1 (MODC+BZLF1) and
MODCs activated with both BZLF1 and EBNA3A (MODC+BZLF1+EBNA3A), respectively.
Patient PG4 with psoriasis inversa and capitis (and arthropathica).
FSC-A = forward scatter area.

3.2.4.2 Cytokine profile

To investigate the functional role of CMV- and EBV-specific T cells in lesional skin, the levels of cytokines, chemokines, and growth factors in cell-free supernatants derived from the co-culture of lesional T cells and activated MODCs were analyzed. MODCs had been stimulated with hCMV cell lysate antigen and recombinant EBV proteins (BZLF1+EBNA3A), respectively. Though the proportion of lesional T cells that had proliferated *in vitro* upon presentation of CMV and EBV antigens was small (range from 0.97 % to 4.34 %), the cytokine levels could be observed to be markedly increased as compared to the supernatant from T cells that had been co-cultured with unstimulated MODCs. In patient PG4 (Figure 18A), the levels of cytokines related to type 1 and type 17 immunity were profoundly elevated, including TNF-α (CMV = x 6.1; EBV = x 6.4), IP-10 (CMV = x 83.4; EBV = x 72.6), IL-8 (CMV = x 84.2; EBV = x 48.3), and MCP-1 (CMV = x 59.9; EBV = x 105.0). In contrast, the cytokine concentrations were relatively evenly increased in patient PG8 (Figure 18B). Here, the highest levels were measured for GM-CSF (CMV = x 19.4; EBV = x 25.8) and IL-13 (CMV = x 9.1; EBV = x 17.7). The included TT control revealed that these cytokine profiles were not specific to CMV or EBV.

A



B

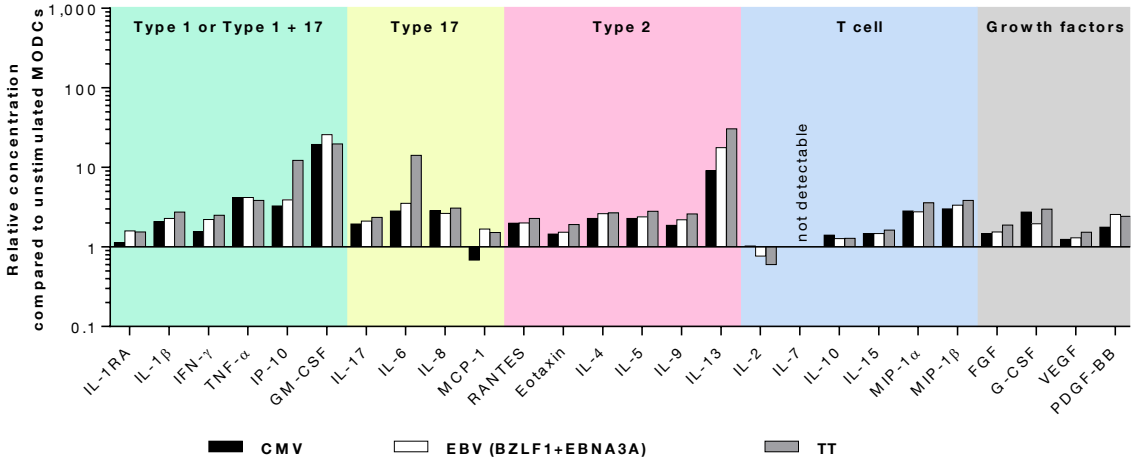


Figure 18: Levels of cytokines in supernatants derived from T cell-MODC co-culture.
MODCs had been activated with hCMV cell lysate antigen (CMV), recombinant EBV proteins (BZLF1+EBNA3A), and tetanus toxoid (TT), respectively.
The results are indicated as relative concentrations compared to unstimulated MODCs.
IL-12 (p70) was not detectable and is therefore not shown.
(A) Patient PG4 with psoriasis inversa and capitis (and arthropathica).
(B) Patient PG8 with SLE.
FGF = fibroblast growth factor; G-CSF = granulocyte colony-stimulating factor; IL-1RA = interleukin-1 receptor antagonist; MIP-1α = macrophage inflammatory protein-1α; MIP-1β = macrophage inflammatory protein-1β; PDGF-BB = platelet-derived growth factor-BB; VEGF = vascular endothelial growth factor.

3.2.5 Investigation of the effects of antiviral treatment of CMV reactivation

The results presented in the previous sections substantiate that reactivation of latent CMV infection may play a clinically relevant and aggravating role for the underlying skin condition in immunocompromised patients with treatment-refractory chronic skin diseases. To elucidate whether patients would benefit from antiviral therapeutic intervention, treatment with (val)ganciclovir was initiated in 4 out of 30 patients from the **"Prospective Study Group, PG"**.

In all these patients (PG1–PG4), the serological profile and/or the results of nucleic acid testing had been interpreted as suggestive of CMV reactivation. HE stainings (as far as examined) had not been suspected of CMV disease during routine histopathology. Indeed, temporary improvement of the skin condition could be observed in one patient (PG1), while three patients (PG2, PG3, and PG4) showed permanent improvement upon antiviral therapy. In the following chapters, the clinical course, diagnostic results, and treatment strategies will be summarized briefly (patients PG1 and PG2) and described in more detail (patients PG3 and PG4), respectively.

3.2.5.1 Cases 1+2: drug-induced exanthema, pemphigus vulgaris

The first case (PG1), a 66-year-old female patient, had developed a drug-induced exanthema during triple therapy with telaprevir, ribavirin, and pegylated interferon alfa-2a for chronic hepatitis C. Though treated with oral glucocorticoids, the lesions worsened and eventually displayed as deep ulcers. Finally, the virological examination showed positive serology for CMV IgG and CMV IgM. CMV DNA (and a small amount of EBV DNA) could be detected in peripheral blood. In addition, the lesional skin biopsy was found to harbor CMV DNA. Antiviral treatment with ganciclovir was initiated, leading to profound improvement of skin lesions.

The second case (PG2), a 45-year-old male patient, was admitted to our hospital for intensified therapy of pemphigus vulgaris (mucocutaneous type). Though the treatment regimen was aggressive, including oral glucocorticoids, azathioprine, and rituximab, lesions did not improve. Eventually, the diagnosis of CMV reactivation could be made based on the detection of CMV DNA in peripheral blood. Serology yielded a borderline result for CMV IgM, whereas CMV IgG was highly positive. Then, antiviral therapy was started, and the skin condition improved quickly.

3.2.5.2 Case 3: pyoderma gangrenosum

The third patient (PG3) was a 61-year-old man who suffered from fast-progressing, treatment-refractory pyoderma gangrenosum proximal to the right medial malleolus. In the following section, the clinical course, diagnostic results, and treatment strategies will be summarized (Table 35) and illustrated with photographs (Figure 19).

Table 35: Clinical course, diagnostic results, and treatment strategies (PG3)

Date	Summary
Day 0 – Day 4	The lesion had appeared some weeks ago and enlarged despite topical wound care and oral prednisolone (200 mg/day) in the last 2 weeks. It was extremely painful (NRS 10/10). The patient showed profound lymphocytopenia (Figure 20, A+B). In contrast, oral thrush could not be detected. The patient was put on intravenous prednisolone (150 mg/day, then tapered off; continued orally after discharge: 80 mg, 60 mg, and 40 mg for 4 days each), infliximab (400 mg), and topical glucocorticoids.
Day 16	The pyoderma had increased in size. Treatment with infliximab (400 mg) was carried out. Tapering of oral prednisolone was continued (30 mg for 8 days, 20 mg for 8 days, then 10 mg).
Day 30	Until then, the ulcer had rapidly enlarged in size and depth. The Achilles tendon was almost uncovered. Given the progression of the skin condition and inflammation, oral cyclosporine (200 mg/day) was introduced. The patient received infliximab (400 mg), and oral prednisolone was continued at 20 mg/day.
Day 36 – Day 59	The patient presented with an uncovered Achilles tendon and massive, therapy-refractory pain (NRS 10/10). He was treated with infliximab (400 mg) on the day of admission. The strong inflammatory activity prompted additional treatment with IVIg (160 g over 6 days, 1 cycle). Though the immunosuppressive therapy had been intensified, the pyoderma increased in size. Fulfilling the criteria of immunosuppression and treatment refractoriness, the patient was tested for CMV and EBV reactivation for the first time on day 41 (i.e., approximately 2 months after the initiation of systemic immunosuppression). Indeed, he showed the serological pattern of CMV reactivation (CMV IgG positive, CMV IgM positive) (Figure 21, A+B). The EBV serostatus was unremarkable. The detection of CMV or EBV DNA in peripheral blood was not possible. However, the lesional skin biopsy (obtained on day 0) was found to harbor CMV DNA. In contrast, EBV DNA (and also HSV-1/2 and VZV DNA) could not be detected. Moreover, CMV IHC showed a weak positive nuclear signal. Therefore, intravenous ganciclovir (800 mg/day for 14 days) was initiated on day 44. In addition, infliximab (500 mg) was administered on day 44 and day 58. In view of the relatively low cyclosporine dose and the concomitant treatment with other immunosuppressants, oral cyclosporine was tapered off. Instead, topical cyclosporine was used. Following antiviral therapy, an improvement of both health status and skin disease, as well as progressive pain relief, could be observed. Finally, the patient was discharged with oral prednisolone (15 mg/day) and topical glucocorticoids.
Day 71	The skin condition had stabilized. The formation of granulation tissue could be seen at the upper pole of the ulcer, and the Achilles tendon was only slightly exposed. The patient received infliximab (500 mg), while oral prednisolone (15 mg/day) and topical glucocorticoids were continued. Topical tacrolimus was added.
Day 85 + Day 99 + Day 113 + Day 141 + Day 171	Until day 85, the skin condition had massively improved. There were numerous islands of granulation tissue. The Achilles tendon was not exposed anymore. In line with this, the CMV IgM ratio had started declining (Figure 21B), and CMV DNA could no longer be detected in lesional tissue obtained through curettage. In the following time, the pyoderma continued to decrease in size and depth. The patient did not suffer from pain anymore. Infliximab therapy (initially 500 mg/2 weeks) was continued: the dose was reduced to 400 mg, and the interval was prolonged to 4 weeks. The dose of oral prednisolone could also be reduced step by step. Topical treatment was switched from tacrolimus to cyclosporine. Topical glucocorticoids could be discontinued temporarily.
…	In the following year, the skin condition steadily improved. The infliximab interval was extended to 8 weeks. Treatment with oral prednisolone (and later also with topical glucocorticoids) could be discontinued.
Day 577 + Day 647	Finally, the infliximab interval was prolonged to 10 weeks. On day 647 (i.e., 20 months after the start of antiviral therapy), CMV IgM could not be detected anymore (Figure 21B). The superficial ulcer was well controlled, and the patient was still free of pain.

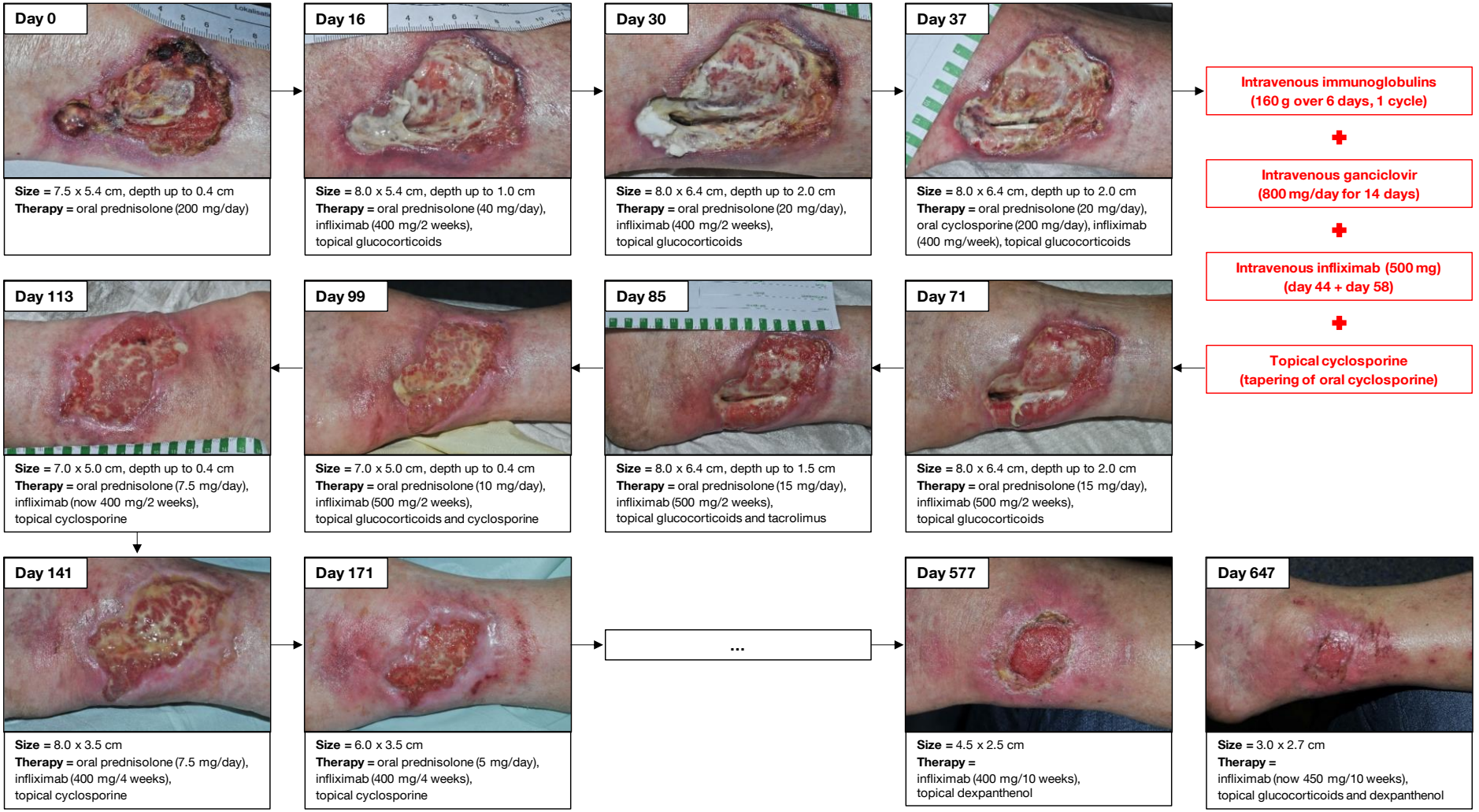


Figure 19: Clinical course, management, and outcome (PG3)

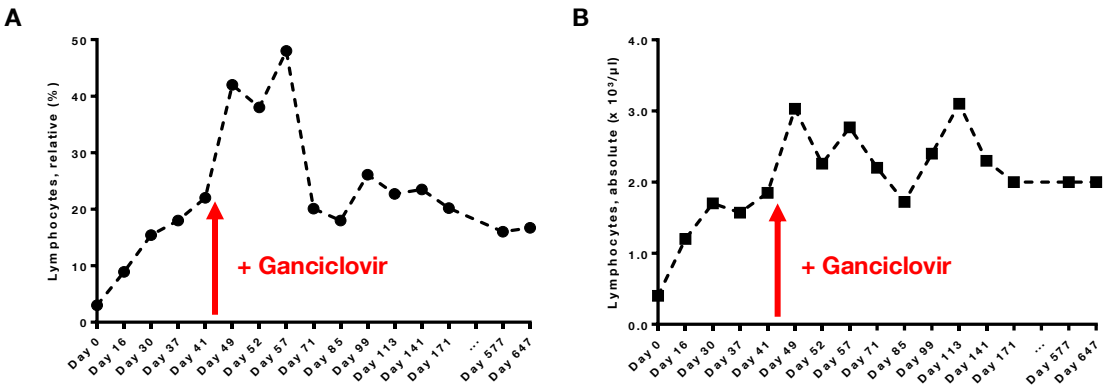


Figure 20: Lymphocyte counts (PG3)

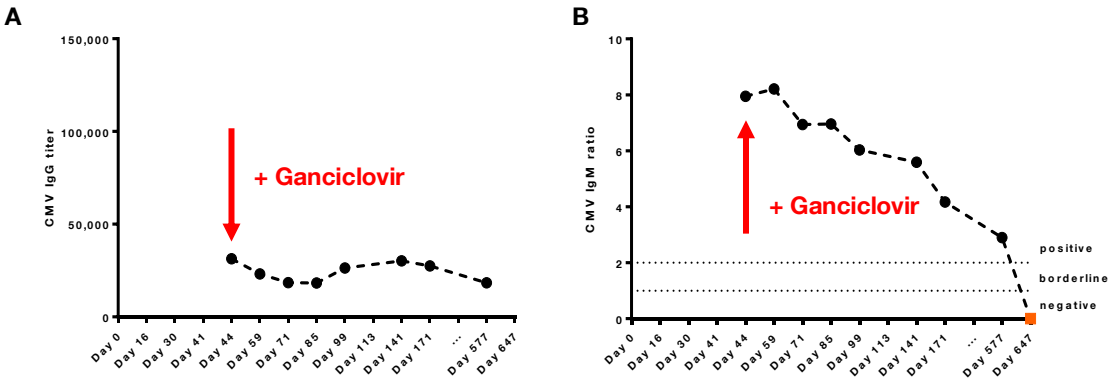


Figure 21: Development of CMV-specific antibody responses (PG3)

3.2.5.3 Case 4: psoriasis inversa and capitis

The fourth patient (PG4) was a 61-year-old woman with an 8-year history of psoriasis inversa and capitis (and arthropathica). The disease was difficult to control, and different systemic therapies had been discontinued over the years because of adverse effects or secondary loss of efficacy. Finally, treatment with ixekizumab had been started (maintenance dose of 80 mg/4 weeks). In the following time, the disease was well controlled, and the Psoriasis Area and Severity Index (PASI) constantly ranged at low levels (2–4). However, the patient presented with an exacerbation of the skin condition 7 months later. In the following section, the clinical course, diagnostic results, and treatment strategies will be delineated (Table 36) and illustrated with photographs (Figure 22).

Table 36: Clinical course, diagnostic results, and treatment strategies (PG4)

Date	Summary
Day 0	The skin lesions had strongly deteriorated 5 weeks ago without identifiable reason (PASI 8.3), including massive burning (NRS 10/10) and itching (NRS 10/10). The involved areas had markedly enlarged with particular affection of axillae, inframammary folds, décolleté, and groins. Treatment with ixekizumab and topical glucocorticoids was continued.
Day 1 – Day 8	The patient received intensified inpatient treatment, including UVB phototherapy (311 nm) and topical glucocorticoids. The relative and absolute lymphocyte counts ranged at high-normal levels and remained relatively stable during the follow-up examinations (Figure 23, A+B). However, there was clinical evidence of oral thrush, which could be confirmed mycologically. Thus, lozenges with amphotericin B were administered. In view of treatment refractoriness and the patient's immunocompromised condition, her CMV and EBV status was assessed. Indeed, CMV IgG and CMV IgM detection was possible in serum (Figure 24, A+B). The EBV serostatus was not remarkable. CMV and EBV DNA could not be demonstrated in peripheral blood. Similarly, CMV and EBV DNA (and also HSV-1/2 and VZV DNA) could not be found in lesional skin.
Day 30	The skin condition had considerably worsened. There was still strong itching, and the oral thrush had persisted. Thus, oral fluconazole was started. Treatment with ixekizumab and topical glucocorticoids was continued. In addition, oral valganciclovir was initiated off-label on day 32 because the skin disease had been refractory to intensive immunomodulating standard treatment during a period of at least 8 weeks and because the patient had shown the serological pattern of CMV reactivation: 1,800 mg/day for 21 days, then 900 mg/day for 7 days.
Day 37	Until then, the skin condition had noticeably improved. Itching had decreased. However, the oral thrush persisted. Therefore, fluconazole treatment was continued (until day 49).
Day 56	The skin lesions were almost entirely under remission less than 4 weeks after initiating antiviral therapy. Given the persisting oral thrush, it was decided to abstain from the scheduled ixekizumab injection.
Day 79	Following treatment with valganciclovir, the CMV IgM ratio had dropped, and the test result was assessed to be borderline (Figure 24B). The oral thrush had disappeared. Therefore, amphotericin B could be tapered off. The skin status was well controlled. Ixekizumab was discontinued, and treatment was switched to guselkumab.
Day 107	The skin condition was still very well controlled (PASI 1.7).

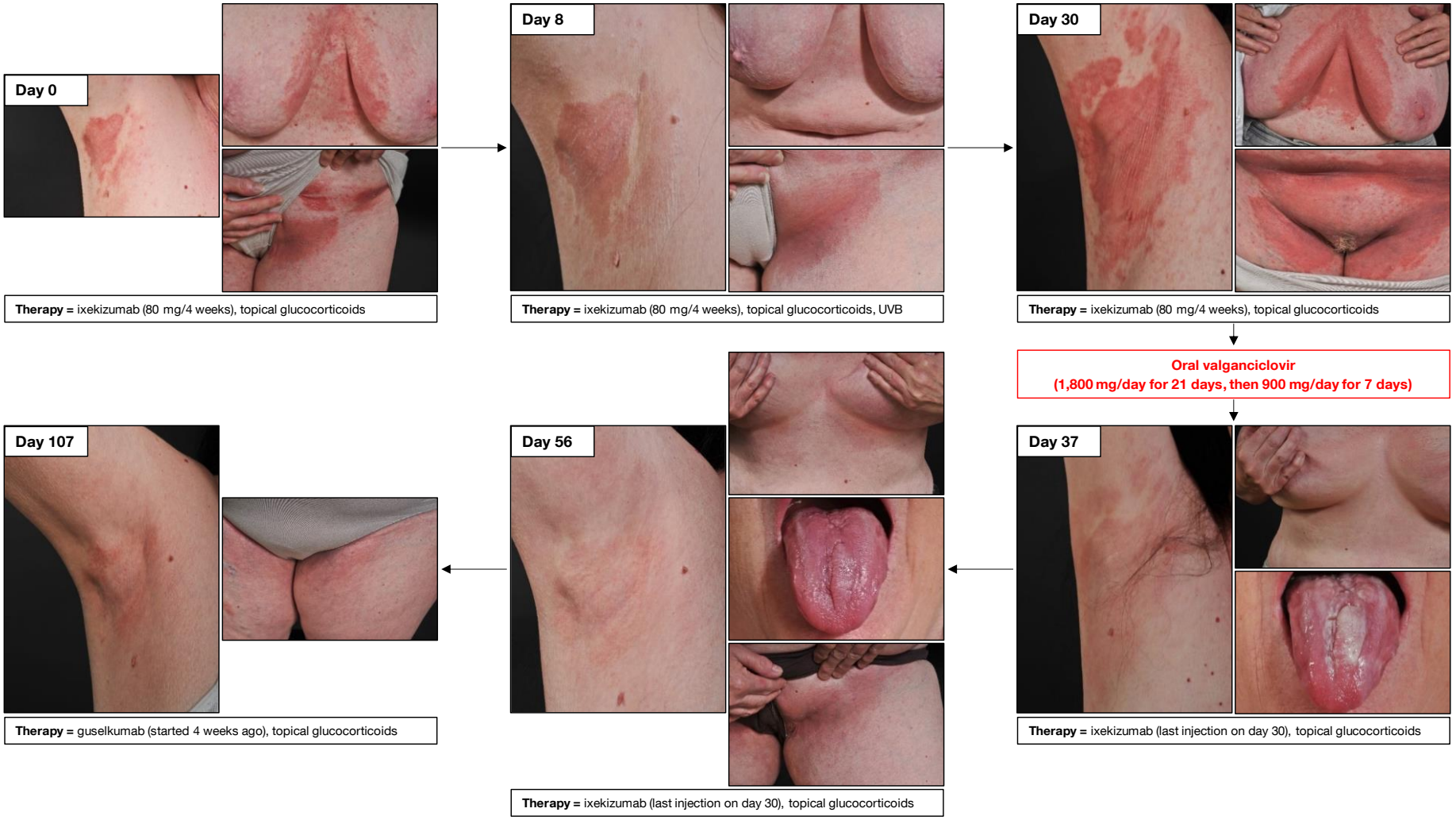


Figure 22: Clinical course, management, and outcome (PG4)

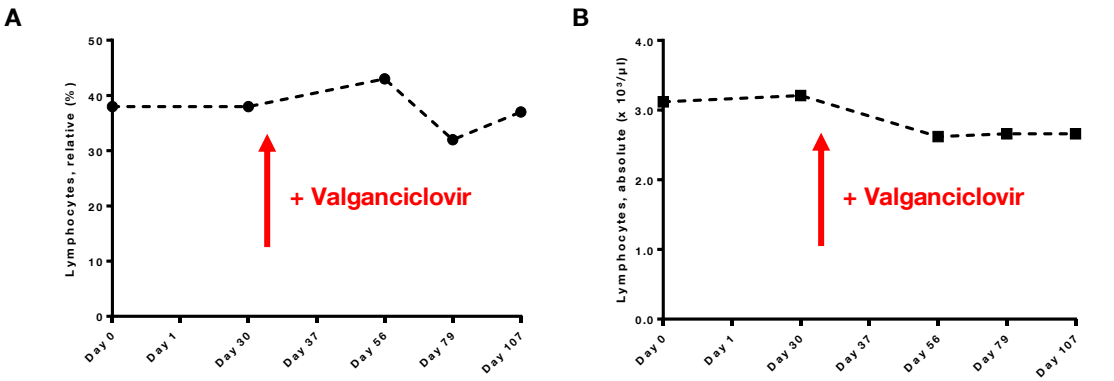


Figure 23: Lymphocyte counts (PG4)

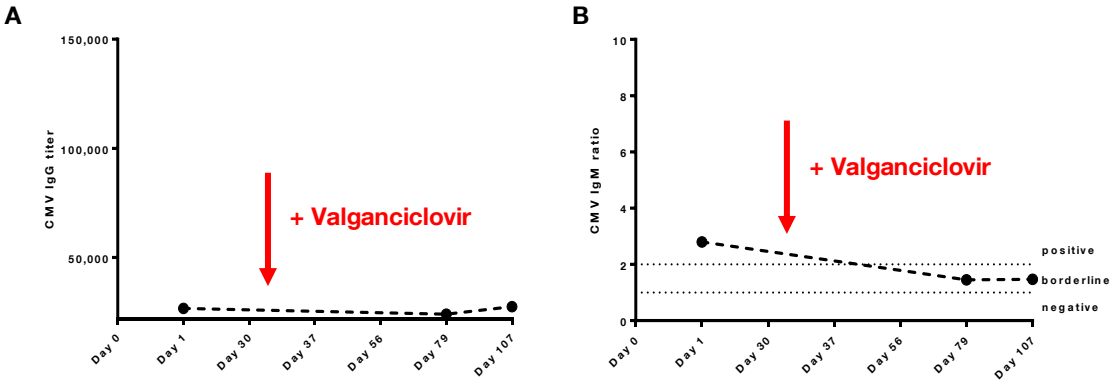


Figure 24: Development of CMV-specific antibody responses (PG4)

3.3 Estimation of the proportion of dermatological patients at risk for CMV reactivation

The reported cases highlight that antiviral treatment of CMV reactivation is warranted in patients with chronic skin diseases who show persistence or progression of skin lesions despite intensive immunosuppressive/immunomodulating standard treatment. To estimate the proportion of patients in whom reactivation of CMV infection might adversely affect the course of the skin disease, two representative inpatient wards at the Department of Dermatology and Allergology of the Technical University of Munich were retrospectively analyzed with regard to diagnoses, treatment regimens, and courses of diseases of the patients.

During the timespan of one month, a total number of 223 patients were admitted. For a minimum of 8 weeks, 29 out of these 223 patients (13.0 %) had been treated with immunosuppressants and/or biologicals that have been implicated with CMV reactivation. Thereof, 8 patients (27.6 %) showed prolonged exacerbation of the skin condition despite intensive immunosuppressive treatment. This means that 3.6 % of all patients did not respond adequately to current gold standard (immunosuppressive) therapy. These patients appear to be at risk for CMV reactivation and thus may benefit from early screening and antiviral treatment.

3.4 Evaluation of the benefit of screening for CMV reactivation and subsequent antiviral treatment

To evaluate the benefit of screening patients at risk for CMV reactivation and initiating antiviral treatment in the case of a positive screening test, the NNS was computed.

“Number needed to screen is defined as the number of people that need to be screened for a given duration to prevent one death or adverse event” (Rembold, 1998).

The following calculations rely on the assumption that reactivation of CMV infection aggravates the underlying skin condition and that antiviral treatment results in an improvement of the skin disease. The absolute risk reduction due to screening (and subsequent antiviral therapy) and the NNS were calculated for different definitions of a positive screening test for CMV reactivation (Table 37). The computation is based on the diagnostic results of the patients from the **“Prospective Study Group, PG”** and the **“Prospective Study Group (PCR), PG(P)”**, respectively. The response rate to antiviral therapeutic intervention was conservatively estimated to range at only 50 %. In fact, temporary or permanent improvement of the skin lesions could be observed in all presented cases upon therapy with (val)ganciclovir (see section 3.2.5).

Table 37: Number needed to screen.
ARR = absolute risk reduction.

Definition of a positive screening test for CMV reactivation	**Number of patients from PG/PG(P) fulfilling criteria**	**ARR**	**NNS**
CMV IgM positive	5 out of 30 patients (16.7 %)	8.3 %	12
CMV DNA in peripheral blood	2 out of 30 patients (6.7 %)	3.3 %	30
CMV DNA in lesional skin	3 out of 14 patients (21.4 %)	10.7 %	10
CMV IgM positive and/or CMV DNA in peripheral blood and/or CMV DNA in lesional skin	7 out of 30 patients (23.3 %)	11.7 %	9

The results illustrate that the NNS strongly depends on the definition of a positive screening test. To summarize, CMV-induced morbidity could be prevented in one patient by screening 9 to 30 patients at risk (i.e., patients showing persistence or progression of skin lesions despite intensive immunosuppressive/immunomodulating therapy) if the detection of CMV reactivation entails antiviral treatment.

4 Discussion

The interaction between microorganisms and autoimmune, inflammatory, and malignant skin diseases has been in the focus of research not only since modern sequencing technologies enabled extensive and rapid characterization of the human microbiome. The decrease in microbiome diversity in patients with atopic dermatitis, which correlates with increased colonization with staphylococcus aureus and disease severity, is one of the most researched examples (Paller et al., 2019). Similarly, the association between acute guttate psoriasis and streptococcal throat infection is well established (Leung et al., 1995).
In addition to the role of bacteria, the pathophysiological relevance of viral infections as potential triggers and aggravators of chronic skin conditions has been increasingly recognized. The viruses that have most commonly been related to chronic skin diseases include hepatitis C virus (lichen planus), HSV (pemphigus), and HIV (psoriasis). Their association with disease onset and exacerbation does not least result from the host antiviral immune response (and the production of proinflammatory cytokines) and the virus-induced immunological dysfunction, respectively. (Georgescu et al., 2019; Senger and Sinha, 2012; Ruocco et al., 2014; Morar et al., 2010)

4.1 Summary and interpretation of results

In addition, there is increasing evidence that the herpesviruses CMV and EBV may play a so far underestimated role as modulators of chronic skin conditions (Grimes et al., 1996; Asadullah et al., 1999; Döcke et al., 2003; Ballanger et al., 2009; Weitz et al., 2011; Larsen et al., 2011).

This study comprehensively investigated the role of CMV and EBV reactivation in immunocompromised patients suffering from (treatment-refractory) chronic skin diseases. To be enrolled in this study, patients had to be diagnosed with an autoimmune or inflammatory skin condition, chronic ulcer, or skin lymphoma. These disease categories had been selected because treatment of autoimmune/inflammatory skin diseases and (advanced) skin lymphoma typically involves immunosuppressive/immunomodulating agents and because ulcers are the most common CMV-associated cutaneous lesions (Drozd et al., 2019). In the following section, the key findings of this study will be summarized and interpreted.

4.1.1 Immunocompromised patients with chronic skin diseases: EBV but not CMV detected in lesional skin

First, the presence of CMV and EBV was screened in lesional skin of patients who suffered from chronic skin diseases and had been treated with biologicals, chemotherapy and/or immunosuppressants for at least 2 weeks prior to the acquisition of the skin biopsy ("Retrospective Study Group, RG"). The results were compared to nonlesional skin of age- and sex-matched control patients, who suffered from skin conditions within the same disease categories but had not been under systemic immunosuppressive/immunomodulating treatment for at least 6 months before the acquisition of the skin biopsy ("Control Group for Retrospective Study Group, CG-RG").

Interestingly, it could be shown that neither patients [RG] nor controls [CG-RG] harbored CMV DNA in lesional and nonlesional skin, respectively. In previous studies, the prevalence of CMV DNA has been investigated by PCR in different skin conditions. For example, Kirby et al. (2000) were not able to detect CMV DNA in both involved and uninvolved skin of patients with chronic plaque psoriasis. Similarly, CMV DNA could not be detected in lesional skin biopsies from patients with cutaneous drug reactions (Özcan et al., 2010) and in both lesional and normal skin biopsies from pemphigus patients (Tufano et al., 1999). This contrasts with the study of Oliveira-Batista et al. (2013), who found that 3 out of 23 patients (13.0 %) harbored CMV DNA in pemphigus vulgaris lesions. Likewise, Drago et al. (2005) detected CMV DNA in lesional skin of patients with bullous pemphigoid (3/22; 13.6 %). Skinner et al. (1995) demonstrated the presence of CMV DNA in a high proportion of lesional alopecia areata samples (9/10; 90.0 %). In contrast, in another study, CMV DNA could not be identified in skin biopsies from patients with the first episode of alopecia areata or disease recurrence (García-Hernández et al., 1998). Finally, Ohtsuka and Yamazaki (2006) demonstrated CMV DNA in scleroderma skin tissue (4/48; 8.3 %). However, the prevalence rate was not significantly different from skin samples of healthy control subjects (5/97; 5.1 %) (Ohtsuka and Yamazaki, 2006).

The presence of EBV DNA could be demonstrated in lesional skin of 14.0 % of the patients [RG]. In contrast, EBV DNA was not detectable in nonlesional skin samples of the controls [CG-RG]. The increased prevalence of EBV DNA (as compared to CMV DNA) in lesional skin of immunocompromised patients may at least in part be related to the higher seroprevalence of EBV in Germany (Abrahamyan et al., 2020; Lachmann et al., 2018). Similar to CMV, the presence of EBV DNA in different skin conditions has been the subject of previous PCR investigations. For example, Özcan et al. (2010) found that patients diagnosed with cutaneous drug reactions did not harbor EBV DNA in lesional skin samples. Similarly, EBV DNA could not be demonstrated in depigmented and/or uninvolved skin from patients with vitiligo, lesional skin from patients with different other skin diseases, and skin from healthy individuals (Grimes et al., 1996). In contrast, Nahidi et al. (2016) found EBV DNA in healthy skin around excised melanocytic nevi (15/31; 48.4 %). Oliveira-Batista et al. (2013) did not detect EBV DNA in mucosal and/or skin swabs

obtained from patients with pemphigus vulgaris, whereas Tufano et al. (1999) demonstrated EBV DNA in lesional skin (but not in normal skin) of a pemphigus patient (1/7; 14.3 %). Finally, Drago et al. (2005) detected EBV DNA in lesional skin of a patient with bullous pemphigoid (1/22; 4.5 %).

The tropism of EBV for lymphocytes and nasopharyngeal epithelial cells has been established (Kasahara and Yachie, 2002). In contrast, skin keratinocytes do not appear to be physiological target cells of EBV infection *in vivo* (Neuhierl et al., 2002). Using EBER ISH, which is the most sensitive method to detect EBV in tissue samples (Marques-Piubelli et al., 2020), we found that EBV was indeed localized in immune cells. However, the demonstration of EBER by ISH was not possible in all PCR-positive samples. In a previous study, the discrepancy between positive EBV PCR and negative EBER ISH results has been attributed to the low copy number of viral genomes per cell and the low number of infected cells per tissue section (Noorali et al., 2002). Anagnostopoulos et al. (1996) argued that EBV-harboring cells might be truly absent in the paraffin sections used to perform EBER ISH as the tissue sections used for DNA isolation were much thicker. In principle, EBERs are present in probably all EBV-infected cells (Skalsky and Cullen, 2015). However, as EBERs are expressed during latent EBV infection (Grywalska and Rolinski, 2015), the detection of EBER positivity merely indicates the presence of EBV-infected cells in the skin infiltrates but does not demonstrate EBV reactivation. In this context, the results of the patients suffering from skin lymphomas have to be interpreted with great caution. Indeed, the role of EBV in cutaneous T cell lymphoma (CTCL) has been extensively investigated, but due to the predominance of studies showing low frequencies or absence of EBV infection, it is not regarded as a likely etiologic agent (Mirvish et al., 2013). Anagnostopoulos et al. (1996) could detect EBV DNA and EBER in lesional skin samples of patients with primary cutaneous T cell lymphoproliferations, though the association with EBV infection was infrequent. The absence of BHLF transcripts, which are produced by infected cells entering the lytic cycle, indicated exclusively latent EBV infection (Anagnostopoulos et al., 1996). In contrast, Shimakage et al. (2001) demonstrated lytic EBV infection-associated ZEBRA protein (BZLF1 product) in tissue samples of CTCL (including mycosis fungoides), interestingly not only in lymphoma cells but also in epithelial squamous cells. In this study, detection of lytic infection-associated EBV EA-D-p52/50 by IHC was possible in both EBER-positive patients, providing evidence of active EBV infection.

4.1.2 Immunocompromised patients with treatment-refractory chronic skin diseases

Grimes et al. (1996) detected CMV DNA in depigmented and/or uninvolved skin from patients with vitiligo (11/29; 37.9 %) who had not been treated with immunosuppressants. Interestingly, the results suggested a correlation between the presence of CMV DNA in skin biopsies and active vitiligo (progression rate 73 % vs. 25 %) of relatively short duration, though not statistically significant (Grimes et al., 1996). Ballanger et al. (2009) found that CMV-seropositive patients with

Sézary syndrome did not harbor CMV DNA in lesional skin biopsies obtained at the time of diagnosis and before treatment. However, in 2 out of 9 patients (22.2 %), the presence of CMV DNA could be demonstrated in biopsies obtained at an advanced stage and following numerous therapies (Ballanger et al., 2009). These patients did not harbor CMV DNA in their blood, but one patient later died of active CMV infection (Ballanger et al., 2009). Similarly, Novelli et al. (2009) found that cutaneous EBV DNA positivity in mycosis fungoides mounted with disease progression. The presence of EBV-specific DNA in skin and blood specimens could be shown to be associated with significantly reduced survival in patients with mycosis fungoides and Sézary syndrome (Novelli et al., 2009).

The findings of Grimes et al. (1996), Ballanger et al. (2009), and Novelli et al. (2009) may indicate particular relevance of CMV and EBV reactivation in progressive and nonimproving skin conditions. This constellation was addressed and comprehensively investigated in the "Prospective Study Group, PG", including patients with chronic skin diseases who had not shown adequate treatment response (i.e., persistence or progression of skin lesions) during a period of at least 8 weeks of intensive therapy with biologicals and/or immunosuppressants.

4.1.2.1 Increased frequency of (serological) CMV and EBV reactivation

First, the presence of CMV- and EBV-specific antibodies was analyzed in the sera of all patients from the "Prospective Study Group, PG" and compared to age- and sex-matched control patients, who suffered from skin conditions within the same disease categories but had not been under systemic immunosuppressive/immunomodulating treatment for at least 6 months before blood withdrawal ("Control Group for Prospective Study Group, CG-PG").

Though there are some limitations, previous CMV exposure can acceptably be estimated for practical purposes using the presence or absence of CMV-specific antibodies in serum (Ljungman et al., 2017). In a nationally representative sample of German adults (samples from 1998), the seroprevalence of CMV-specific antibodies (IgG, IgM, and IgA) has been demonstrated to mount with age: from 31.8 % to 63.7 % in males and from 44.1 % to 77.6 % in females when comparing the 18–29 year to the 70–79 year age group, respectively (Lachmann et al., 2018). In this study, the frequency of CMV IgG was found to be increased in patients [PG] as compared to controls [CG-PG] (70.0 % vs. 62.2 %), though statistically not significant. Given the demographic characteristics of patients and controls, the seroprevalence rates are within the range of the aforementioned nationally representative sample. Hence, it could be identified that a large proportion of patients were latently infected with CMV and thus at risk for reactivation. Interestingly, the frequency of CMV IgM was higher in patients [PG] as compared to controls [CG-PG] (16.7 % vs. 5.6 %). However, this difference was not statistically significant, most likely due to the small number of patients. The simultaneous detection of CMV IgG in all CMV IgM-positive individuals hinted at reactivation of latent CMV infection. In fact, CMV reactivation might be even more common in the patient group [PG] because immunosuppression may lead to false-negative

serological results (Dioverti and Razonable, 2016; Ljungman et al., 2017). In contrast, it has to be considered that CMV-specific antibodies might have been passively transferred to the patients (Ljungman et al., 2017). Indeed, 3 out of 5 patients with detectable CMV IgM had been treated with IVIg, potentially leading to false-positive results. It is also noteworthy that CMV IgM may be false positive in some patients with acute EBV infection (Storch, 2000). However, all patients [PG] and controls [CG-PG] who tested positive for CMV IgM did not have detectable EBV-VCA IgM. In addition, all but one patient [PG] showing CMV IgM positivity did not harbor EBV DNA in peripheral blood. Finally, it has to be considered that CMV IgM may persist long after the resolution of symptoms and thus not be indicative of active CMV infection (Dioverti and Razonable, 2016). In summary, the clinical use of serologic tests to diagnose acute CMV infection in the immunosuppressed host has relevant limitations (Dioverti and Razonable, 2016). In this study, the serological diagnosis of CMV reactivation could be corroborated by the detection of CMV DNA in peripheral blood in 1 out of 5 patients [PG]. In addition, CMV DNAemia could be demonstrated in a patient who had shown CMV IgG positivity and borderline CMV IgM.

In this study, 86.7 % of patients [PG] and 87.8 % of controls [CG-PG] showed latent EBV infection, as documented by the detection of EBNA1 IgG in serum. These results are within the range of previously published data from a large hospital population from Berlin and Northern Germany (samples from 2014–2016) (Abrahamyan et al., 2020). The frequency of EBV-VCA IgM was higher among the patients [PG] as compared to the controls [CG-PG] (6.7 % vs. 0.0 %). The presence of EBV-VCA IgM was confined to patients with detectable EBNA1 IgG, hinting at reactivation of latent EBV infection. In the immunocompromised host, serologic testing (for EBV infection) can be confounded by the patient's incapability to mount adequate antibody responses and the presence of passively transferred antibodies from blood products (Nowalk and Green, 2016). Indeed, both patients with positive EBV-VCA IgM tests had been treated with IVIg and did not harbor EBV DNA in peripheral blood, which creates doubts regarding the diagnosis of EBV reactivation. In contrast, the detection of EBV DNA was possible in the peripheral blood of three other patients showing positive EBNA1 IgG but borderline/negative EBV-VCA IgM results.

4.1.2.2 Detection of CMV and EBV DNA/proteins in lesional skin

The serological results indicated a trend for higher rates of CMV and EBV reactivation among the patients [PG] compared to the controls [CG-PG]. However, whether this finding is related to immunosuppression, treatment refractoriness (and increased inflammatory disease activity), or both factors remains open. The potential shortcomings of serology have been addressed in the previous section. The fact that the detection of virus-specific IgM is only an indirect indicator of lytic viral activity represents an additional major limitation that must be considered. To shed light on the potential impact of CMV and EBV reactivation on skin pathology, the presence of virus-specific DNA and proteins in skin samples was investigated.

Indeed, CMV and EBV DNA could be detected in lesional skin of immunocompromised patients with therapy-refractory chronic skin diseases [PG(P)] (CMV: 21.4 %; EBV: 30.8 %). In contrast, CMV and EBV DNA were not found in nonlesional skin of age- and sex-matched control patients, who suffered from skin conditions within the same disease categories but had not been under systemic immunosuppressive/immunomodulating treatment for at least 6 months before the acquisition of the skin biopsy [CG-PG(P)] (CMV: 0.0 %; EBV: 0.0 %). The absence of CMV and EBV DNA in nonlesional skin of nonimmunocompromised control patients (analogous to the retrospective analysis) hinted at a clear impact of both cutaneous inflammation and immunosuppression on the presence of virus-specific DNA in the skin. Interestingly, CMV DNA was not detectable in lesional skin of immunocompromised patients with chronic skin diseases in the retrospective analysis [RG]. This implies that the third condition – namely, the persistence or progression of skin lesions despite intensive immunosuppressive/immunomodulating treatment – is pivotal for the presence of CMV DNA in the skin. It is important to note that the high sensitivity of the PCR technique might entail false-positive results if tissue samples are minimally contaminated with blood that contains CMV DNA (Tosti et al., 1996). However, detecting CMV DNAemia was not possible in 2 out of 3 patients who harbored CMV DNA in lesional skin [PG(P)]. In addition, there is no widely accepted viral load threshold to predict CMV disease (Dioverti and Razonable, 2016). Finally, CMV NAT assays (particularly those targeting CMV DNA) are associated with the risk of detecting inactive, nonreplicating CMV infection (Dioverti and Razonable, 2016). Hence, the demonstration of CMV (but also EBV) DNA in lesional skin is not per se indicative of CMV (EBV) reactivation but might be related to latent viral infection.

To provide direct evidence of active viral infection, the presence of lytic infection-associated gene products was investigated by IHC in the available FFPE samples. Concretely, we stained FFPE samples of patients positive for CMV DNA in lesional skin [PG(P)] for CMV immediate early and early antigens, which resulted in a weak positive nuclear signal in 1 out of 2 tissue samples. In addition, we performed IHC for EBV EA-D-p52/50 in patients harboring EBV DNA in lesional skin [PG(P)]. Here, 3 out of 4 tissue samples stained positive. On the one hand, the absence of gene products in PCR-positive cases may reflect nonproductive (i.e., latent) infection, which means that lytic infection-associated viral proteins were truly absent in lesional skin. On the other hand, the increased sensitivity of TaqMan probe-based real-time PCR as compared to IHC in the detection of CMV is well established (Drebber et al., 2011). It is important to note that the discrepancies between positive PCR and negative IHC results may also be related to the focal distribution of virus material. Indeed, different specimens representing a fraction from the same skin biopsy or another lesional skin biopsy obtained on the same day (or another day) had been used for the respective experiments. AbdullGaffar et al. (2008) recommended investigating both multiple, deeper serial sections per HE slide and multiple slides in order not to miss the diagnosis of cutaneous CMV infection because the characteristic viral cytopathic changes were shown by a very small number of endothelial cells. To avoid false-negative results, this approach should

also be applied to the detection of CMV antigen by IHC (AbdullGaffar et al., 2008). However, due to limited sample material, it was not possible to stain more slides in this study. Finally, the PCR results are unlikely to be false positive as negative (and positive) controls had been included in the experiments.

To summarize, the immunohistochemical stainings substantiate the reactivation of latent CMV and EBV infection in lesional skin. The results give rise to the hypothesis that CMV and EBV antigens in the skin may be able to trigger immune responses and thus aggravate ongoing cutaneous inflammation, which may impede the improvement of the underlying skin disease upon immunosuppressive/immunomodulating standard treatment.

4.1.2.3 Demonstration of CMV- and EBV-specific T cells in lesional skin: the model of a vicious circle

To corroborate this assumption, T cells isolated from lesional skin biopsies were stimulated with viral antigens. The frequency and the phenotype of CMV-and EBV-specific T cells and activation-induced cytokine production were analyzed. This experiment was conducted with the biomaterial of two patients whose laboratory tests had been interpreted as suggestive of CMV and EBV reactivation, respectively.

Sylwester et al. (2016) demonstrated that CMV-specific T cell immunity could not be investigated representatively by measuring only a few randomly selected epitope-specific responses (e.g., by stimulation with single antigenic peptides). Instead, the analysis had to include numerous target proteins to permit a meaningful quantitative comparison between different individuals (Sylwester et al., 2016). For this reason, T cell stimulation experiments were carried out with a clarified cell lysate that contains different CMV antigens from all stages of the replication life cycle. To assess EBV-specific T cell immunity, lesional skin-infiltrating T cells were stimulated with recombinant EBV proteins (BZLF1+EBNA3A). Indeed, the presence of a distinct population of CMV- and EBV-specific T cells in lesional skin of both patients examined in this study could be revealed. It is important to note that EBNA3A is a latent infection-associated nuclear antigen. To explicitly investigate the T cell response to EBV reactivation, lytic infection-related antigens should be used. Therefore, the T cell proliferation assay was repeated with BZLF1 (a lytic infection-associated immediate early antigen) alone, albeit only in the case of one patient. The results highlighted that both EBV antigens contributed to skin inflammation with a more pronounced effect of the lytic phase antigen BZLF1. The concentrations of cytokines, chemokines, and growth factors in cell-free supernatants derived from the co-culture of lesional T cells and activated MODCs were measured as a read-out for the function of CMV- and EBV-specific T cells. Though the proportion of lesional T cells that had proliferated *in vitro* upon presentation of viral antigens was small, the levels of different proinflammatory cytokines were profoundly increased. These included cytokines that have been widely accepted as the disease-driving cytokines in the pathogenesis of chronic skin diseases (Eyerich and Eyerich, 2018).

To conclude, the highly inflammatory environment and the use of immunosuppressants in patients with treatment-refractory chronic skin diseases may prompt reactivation of CMV infection. The production of proinflammatory cytokines, e.g., IL-1β, IL-6, IL-8, IP-10, and TNF-α, by CMV-infected cells has been shown before (Cheeran et al., 2003; Botero et al., 2008; Alcendor et al., 2012). In addition, the release of disease-aggravating cytokines by lesional skin-infiltrating T cells in response to CMV antigens, which could be demonstrated in this study, may have a particularly amplifying effect on skin inflammation.

This model of a vicious circle has first been addressed in transplantation medicine. Fietze et al. (1994) postulated that there was a mutual relationship between graft rejection and CMV infection in solid organ transplant recipients. In a recent review, Forte et al. (2020) outlined that graft rejection, other infections, or antilymphocyte treatment, all of which were associated with high systemic levels of inflammatory mediators (particularly TNF-α) and reduced immune function, had been proposed to induce CMV reactivation. This mechanism would result in an amplifying loop in which CMV infection enhances the expression of MHC genes, thus increasing the immunogenicity of the graft and the expression of inflammatory cytokines (Forte et al., 2020). This would further drive reactivation and rejection (Forte et al., 2020).

In dermatology, Ballanger et al. (2009) suggested that the immunosuppressive state in advanced Sézary syndrome and the skin inflammation might predispose to CMV reactivation, which, in turn, might increase cutaneous immunosuppression and sustain cutaneous inflammation, thus interfering with the evolution of this disease. Chai and Oh (2020) pointed out that active CTCL might predispose to localized CMV reactivation ("locus minoris resistentiae") and suggested that CMV infection might complicate CTCL, particularly in patients who showed aggressive disease and/or were refractory to different treatment regimens. Likewise, Asadullah et al. (1999) and Döcke et al. (2003) argued that the systemic reactivation of CMV infection might not only be a consequence of active psoriasis/atopic dermatitis and the associated systemic inflammation. Instead, the inflammatory response resulting from active CMV infection might also trigger psoriasis/atopic dermatitis exacerbation (Asadullah et al., 1999; Döcke et al., 2003). Indeed, Asadullah et al. (1999) found a strong positive correlation between the plasma concentration of bioactive TNF-α and the frequency of CMV antigen-positive PBMCs in patients with psoriasis. Interestingly, the clearance of CMV antigenemia could be observed in all patients investigated after clinical response to topical antipsoriatic treatment and phototherapy (Asadullah et al., 1999). Similarly, effective anti-eczematous treatment resulted in the almost complete clearance of CMV antigenemia in patients with atopic dermatitis (Döcke et al., 2003). The more recent study by Weitz et al. (2011) revealed a significant positive correlation between the disease severity and the frequency of CMV antigen-positive cells in the peripheral blood of patients with psoriasis.

Finally, the model of a vicious circle has also been proposed in the context of EBV infection. Larsen et al. (2011) suggested that autoimmune activation of B cells during SLE flares induced frequent EBV reactivation, which, in turn, contributed to perpetuating immune activation.

4.1.2.4 Improvement of skin lesions upon antiviral treatment of patients with CMV reactivation

The occurrence of CMV disease in patients with chronic skin diseases has been described to affect different organs, including gastrointestinal (Ozaki et al., 2013), pulmonary (Leshem et al., 2014), and retinal (Laws et al., 2014) involvement. However, CMV reactivation in dermatology patients may also affect the skin condition. In a small longitudinal study of immunocompromised patients with therapy-refractory chronic skin diseases (drug-induced exanthema, pemphigus vulgaris, pyoderma gangrenosum, and psoriasis inversa and capitis) showing CMV reactivation, the effects of anti-CMV intervention were investigated. To summarize, the evidence put forward in the presented cases does not prove that CMV reactivation impeded the healing of skin lesions. However, the rapid and dramatic improvement of the skin condition in response to antiviral treatment and (to a greater or lesser extent) the diagnostic results strongly support a disease-modifying role of the virus. The literature on this topic is scarce and relies mainly on anecdotal evidence. There are, for example, reports of immunocompromised patients with bullous pemphigoid (Harada et al., 2016), cutaneous ulcer (Nolan et al., 2007), generalized pustular psoriasis (Ali et al., 2014), and mycosis fungoides (Chai and Oh, 2020) whose disease course was complicated by cutaneous or mucosal CMV infection/disease. The treatment of these patients included antiviral agents, and the outcomes were favorable (Harada et al., 2016; Nolan et al., 2007; Ali et al., 2014; Chai and Oh, 2020).

The role of immunosuppressive/immunomodulating agents

In this study, the patients diagnosed with and treated for CMV reactivation had undergone a broad spectrum of systemic therapy regimens, including glucocorticoids, azathioprine, cyclosporine, infliximab, ixekizumab, and rituximab. In the following subsection, the association between immunosuppressive/immunomodulating agents and CMV reactivation will be delineated using the examples of glucocorticoids, infliximab, and ixekizumab.

The link with CMV reactivation appears to be strongest for the use of glucocorticoids, which are highly important in managing disease flares. Their mechanism of action includes antiproliferative effects, anti-inflammatory effects (mediated, e.g., by suppression of transcription factor NF-κB and reduced expression of proinflammatory cytokines), and immunosuppressive effects (affecting mostly cell-mediated but also humoral immunity) (Schreml and Gollnick, 2018). Corticosteroid treatment has long been associated with the development of CMV-related infectious complications (Segal and Sneller, 1997). In general, the risk of infectious complications mounts with steroid dose and duration of treatment (Klein et al., 2001). More recently, Lim et al. (2018) found that methylprednisolone was independently related to CMV infection in patients prescribed potent immunosuppressants for glomerulonephritis. In the study of Ota et al. (2021), the initial prednisolone dose was identified to be an independent risk factor for CMV reactivation in patients with rheumatic diseases undergoing remission induction treatment. In patients with rheumatic

diseases, the daily steroid dose has also been shown to be significantly associated with CMV antigenemia (Ogata et al., 2022), and increased maximum and cumulative prednisolone doses (in the preceding 3 months) were identified to be significant risk factors for CMV reactivation (Shimada et al., 2022).

Infliximab is a monoclonal antibody that inhibits TNF-α and its interaction with cell surface TNF receptors (Schreml and Gollnick, 2018). In general, TNF-α is a pleiotropic cytokine, which has been established to be involved in the pathogenesis of numerous inflammatory diseases (Davis et al., 2020). On the one hand, TNF-α is known to trigger proinflammatory signaling pathways leading to the activation of transcription factors (e.g., NF-κB) that induce MIEP activation and IE gene expression (Heald-Sargent et al., 2020). Indeed, the inhibition of TNF-α action and/or release has been proposed to prevent TNF-α-induced CMV reactivation (Fietze et al., 1994). On the other hand, TNF-α plays an important role in the defense against viral disease (Davis et al., 2020). Accordingly, the impact of TNF-α antagonists is difficult to dissect. Davis et al. (2020) outlined that reactivation of CMV infection had been reported in association with infliximab treatment. However, the reported cases have generally occurred in the context of combined immunosuppressive therapies, which hampers the estimation of the exact attributable risk (Davis et al., 2020). Neither routine antiviral prophylaxis nor monitoring of CMV reactivation is currently recommended (Ho et al., 2020).

Ixekizumab is a monoclonal antibody that inhibits IL-17A effects (Schreml and Gollnick, 2018). IL-17 is a major proinflammatory mediator in the pathogenesis of psoriasis and psoriatic arthritis (Schreml and Gollnick, 2018). Ixekizumab has not been associated with the risk of CMV infection so far (Ho et al., 2020). Accordingly, Chiu et al. (2016) could not detect CMV DNA in PBMCs or plasma of psoriasis patients at baseline and following treatment with secukinumab, which is another anti-IL-17A monoclonal antibody.

To sum up, the cases reported in this thesis highlight that not only heavily immunocompromised individuals (e.g., transplant recipients) may be affected by CMV reactivation. Interestingly, there is also evidence suggesting antiviral properties, including anti-CMV activity, of different immunosuppressive agents (Brennan et al., 2013). Hence, the impact of immunosuppressive/immunomodulating treatment on CMV reactivation might be more complex than generally assumed.

Diagnostic strategies

In this study, the diagnostic approach to CMV reactivation in immunocompromised patients with therapy-refractory chronic skin diseases included serology, blood and tissue PCR, IHC, and HE histopathology. The major limitations of these laboratory assays have already been addressed in the previous sections and/or will be outlined in this subsection. In general, the development of cutaneous CMV disease must be distinguished from subclinical CMV reactivation. Unfortunately, Ljungman et al. (2017) do not provide a specific definition of “cutaneous CMV disease”

(proven, probable, possible). However, the diagnostic criteria of (proven) “other CMV end-organ diseases” may be used, which means that the detection of CMV in biopsy material (i.e., lesional skin) – by virus isolation, rapid culture, IHC, or DNA hybridization – is needed to diagnose (proven) “cutaneous CMV disease” (Ljungman et al., 2017). In contrast, lesional evidence of CMV is lacking in the case of subclinical CMV reactivation, which may be indicated by CMV DNAemia and/or antigenemia. Nonetheless, subclinical CMV reactivation may affect the skin condition.

Important insights into the pathophysiological relevance of subclinical active CMV infection and its implications for general inflammatory processes could be gained in transplantation medicine. Reinke et al. (1994) found that there was an expansion of memory-type CD8-positive T cells in peripheral blood and an association with symptomless CMV infection (indicated by the detection of CMV DNA/RNA in PBMCs or CMV antigenemia) in renal allograft recipients with late-acute rejection who did not respond to conventional anti-rejection treatment. The detection of CMV in the grafts of these patients was rare, even with PCR. The authors proposed that the strong – possibly antiviral (Alexopoulos et al., 1995) – memory-type T cell response might prevent the development of the typical CMV disease pattern but result in graft injury. (Reinke et al., 1994)
These assumptions could be corroborated with regard to chronic skin diseases:
Indeed, Asadullah et al. (1999) could only rarely detect CMV DNA in psoriatic lesions. Their results gave no evidence that cutaneous CMV infection might be responsible for prominent CMV antigenemia. Instead, the infiltration with CMV-positive PBMCs may be the reason for the detection of CMV DNA in lesional skin in a few cases. (Asadullah et al., 1999)
Döcke et al. (2003) pointed out that the infrequence of cutaneous CMV infection did not rule out the pathophysiological relevance of this herpesvirus in inflammatory skin conditions. The authors suggested that the situation might be similar to cutaneous graft-versus-host disease and late graft injury in renal transplant recipients. Though the viral infection is not essential, and CMV has rarely or not at all been demonstrated in the respective target organs, the impact of active CMV infection is well established in these conditions. (Döcke et al., 2003)
Accordingly, Weitz et al. (2011) suggested that the impact of CMV infection on psoriasis severity was rather due to the systemic effects of anti-CMV immune response than CMV replication in lesional skin. The authors proposed that there might be an interactive relationship between psoriasis and CMV infection, which might be mediated by CD8-positive T cells in peripheral blood. The reduced frequencies of circulating CMV-specific T cells in CMV-seropositive psoriasis patients as compared to CMV-seropositive healthy controls might reflect insufficient anti-CMV immune response in psoriasis. However, this finding might also result from the sequestration of CMV-specific T cells to the affected skin with potential involvement in lesional immunopathology. This would be in accordance with the transient expansion of peripheral CMV-specific T cells that could be observed under effective antipsoriatic treatment. (Weitz et al., 2011)
Indeed, we were able to demonstrate the presence of CMV-specific T cells in lesional skin of a patient with psoriasis inversa and capitis (see section 4.1.2.3).

In this study, the serological profile and/or the results of CMV NAT of patients PG1–PG4 were interpreted as suggestive of CMV reactivation. HE stainings (as far as examined) had not been suspected of CMV disease during routine histopathology. However, it has to be considered that the histopathological findings resulting from cutaneous CMV infection are not clustered together in an epithelium but are usually present in scattered mesenchymal or inflammatory cells (Resnik et al., 2000). The presence of cytologic changes is often limited to a few cells in any one sample, and there is a wide range of CMV-induced cellular changes, which depend on the stage of infection (Resnik et al., 2000). Choi et al. (2006) investigated the histopathological findings in non-AIDS, immunocompromised patients diagnosed with cutaneous CMV infection based on positive immunohistochemical staining. Indeed, the characteristic owl's eye-shaped intranuclear inclusions could only be seen in one-third of biopsies (Choi et al., 2006).

In patient PG1, both CMV IgG and CMV IgM could be detected. This result might potentially have been impacted by IVIg treatment (Ljungman et al., 2017). However, the demonstration of CMV DNA in both peripheral blood and lesional skin corroborated CMV reactivation. In view of the presence of CMV DNAemia, it has to be considered that the detection of CMV DNA in lesional skin might have been related to blood contamination (Tosti et al., 1996) or infiltration with CMV-positive PBMCs (Asadullah et al., 1999). In this patient, CMV IHC could not be carried out. Hence, it is intricate to differentiate between cutaneous CMV disease and subclinical CMV reactivation.

In patient PG2, the results of the CMV IgG and CMV IgM tests were highly positive and borderline, respectively. The demonstration of CMV DNAemia hinted at CMV reactivation. In the first instance, these findings are consistent with subclinical CMV reactivation. However, it must be considered that lesional skin of this patient could not be investigated.

In patient PG3, the detection of CMV DNA in lesional skin in conjunction with supportive serologic evidence (i.e., detection of CMV IgG and CMV IgM) indicated CMV reactivation. The passive transfer of CMV IgM to this patient by IVIg treatment (Ljungman et al., 2017) is theoretically imaginable. Still, it appears unlikely because detection of CMV IgM was possible over a long period. The presence of CMV DNA in lesional skin was presumably not related to blood contamination or infiltration with CMV-infected PBMCs because CMV DNA was – albeit with a time lag – not detectable in peripheral blood. Besides, CMV IHC showed a weak positive nuclear signal. It is important to note that the detection of CMV in lesional skin may be of pathogenetic relevance in the absence of demonstrable CMV DNAemia. Indeed, the diagnosis of (proven) "other CMV end-organ diseases" demands the detection of CMV in biopsy material (e.g., by IHC) (Ljungman et al., 2017) but not in peripheral blood. In addition, the detection of high CMV DNA levels in the relevant tissue via quantitative NAT (e.g., PCR) is newly accepted as "possible CMV end-organ disease" in some end-organ disease categories, especially when CMV DNA is not detectable in blood obtained at the same time (Ljungman et al., 2017). In the synopsis of all diagnostic results, the case of patient PG3 may probably be classified as cutaneous CMV disease.

In patient PG4, the detection of CMV IgM (in conjunction with CMV IgG positivity) indicated putative CMV reactivation. However, this was the sole diagnostic evidence for acute CMV infection.

In this context, it is notable that CMV IgM may persist long after the resolution of symptoms and thus not be indicative of active CMV infection (Dioverti and Razonable, 2016). In summary, the diagnostic findings are neither fully consistent with cutaneous CMV disease nor subclinical CMV reactivation. Hence, the diagnosis has to be critically scrutinized in this patient.

Finally, Kim et al. (2015) emphasized that coinfections frequently occurred with CMV infection and had to be included in the differential diagnoses. Indeed, bacterial coinfections have been described, e.g., in pyoderma gangrenosum (Tsutsumi et al., 2021). In addition, simultaneous infections with CMV and HSV/VZV have been reported in patients with pemphigus vulgaris (Pushkarevskaya et al., 2017; Chiu et al., 2013). Interestingly, Kalra et al. (2005) demonstrated a high rate of HSV colonization of recalcitrant oral erosions in patients with pemphigus vulgaris. There was a reduction in the size of oral lesions in all HSV antigen-positive patients who were treated with aciclovir (Kalra et al., 2005). Likewise, Kumar et al. (2017) identified therapy refractoriness of oral lesions in patients with pemphigus vulgaris to be significantly associated with HSV DNA positivity in oral tissue scrapings. However, detection of HSV-1/2 and VZV DNA was not possible in the lesional skin samples that could be investigated in this study (patients PG3 and PG4), which corroborated the pathophysiological relevance of CMV infection.

Treatment and outcome

In general, treatment of CMV disease consists of administration of antiviral drugs and (if arguable) reduction of immunosuppressive agents (Dioverti and Razonable, 2016). It is important to note that antiviral treatment may also be beneficial in the case of subclinical CMV reactivation. Indeed, Reinke et al. (1994) reported stable improvement of graft function following ganciclovir treatment without additional conventional anti-rejection therapy in 17 out of 21 patients with late-acute renal allograft rejection and symptomless CMV antigenemia. Interestingly, Asadullah et al. (1999) have already speculated that treatment of CMV infection might become a novel adjuvant therapeutic approach in some psoriatic patients.

In this study, the initiation of treatment with ganciclovir upon detection of CMV DNA in peripheral blood and/or lesional skin of patients PG1, PG2, and PG3 resulted in profound improvement of the skin condition. Though CMV DNA had neither been detected in peripheral blood nor lesional skin, patient PG4 was treated off-label with valganciclovir in view of refractoriness to intensive immunomodulating standard treatment. Indeed, ixekizumab has not been associated with the risk of CMV infection so far (Ho et al., 2020), and there is little diagnostic evidence for acute CMV infection in this case. However, the resulting dramatic improvement of skin lesions hints at the involvement of CMV in the exacerbation of the skin disease. Ljungman et al. (2017) pointed out that other viruses might also respond to antiviral treatment if the antiviral agent was not CMV-specific. Indeed, (val)ganciclovir does not only show anti-CMV activity but also activity against HSV and VZV (Ho et al., 2020). Hence, the improvement of the patients' skin lesions upon (val)ganciclovir might have been related to the control of HSV and/or VZV infection. However, at

least in patients PG3 and PG4, detection of HSV-1/2 and VZV DNA in lesional skin had not been possible. In all patients reported in this study, there was a temporal correlation between antiviral therapy and improvement of skin lesions, while immunosuppressive/immunomodulating treatment had not been altered substantially. Still, it is not possible to draw conclusions as to causal relations. First, potential anti-CMV effects of IVIg have to be considered in patients PG1 and PG3. In addition, the improvement of the pyoderma gangrenosum in patient PG3 might, at least partially, have been related to a delayed onset of the infliximab effect. Indeed, CMV DNA was detectable in lesional skin obtained prior to the initiation of infliximab. Interestingly, Saito et al. (2006) and Moscarelli et al. (2011) suggested that high TNF-α levels in the wound bed environment stimulated reactivation of latent CMV infection. In addition, Ortigosa et al. (2014) demonstrated that IFN-γ and lymphoproliferative responses to CMV in psoriasis patients were not reduced by infliximab treatment. Instead, immunoreactivity to CMV lysate was either not affected or slightly enhanced (Ortigosa et al., 2014). Therefore, infliximab treatment in patient PG3 was continued. The combined therapeutic regimen (including an antiviral agent and a TNF-α antagonist) eventually showed a favorable effect on the lesion. In patient PG2, the delayed onset of the immunosuppressive effects of azathioprine might have contributed to the improvement of the skin condition. In addition, the potential role of UVB phototherapy in patient PG4 is not clear.

To conclude, the cases reported in literature (Harada et al., 2016; Nolan et al., 2007; Ali et al., 2014; Chai and Oh, 2020) and in this study highlight the high practice variation in the management of cutaneous CMV disease and subclinical CMV reactivation with skin involvement, respectively. Indeed, there are no guidelines that specifically address the indication, type, and duration of antiviral treatment and the handling of immunosuppressive/immunomodulating therapies in this context.

4.1.3 Potential reduction of morbidity and health care costs through early detection of CMV reactivation

The retrospective analysis of two representative inpatient wards revealed that 13.0 % of all patients admitted had been treated with biologicals and/or immunosuppressants that have been implicated with CMV reactivation for a minimum of 8 weeks. Thereof, 27.6 % (i.e., 3.6 % of all patients) showed prolonged exacerbation of the skin condition despite intensive immunosuppressive treatment. In this patient group, CMV reactivation may be a major factor contributing to the course of disease. However, it must be considered that the percentages might have changed since the time of data collection, e.g., due to the introduction of novel therapeutic agents.

To evaluate the benefit of screening for CMV reactivation and subsequent antiviral treatment, the NNS was calculated. It could be found that – depending on the definition of a positive screening test – only 9 to 30 patients at risk (i.e., patients who show persistence or progression of skin

lesions despite intensive immunosuppressive/immunomodulating therapy) would need to be screened to prevent CMV-induced morbidity in one patient.
However, it is essential to note that the NNS is expression of an absolute (not a relative) risk, which is specific to the population (including age, underlying incidence of disease, and treatment that people were already receiving) and intervention. Thus, it cannot be generalized from the group of people that has been studied to the population at large. The results might be unstable, with small changes in factors of influence having large effects on the estimate. (Law, 2001)

To sum up, screening patients at risk may be beneficial to reduce individual suffering and medical resource utilization. The costs for blood- and/or tissue-based screening are probably multiple times lower than those resulting from prolonged and repeated hospitalization and administration of immunosuppressive medication.

4.2 Potential lessons from inflammatory bowel disease research

Interestingly, the topic of this doctoral thesis has broadly been addressed in gastroenterological research. In recent years, important insights have been gained regarding the role of CMV infection in inflammatory bowel disease (IBD). The prevalence of CMV intestinal disease among IBD patients ranges between 2 % and 38 % (Lamb et al., 2019). The high variation is attributed to the fact that the definitions of CMV infection and CMV intestinal disease are widely heterogeneous (Lamb et al., 2019). Nakase et al. (2010) argued that the decreased frequency of CMV infection in patients with Crohn's disease compared to ulcerative colitis (UC) patients might be related to the differences in immune response between both conditions. Likewise, the different immune response patterns underlying chronic skin diseases might impact the susceptibility to CMV reactivation. However, due to the small number of patients, this study does not allow conclusions regarding this question.

The aforementioned model of an amplifying loop has also been established in IBD patients. Mourad et al. (2020) outlined that the use of corticosteroids in the setting of UC-related inflammation and treatment with immunosuppressants might result in CMV reactivation, leading to increased inflammation and migration of CMV-infected monocytes and macrophages into the inflamed tissue. This might, in turn, propagate more virus replication and worsen intestinal inflammation (Mourad et al., 2020).
Importantly, Mourad et al. (2020) delineated the concept of high-grade and low-grade CMV colitis. In patients with high-grade CMV colitis ("true CMV colitis"), as defined by high tissue viral load and inclusion bodies, CMV may be responsible for gut inflammation, whereas UC-related inflammation is of minor relevance. The administration of antiviral agents may be effective in treating inflammation in these patients and may change the clinical course. In cases of low-grade

CMV colitis, the underlying IBD colitis is considered the main driver of gut inflammation. Instead of acting as a pathogen, CMV is most likely an innocent bystander or a marker of disease severity. The aggressive optimization of immunosuppressive treatment may help to control inflammation in these patients. (Mourad et al., 2020)
Accordingly, detectable CMV in lesional skin may be the culprit for skin inflammation but also represent an innocent bystander or reflect disease severity with the respective implications for the treatment of choice.

The challenge that gastroenterologists face is to distinguish an acute UC flare from true CMV colitis (Mourad et al., 2020). The situation for dermatologists is similar. The aggravation of skin lesions during immunosuppressive treatment may be due to an exacerbation of the underlying skin disease or related to CMV reactivation. Hence, the knowledge from IBD research regarding diagnostics (and treatment) may be transferable to dermatology.
The British Society of Gastroenterology (BSG) consensus guidelines on the management of IBD in adults from 2019 suggest investigation of colonic biopsies for CMV disease in patients with moderate to severe colitis, particularly in case of corticosteroid-refractory disease, using HE staining and preferably also IHC or quantitative tissue PCR (Lamb et al., 2019). Interestingly, Tandon et al. (2017) found that the overall sensitivity of blood-based tests (pp65 antigenemia assay, blood PCR) and HE histopathology to detect colonic CMV reactivation in IBD patients (in comparison to IHC and tissue PCR as reference standards) ranged at only 50.8 % and 12.5 %, respectively. The diagnostic discrepancies in the patients reported in this study might also be related to the poor sensitivity of blood-based tests and HE histopathology. However, comprehensive studies investigating the sensitivity of blood-based tests, HE histopathology, IHC, and tissue PCR in the diagnosis of cutaneous CMV disease are lacking. Besides, McCurdy et al. (2015) found that the location and number of biopsies were relevant in assessing IBD patients for CMV disease. For UC patients, for example, flexible sigmoidoscopy with a total number of 11 biopsies was recommended to accomplish an 80 % probability of a single positive biopsy (as determined by IHC or ISH) (McCurdy et al., 2015). Hence, the investigation of a higher number of lesional skin biopsies per patient in this study might have allowed the detection of CMV protein (and/or DNA) in additional patients.
The BSG consensus guidelines on the management of IBD in adults from 2019 propose ganciclovir treatment of colonic CMV reactivation in patients hospitalized with IBD exacerbation while conventional therapy with corticosteroids or rescue medication is continued (Lamb et al., 2019). The additional cessation of all immunosuppressive agents is recommended in the rare case of systemic CMV disease (Lamb et al., 2019). Mourad et al. (2020) outlined that the data on the association between TNF-α antagonists and the risk of CMV infection/reactivation in IBD patients were inconsistent. Importantly, it was found that TNF-α antagonists did not adversely affect the outcome of UC patients with CMV reactivation (Mourad et al., 2020). TNF-α blockers

might theoretically reduce CMV reactivation (Mourad et al., 2020). Indeed, favorable development of tissue CMV copy number has been demonstrated in UC patients with low-grade CMV infection following initiation or optimization of infliximab treatment without administration of antiviral agents (Boivineau et al., 2020). These findings are in accordance with the beneficial effects of the combined treatment regimen, including ganciclovir but also infliximab, in patient PG3.

To summarize, the insights from IBD research may enable a greater understanding of the role of CMV reactivation and its pathogenetic link with therapy refractoriness in immunocompromised patients with chronic (inflammatory) skin diseases. Interestingly, Mourad et al. (2020) emphasized that it was important to maintain a high clinical suspicion for CMV infection/reactivation in UC patients who show worsening gastrointestinal symptoms regardless of their immunosuppression status. Indeed, Kim et al. (2010) detected CMV IE antigen by immunoperoxidase staining on colonic biopsies from patients with active UC who were newly diagnosed and had not been treated with steroids or immunosuppressants before endoscopy. In view thereof, the role of CMV reactivation in newly diagnosed and untreated patients with chronic skin diseases may be worth future investigations. Finally, it could be shown that CMV infection in hospitalized UC patients was significantly associated with longer hospital stays and higher total charges (Grossberg et al., 2018). This finding substantiates that awareness of CMV reactivation helps reduce medical resource utilization – presumably not only in gastroenterology but also in dermatology.

4.3 Limitations of this study, major obstacles to research success, and potential approaches in future research

In the following chapter, the identifiable limitations of this study will be outlined, and the methods used will be critically appraised. In addition, the major obstacles to research success and potential approaches in future research will be delineated. Finally, the question of causality and coincidence will be addressed.

In general, the heterogeneity of the definitions of CMV infection, reactivation, and disease in different scientific papers hampered the comparison of research results.

4.3.1 Study design

The following subsection will focus on shortcomings related to the study design and identify possible approaches in future research.

First of all, the design of this study was retrospective for the most part, which represents one of the major limitations. The patients from the “Retrospective Study Group” and all control patients were recruited retrospectively. In addition, the analysis of two representative inpatient wards was carried out retrospectively. Hence, the respective medical records had to be reviewed to obtain

the relevant clinical and laboratory data. However, the documentation in the patient files was not always clear, complete, and comprehensive.

To be enrolled in the “Retrospective Study Group”, patients had to be treated with biologicals, chemotherapy and/or immunosuppressants for at least 2 weeks prior to the acquisition of the skin biopsy. The resulting group of patients was highly heterogeneous regarding the duration of immunosuppressive/immunomodulating treatment, which limited the validity of the results. In general, the definition of inclusion criteria regarding the duration of immunosuppressive/immunomodulating therapy is challenging because the time until the onset of the immunosuppressive/immunomodulating effects varies widely.

To be included in the “Prospective Study Group”, patients had to show inadequate treatment response during a period of at least 8 weeks of intensive therapy with biologicals and/or immunosuppressants. Interestingly, the presence of CMV DNA (and protein) could be demonstrated *ex post* in a lesional skin biopsy that had been obtained only 14 days after the initiation of glucocorticoids (patient PG3). Indeed, there are also reports of CMV disease associated with short-time glucocorticoid treatment in literature (Casals et al., 2003; Weile et al., 2009). Sekiguchi et al. (2020) found that the mean interval between the initiation of prednisolone treatment and the diagnosis of CMV reactivation in dermatomyositis patients ranged at 6.1 weeks. Similarly, the median time from the initiation of immunosuppressive therapy until the onset of CMV antigenemia in patients with rheumatic diseases ranged at 30 days (Ogata et al., 2022). These findings highlight that vigilance is reasonable even in the case of short-time immunosuppressive treatment. In fact, morbidity and health care costs might have been prevented if the lesional skin biopsy of patient PG3 had been screened for CMV earlier in the disease course.
Besides this aspect, the reliance on prospective data helped circumvent some of the aforementioned problems associated with retrospective study design.
The treatment response was assessed by at least two experienced board-certified dermatologists. To increase the level of objectivity in the evaluation of treatment refractoriness, more specific inclusion criteria (e.g., related to the temporal development of disease activity scores) may be defined in future studies.

The results of this study corroborated the association between systemic immunosuppressive/immunomodulating therapy and CMV (and EBV) reactivation. Though not the focus of this research project, topical medication, which is a key element in treating chronic skin conditions, might also be linked to increased risks of CMV (and EBV) reactivation.
Topical glucocorticoids are the most commonly administered subgroup of topical dermatological agents. It is well known that, depending on the type of dressing used, large-scale and long-term topical application of highly potent glucocorticoids for the treatment of skin diseases may result in systemic adverse effects. In addition, the development of local – bacterial, fungal, or viral – infections may be observed in the context of long-term application. The prime example is

the occurrence of eczema herpeticum following long-term treatment of atopic dermatitis with highly potent topical glucocorticoids. (Garbe and Staubach-Renz, 2018)
Interestingly, the development of CMV retinitis (Kaplan et al., 2019; Scoles et al., 2020) and CMV esophagitis (Suzaki et al., 2020) has also been reported in the setting of (prolonged) topical glucocorticoid treatment. Similarly, topical cyclosporine has been related to the occurrence of CMV anterior uveitis (Siak and Chee, 2018). Therefore, investigation of the potential effects of topical immunosuppressive/immunomodulating agents on CMV (and EBV) reactivation in patients with chronic skin diseases may be considered in future studies.

Finally, the results of the "Prospective Study Group" were compared to age- and sex-matched control patients. Due to the limited availability of biomaterial, two different control groups had to be established to investigate serum and tissue samples, respectively. In future studies, blood and skin samples should be obtained from the same control patient for more in-depth comparisons. In addition, the presence of CMV and EBV DNA should also be examined in the peripheral blood of the control patients, which was not done in this study. To be able to make more sophisticated intra- and interindividual comparisons, the study groups and control groups could be designed as follows:

- patients with skin disease (lesional skin vs. nonlesional skin):
  immunocompromised vs. nonimmunocompromised, nonresponder vs. responder
- patients without skin disease (nonlesional skin):
  immunocompromised vs. nonimmunocompromised

Thus, the individual and combined effects of inflammation, immunosuppression, and therapy refractoriness on CMV and EBV reactivation may be elucidated.

4.3.2 Laboratory investigations

In the following subsection, the laboratory methods used will be critically appraised, the major obstacles related to laboratory work will be identified, and potential approaches in future investigations will be delineated.

In general, the investigation of different types of biomaterials (e.g., punch biopsy vs. swab) and the lack of standardization regarding laboratory methods (e.g., antibody titer/viral load reporting) limited the comparability of results with other studies.

The major obstacle in this research project was the acquisition of tissue samples for laboratory studies. The skin appears to be easily accessible, particularly as compared to internal organs. However, in clinical practice, skin biopsies are usually obtained at the time of diagnosis and only rarely during immunosuppressive/immunomodulating treatment. For this reason, the total number of patients included in the "Retrospective Study Group" and particularly in the "Prospective Study Group (PCR)" is relatively small, which represents one of the major shortcomings of this study. To be able to assess the presence of CMV and EBV in the skin of a higher number of

patients, the acquisition of biomaterial needs to be facilitated. For example, lesional microbiopsies and virological swabs may be investigated in future studies.

The general limitations of CMV and EBV serology have been addressed in the previous sections. The shortcomings of this study include the lack of paired serum samples in most patients. Measuring two or more sequential samples might have allowed a more reliable interpretation of the serostatus, particularly the demonstration of significant changes in activity and seroconversion. However, due to IVIG treatment in a substantial proportion of patients, the additional knowledge gained by the analysis of paired serum samples would have been limited.
For cost reasons, the assessment of the EBV serostatus in this study was restricted to a small antibody panel, including EBNA1 IgG and EBV-VCA IgM, which represents an additional major limitation. Future studies may examine the presence of different other antibodies (e.g., EBV-VCA IgG and EBV early antigen IgG) for a more comprehensive analysis.
In this study, the demonstration of CMV IgM (EBV-VCA IgM) in conjunction with CMV IgG (EBNA1 IgG) positivity was interpreted as suggestive of CMV (EBV) reactivation. It is worth noting that the detection of virus-specific IgM may not only be related to recent viral reactivation but also to recent primary CMV infection (Dioverti and Razonable, 2016). Similarly, positivity for both EBNA1 IgG and EBV-VCA IgM may result from shortly past primary infection instead of EBV reactivation (Gulley and Tang, 2008). In principle, testing CMV IgG avidity would have allowed to differentiate between primary infection and reactivation of CMV infection (Dioverti and Razonable, 2016). However, due to the relatively high age of the patients and controls included in this study, primary CMV (EBV) infection appears unlikely. Furthermore, it does not make much difference in terms of the research question because both constellations represent active CMV (EBV) infection. The same applies to the differentiation between reinfection and reactivation of latent infection.
Finally, the proportion of borderline IgM test results was relatively high in relation to the frequency of positive IgM tests, which hampered the interpretation of the patients' serological profiles. Regarding CMV-specific serology, the confirmation of the sample assessment as "borderline" by repeat testing pointed to viral infection, as delineated in the manufacturer's instructions. In this case, a second sample had to be obtained after an interval of at least 7 days and analyzed together with the first sample. Regarding "borderline" EBNA1 IgG/EBV-VCA IgM results, the guidelines from the manufacturer recommended repeating the test 2–4 weeks later using a fresh sample. If the result of the second test was classified as "borderline" again, the specimen had to be considered negative. However, this modus operandi was not feasible in this study, among other reasons, because the control patients had been recruited retrospectively.

The detection of CMV and EBV DNA in lesional skin may be related to latent viral infection, as outlined previously in this thesis. To provide further evidence for the involvement of CMV and EBV reactivation in the development of therapy-refractory skin lesions, more extensive investigation of viral gene expression is needed.

For example, total RNA may be isolated from lesional skin biopsies, and RT-PCR may be carried out to examine lytic infection-associated viral gene transcripts. However, it has to be considered that the analysis of a tissue extract does not provide information on the localization of viral RNA. In contrast to PCR, ISH specifically localizes viral nucleic acid sequences in a particular cell type (Drago et al., 2000). RNAscope is a relatively new RNA ISH (RISH) technology based on a special target probe design strategy, which allows single-molecule visualization and signal mapping to individual cells (Wang et al., 2012). Roe et al. (2019) demonstrated increased sensitivity of RNAscope RISH compared to IHC in detecting CMV in the clinical setting. The results of CMV RISH were concordant in 100 % of the samples determined to be positive for CMV by IHC (Roe et al., 2019). Additionally, CMV RISH detected positive cells in 50 % of the samples that had not been stained by CMV IHC (Roe et al., 2019). In this study, the presence of CMV DNA could be demonstrated by PCR in RNAlater preserved lesional skin biopsies from patients PG3 and PG5. The immunohistochemical staining of the corresponding FFPE samples for CMV immediate early and early antigens yielded only a weak positive nuclear signal and was negative, respectively. In both cases, the results of RNAscope would be of high interest.

The demonstration of CMV- and EBV-specific T cells in lesional skin and their phenotypic and functional analysis represent a novelty of this research project. The major identifiable limitation is the low number of patients investigated and the lack of control patients. In this regard, the examination of lesional T cells of patient PG23, who did neither harbor CMV- or EBV-specific antibodies in serum nor CMV or EBV DNA in peripheral blood/lesional skin, would have been particularly interesting. The analysis of lesional skin-derived T cells of patients PG1–PG3 would also have been desirable. In contrast to patient PG4, there are no doubts regarding the diagnosis of CMV reactivation in these patients. Accordingly, the cytokine profiles measured in the multiplex immunoassay would possibly have been more specific to CMV. However, in patients PG1–PG3 and patient PG23, lesional skin could either not be obtained, or T cells did not grow well.

In general, this study relied on laboratory methods that could either easily be implemented in a nonvirological research group at a nonvirological research institute or were part of routine diagnostics at the cooperating institutes. However, basic experimental research must be carried out to gain further insights into the pathogenetic role of CMV and EBV reactivation in chronically inflamed skin. In the setting of an *in vitro* experiment, virus or viral peptides may be introduced to skin biopsies. The effects of this intervention may be monitored histologically, comparing infected and noninfected lesional skin of the same donor. In addition, different laboratory techniques (including RT-PCR, RISH/RNAscope, IHC, and ELISA) may be used to study the viral gene expression and the activity of both innate immune cells and virus-specific T cells in infected and noninfected inflamed skin. Thus, infection-related changes (e.g., in T cell markers and cytokine/chemokine release) may be revealed. Future research may also be built on the methods of Astegiano et al. (2012), who developed an *in vitro* model of CMV infection in dermal fibroblasts and epidermal keratinocytes. The absence of CMV infection in keratinocytes at 10 days post-

infection was attributed to their nonpermissiveness for CMV replication (Astegiano et al., 2012). Hence, it may be particularly promising to investigate the role of dermal fibroblasts in more detail. Arvia et al. (2020) established a wound-healing assay to assess the migration ability of parvovirus B19-infected normal human dermal fibroblasts compared to mock-infected cells. This experiment may be modified to elucidate the impact of CMV and EBV infection on the phenotype of dermal fibroblasts and wound healing.

4.3.3 Causality vs. coincidence

The cases presented in this thesis highlight that CMV reactivation is a potentially deleterious complication in immunocompromised patients suffering from treatment-refractory chronic skin diseases. In patients PG1–PG4, antiviral treatment with (val)ganciclovir reduced skin inflammation and speeded up improvement of health status. However, it is essential to note that generalizations cannot be drawn from single cases. The diagnostic results obtained in this study do not provide conclusive evidence of an etiologic link between CMV infection and aggravation of the skin condition. In addition, the findings of this study do not prove that the induction of remission was causally related to antiviral therapy. The observed temporal correlation between antiviral intervention and improvement of skin lesions may only be coincidental. For further details, see section 4.1.2.4.

It also must be mentioned that detecting CMV IgM in serum or CMV DNA in lesional skin might not always be clinically relevant. In this study, patient PG5 harbored CMV DNA in lesional skin, and patients PG6 and PG7 tested positive for CMV IgM in serum. Interestingly, these patients' skin lesions and/or health status stabilized or improved after all, although antiviral treatment had not been carried out. Hence, these patients were obviously not subject to CMV reactivation, or CMV reactivation was not the main driver of skin inflammation and treatment refractoriness. In the case of patient PG5, the presence of CMV DNA in lesional skin may have been related to CMV DNA shedding, which would be in accordance with the negative CMV IHC result. Indeed, detection of CMV DNA shedding was possible in one or more body sites (including nose, skin, oral cavity, and vagina) with a frequency of 7–8 % among asymptomatic adults (Forte et al., 2020). Likewise, high-throughput metagenomic DNA sequencing revealed that 5.9 % of generally healthy adults who were not symptomatic for acute infections harbored CMV DNA in skin swabs collected from the retroauricular crease (Wylie et al., 2014). The limitations of the CMV IgM test have already been delineated. In particular, IVIg therapy may have impacted the serological result (Ljungman et al., 2017) in the case of patient PG7. To sum up, these cases highlight that treatment refractoriness does not necessarily result from CMV reactivation, even if the diagnostic results are suspicious. In fact, different clinical, demographic, and social factors could be related to reduced effectiveness of biologic therapies in psoriasis patients (Warren et al., 2019). The loss of treatment response to biologicals may, for example, also result from anti-drug antibody formation. Finally, innovative treatment strategies targeting other molecular pathways

may be beneficial in some patients. For instance, tocilizumab, an IL-6 receptor antagonist, has been found to be effective in patients with systemic sclerosis showing therapy-refractory joint and skin involvement (Panopoulos et al., 2022).

To validate our assumptions and to prove the existence of a causal association between CMV (and EBV) reactivation and therapy refractoriness in immunocompromised patients with chronic skin diseases, further studies are required:
First, the real epidemiologic relevance of CMV (and EBV) reactivation in dermatological patients must be determined in a larger cohort of patients. In particular, longitudinal studies investigating the relation between disease activity, viral reactivation, and treatment response over a long time are needed.
To shed more light on the impact of CMV (and EBV) reactivation on skin pathology, additional basic scientific research has to be carried out. Potential approaches have been described in the previous section (see section 4.3.2).
Moreover, the benefit of active (e.g., serology- and/or PCR-based) surveillance strategies and antiviral treatment of CMV reactivation has to be evaluated in clinical pilot studies. In particular, clearly defined criteria are needed for both indication and mode of antiviral therapy. In this way, the precise subgroup(s) of patients that is/are likely to benefit from testing and antiviral treatment may ultimately be identified. It is important to note that the administration of antiviral agents may be associated with undesirable effects. The major side effect of ganciclovir is myelosuppression (Dioverti and Razonable, 2016). Even though it usually disappears after discontinuation of the drug (Dioverti and Razonable, 2016), this adverse drug reaction might be critical in patients already being immunocompromised. Therefore, future studies should not only address the efficacy but also the safety of antiviral treatment.
Though the potential cost-saving implications are evident, a detailed cost-benefit analysis with a larger sample size has to be conducted before establishing recommendations regarding CMV screening in particular patient groups.

4.4 Novel aspects and outlook

The current gold standard in the management of patients with chronic skin diseases consists of initiating systemic immunosuppressive/immunomodulating agents in addition to topical treatment. This usually results in mild to severe immunosuppression. The monitoring of these patients during the disease course is limited to regular laboratory workup, including blood cell counts and serum parameters (liver and kidney function). In contrast, potential factors negatively influencing the skin condition – apart from the underlying skin disease itself – are rarely considered. In particular, the possible deleterious effects of CMV reactivation with its predilection to occur within inflammatory environments have not been sufficiently recognized so far.

Indeed, the updated S2K guidelines on the management of pemphigus vulgaris and foliaceus initiated by the European Academy of Dermatology and Venereology recommend close monitoring of potential adverse effects related to immunosuppressive treatment, including CMV reactivation (Joly et al., 2020). Even though there is apparently increasing awareness of the relevance of CMV reactivation in immunocompromised dermatological patients, it is essential to note that reactivation of CMV infection may not only present with clinical findings commonly related to this herpesvirus (e.g., gastrointestinal or pulmonary involvement). Instead, the skin condition may be affected by either cutaneous CMV disease or subclinical CMV reactivation. The recommendations that have been made in this regard so far mostly come from anecdotal reports and are primarily related to bullous autoimmune dermatoses and ulcers. For example, Gee et al. (2012) recommended evaluating patients with bullous autoimmune diseases for concurrent cutaneous viral infection in case of changing or atypical skin and/or mucosal lesions, new skin pain, constitutional symptoms, rapid progression and/or lack of treatment response. The authors advocated a low threshold to perform additional diagnostic tests, to obtain repeat biopsies, and to consider empiric treatment in severe cases, including antiviral agents and modification of immunosuppression (Gee et al., 2012). In addition, Pushkarevskaya et al. (2017) suggested considering viral infection or reactivation in the differential diagnosis of new skin and mucosal lesions in immunosuppressed patients with bullous autoimmune diseases, particularly in the case of relapse or treatment refractoriness. In terms of ulcers, Chai and Oh (2020) emphasized the need to take CMV infection into account if skin ulcers of immunocompromised patients did not respond to antibiotic treatment. Similarly, Ali et al. (2014) recommended considering atypical infections if ulcers were deteriorating or did not respond to treatment, especially in immunocompromised patients.

The results of this research project support the hypothesis that skin inflammation and immunosuppression predispose to CMV reactivation, which, in turn, hinders the efficacy of immunosuppressive/immunomodulating treatment and even worsens the skin pathology. The case series from our clinic highlights that it is essential to consider CMV reactivation in the differential diagnosis of therapy-refractory skin lesions in the immunologically incompetent host. Though CMV infection may reinforce inflammatory processes, the diagnosis and treatment of CMV reactivation are frequently delayed by a lack of suspicion. However, as efficient therapies are available to control CMV replication, it is critical to establish the diagnosis early in the disease course and to promptly implement antiviral treatment. Thus, the vicious circle of skin inflammation and CMV reactivation may be broken, and the skin condition may eventually be controlled.

In daily clinical practice, laboratory testing for CMV reactivation is rarely carried out. This may, at least in part, be related to the limitations of serology, which have been outlined in the previous sections of this thesis. In fact, the detection of CMV-specific antibodies in serum is inexpensive, little invasive, and well established. However, the results do not allow reliable conclusions on the pathogenetic effects of CMV in the respective tissue (i.e., lesional skin). Therefore, tissue-based

diagnostic approaches (in addition to blood-based tests) may be valuable. In contrast to other studies investigating CMV in peripheral blood, we demonstrated the presence of CMV DNA/proteins and CMV-specific T cells in lesional skin of immunocompromised patients with treatment-refractory chronic skin diseases. The screening of lesional skin biopsies allows early initiation of antiviral therapy in the case of CMV reactivation. Thus, long-term hospitalization and health care costs may be reduced as skin lesions may improve faster than without antiviral treatment. However, the need to invasively obtain a skin biopsy represents the major disadvantage of this diagnostic approach. In this context, the implementation of novel tissue acquisition procedures (e.g., microbiopsies) may be helpful.

To summarize, this study comprehensively addressed the pathogenetic relevance of CMV reactivation in immunocompromised patients with chronic skin diseases. It is a combination of comparative studies (PCR, serology), histopathological examinations, *in vitro* experiments, a case series, and an economic analysis. The major strength of this study is the investigation of many different types of biomaterials using a wide array of laboratory techniques. The results extend earlier observations suggesting that immunocompromised patients with therapy-refractory chronic skin diseases may be at risk for CMV reactivation. These patients should be monitored closely and may benefit from antiviral treatment. Future research efforts attempting to further our understanding of this complication are needed, not least because more and more systemically acting drugs with immunosuppressive/immunomodulating properties (e.g., biologicals and small molecules) are administered in dermatology. This may finally result in the development of clinical guidelines regarding the screening, diagnosis, and treatment of CMV reactivation in immunocompromised patients with (therapy-refractory) chronic skin diseases. Last but not least, preventive measures appear to be reasonable. For example, using more targeted therapeutic drugs instead of nonspecific immunosuppressants (particularly glucocorticoids) may be helpful to avoid infectious complications.

Interestingly, Miehsler et al. (2018) pointed out that there were initial indications of a potential link between EBV and refractory colitis (analogous to CMV). However, it remains unclear whether this is merely an epiphenomenon (or whether there is a causal association) and if antiviral treatment is reasonable also in this respect, particularly as EBV is frequently detectable in inflamed gastric and ileocolonic mucosa (Miehsler et al., 2018). The same questions arise in relation to immunocompromised patients with therapy-refractory chronic skin diseases. The results of this study, particularly the demonstration of lytic EBV infection-associated protein and EBV-specific T cells in lesional skin, support a disease-modifying role of EBV reactivation, which should be further investigated. However, no antiviral therapeutic has been approved for EBV infection until now. Though some antiviral drugs have been demonstrated to inhibit EBV replication *in vitro*, the clinical success was limited (Andrei et al., 2019).

Finally, the recent insights regarding the involvement of EBV infection in the pathogenesis of multiple sclerosis (Bjornevik et al., 2022; Schneider-Hohendorf et al., 2022) highlight that there is still a lot to be explored about the herpesviruses EBV and CMV. Even though CMV seroprevalence has significantly declined over the last three decades (Hoehl et al., 2020), the implications of CMV infection will continue to challenge clinicians and patients in the foreseeable future. To date, no vaccine is available.

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Publication Resulting from This Thesis

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Appendix

Table A1: Detailed characteristics of patients in the “Prospective Study Group, PG”
(Table continues on the next page.)

ID	Sex	Age	Diagnosis	Biologicals/ immunosuppressants	IVIg	Oral thrush	%LYMPH	#LYMPH	CMV IgG	CMV IgM	EBNA1 IgG	EBV-VCA IgM	CMV PCR [EDTA blood]	EBV PCR [EDTA blood]
PG1	F	66	Drug-induced exanthema	GC	Yes	Yes	22.8	0.48	+	+	+	-	+ (2660 IU/ml)	+ (small amount)
PG2	M	45	Pemphigus vulgaris	GC, AZA, RTX	No	No	7.5	0.90	+	±	+	-	+ (644 IU/ml)	ND
PG3	M	62	Pyoderma gangrenosum	GC, CsA, IFX	Yes	No	18.0	1.57	+	+	+	-	-	-
PG4	F	61	Psoriasis inversa and capitis (and arthropathica)	IXE	No	Yes	38.0	3.12	+	+	+	-	-	- (*)
PG5	F	88	Bullous pemphigoid	GC, AZA	No	No	19.0	1.60	+	-	+	-	-	-
PG6	F	78	Systemic sclerosis	GC, MPA	No	No	12.0	0.78	+	+	-	-	-	-
PG7	F	70	Lichen sclerosus	MTX	Yes	Yes	24.5	2.40	+	+	+	-	-	-
PG8	F	42	SLE	GC	No	Yes	14.9	0.80	+	±	-	-	-	-
PG9	F	47	PAPASH syndrome	IFX, MTX	No	No	35.5	2.50	-	-	+	-	-	+ (23 geq/10^5 cells)
PG10	F	50	Pemphigus vulgaris	GC	Yes	No	15.8	2.10	+	-	+	±	-	+ (small amount)
PG11	F	40	Pyoderma gangrenosum	GC, CsA	Yes	No	16.5	2.00	+	-	+	+	-	-
PG12	F	71	Psoriasis vulgaris (and arthropathica)	GC	No	No	20.4	2.10	-	-	-	-	-	-
PG13	F	51	Pemphigus vulgaris	GC, AZA	Yes	No	13.3	1.50	-	-	+	+	-	-
PG14	F	43	Pyoderma gangrenosum	GC, ADA	Yes	No	10.9	1.10	+	-	+	-	-	-
PG15	M	75	Dermatomyositis	GC	Yes	No	8.5	0.60	+	-	+	-	-	-
PG16	F	52	Pemphigus vulgaris	GC	Yes	No	34.2	1.60	+	-	+	-	-	-
PG17	M	97	Bullous pemphigoid	GC, MPA	No	No	14.0	2.00	-	-	+	-	-	-
PG18	F	42	SLE	BEL	No	Yes	22.6	2.70	+	-	+	-	-	-
PG19	F	75	Linear IgA bullous dermatosis	RTX	Yes	No	22.4	1.30	+	-	+	-	-	-
PG20	F	67	Pemphigus vulgaris	GC, AZA	Yes	No	32.0	2.40	+	-	+	-	-	-

Table A1: Detailed characteristics of patients in the “Prospective Study Group, PG”
(Table continued from the previous page.)

ID	Sex	Age	Diagnosis	Biologicals/ immunosuppressants	IVIg	Oral thrush	%LYMPH	#LYMPH	CMV IgG	CMV IgM	EBNA1 IgG	EBV-VCA IgM	CMV PCR [EDTA blood]	EBV PCR [EDTA blood]
PG21	M	49	Pyoderma gangrenosum	GC	Yes	No	17.0	2.86	+	-	+	-	-	-
PG22	F	60	Systemic sclerosis	GC, RTX	No	No	18.8	1.80	+	-	+	-	-	-
PG23	M	70	Psoriasis vulgaris (and arthropathica)	IFX	No	No	17.8	2.40	-	-	-	-	-	-
PG24	F	74	EBA	GC	Yes	No	9.0	0.27	+	-	+	-	-	-
PG25	F	48	SLE	GC	No	No	24.0	2.96	-	-	+	-	-	-
PG26	F	47	SLE	GC, BEL	No	No	28.2	1.10	-	-	+	-	-	-
PG27	M	64	Dermatomyositis	GC, MPA	Yes	No	13.1	1.10	+	-	+	-	-	-
PG28	F	44	Erythroderma	GC, IFX	No	No	12.0	1.42	-	-	+	-	-	-
PG29	M	44	SLE	BEL, MTX	No	No	24.0	1.24	-	-	+	-	-	-
PG30	F	61	Drug-induced exanthema	GC	No	No	16.0	1.11	+	-	+	-	-	-

Abbreviations: *ADA = adalimumab; AZA = azathioprine; BEL = belimumab; CMV = cytomegalovirus; CsA = cyclosporine; EBA = epidermolysis bullosa acquisita; EBNA1 = Epstein-Barr virus nuclear antigen 1; EBV = Epstein-Barr virus; EDTA = ethylenediaminetetraacetic acid; F = female; GC = glucocorticoid; geq = genome equivalents; IFX = infliximab; IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; IU = International Unit; IVIg = intravenous immunoglobulins; IXE = ixekizumab; M = male; MPA = mycophenolic acid; MTX = methotrexate; ND = not done; PAPASH = pyoderma gangrenosum, acne, psoriasis, arthritis, and suppurative hidradenitis; PCR = polymerase chain reaction; RTX = rituximab; SLE = systemic lupus erythematosus; VCA = viral capsid antigen; + = positive; ± = borderline; - = negative; * = PCR analysis of serum (instead of EDTA blood); %LMYPH = lymphocytes, relative (%); #LMYPH = lymphocytes, absolute (x 10^3/µl).*

Table A2: Detailed characteristics of patients in the “Prospective Study Group (PCR), PG(P)”

ID	Sex	Age	Diagnosis	Biologicals/ immunosuppressants	Oral thrush	%LYMPH	#LYMPH	CMV PCR [lesional skin]	EBV PCR [lesional skin]	HSV-1/2 PCR [lesional skin]	VZV PCR [lesional skin]	CMV IHC [lesional skin]	EBER ISH [lesional skin]	EBV IHC [lesional skin]
PG1	F	66	Drug-induced exanthema	GC	Yes	22.8	0.48	+ (viral DNA load unknown)	ND	ND	ND	ND	ND	ND
PG3	M	61	Pyoderma gangrenosum	GC	No	3.0	0.40	+ (11.95 mIU/cell)	-	-	-	+ (weakly)	ND	ND
PG4	F	61	Psoriasis inversa and capitis (and arthropathica)	IXE	Yes	38.0	3.12	-	-	-	-	ND	ND	ND
PG5	F	88	Bullous pemphigoid	GC, AZA	No	19.0	1.60	+ (58.43 mIU/cell)	+ (6.74 mIU/cell)	-	-	-	-	+
PG8	F	42	SLE	GC	Yes	14.9	0.80	-	+ (0.02 mIU/cell)	-	-	ND	-	+
PG11	F	40	Pyoderma gangrenosum	GC, CsA	No	16.5	2.00	-	+ (0.13 mIU/cell)	-	-	ND	-	-
PG12	F	71	Psoriasis vulgaris (and arthropathica)	GC	No	20.4	2.10	-	+ (0.53 mIU/cell)	-	-	ND	-	+
PG14	F	43	Pyoderma gangrenosum	GC, ADA	No	15.3	1.60	-	-	-	-	ND	ND	ND
PG16	F	52	Pemphigus vulgaris	GC	No	34.2	1.60	-	-	-	-	ND	ND	ND
PG23	M	70	Psoriasis vulgaris (and arthropathica)	IFX	No	17.8	2.40	-	-	-	-	ND	ND	ND
PG25	F	48	SLE	GC	No	24.0	2.96	-	-	-	-	ND	ND	ND
PG27	M	64	Dermatomyositis	GC, AZA	No	7.7	0.90	-	-	-	-	ND	ND	ND
PG28	F	44	Erythroderma	GC, IFX	No	9.9	1.60	-	-	-	-	ND	ND	ND
PG29	M	44	SLE	BEL, MTX	No	24.0	1.24	-	-	-	-	ND	ND	ND

Abbreviations: *ADA = adalimumab; AZA = azathioprine; BEL = belimumab; CMV = cytomegalovirus; CsA = cyclosporine; DNA = deoxyribonucleic acid; EBER = Epstein-Barr virus-encoded RNA; EBV = Epstein-Barr virus; F = female; GC = glucocorticoid; HSV = herpes simplex virus; IFX = infliximab; IHC = immunohistochemistry; ISH = in situ hybridization; IXE = ixekizumab; M = male; mIU = milli-International Unit; MTX = methotrexate; ND = not done; PCR = polymerase chain reaction; SLE = systemic lupus erythematosus; VZV = varicella-zoster virus; + = positive; - = negative; %LMYPH = lymphocytes, relative (%); #LMYPH = lymphocytes, absolute (x 10^3/µl).*