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PII: S0022-202X(21)02161-8

DOI: https://doi.org/10.1016/j.jid.2021.07.171

Reference: JID 3096

To appear in: *The Journal of Investigative Dermatology*

Received Date: 20 January 2021

Revised Date: 24 June 2021

Accepted Date: 5 July 2021

Please cite this article as: Speth P, Jargosch M, Seiringer P, Schwamborn K, Bauer T, Scheerer C, Protzer U, Schmidt-Weber C, Biedermann T, Eyerich S, Garzorz-Stark N, Immunocompromised patients with therapy-refractory chronic skin diseases show reactivation of latent EBV and CMV infection, *The Journal of Investigative Dermatology* (2021), doi: https://doi.org/10.1016/j.jid.2021.07.171.

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Immunocompromised patients with therapy-refractory chronic skin diseases show reactivation of latent EBV and CMV infection

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ABSTRACT

Reactivation of latent Epstein-Barr virus (EBV) and/or Cytomegalovirus (CMV) infection is a dreaded complication in immunocompromised patients receiving hematopoietic stem cell transplantation. Evidence is sparse if subclinical reactivation of viral infection may also be of clinical relevance in dermatological patients. We screened patients (n= 206) suffering from chronic skin diseases for subclinical reactivation of EBV and CMV infection. We found that immunocompromised patients with therapy-refractory chronic skin diseases showed higher rates of subclinical reactivation of CMV and EBV infection (6.7 % vs. 0 % for EBV and 16.7 % vs. 5.6% for CMV) and higher prevalence of virus specific DNA in skin tissue (30.8 % vs. 0% for EBV and 21.4% vs. 0% for CMV) as compared to non-immunocompromised patients with chronic skin diseases. T cells isolated from lesional skin exhibited up to 14-fold increased proliferation with production of Th1 and Th17 cytokines upon stimulation with viral proteins providing evidence for possible aggravation of the underlying skin diseases by viral infection. Improvement of skin lesions in patients with reactivation of CMV infection (n=4) was observed upon anti-viral treatment. Our data suggests that subclinical reactivation of EBV and/or CMV infection is an under-recognized condition in the dermatological patient population with chronic skin diseases.

Key words: Epstein-Barr virus, Cytomegalovirus, chronic skin diseases, reactivation of latent viral infection, immunosuppression, immunomodulation

Abbreviations: EBV: Epstein-Barr virus, CMV: Cytomegalovirus (CMV), IgM: Immunoglobulin M, IgG: Immunoglobulin G, IVIg: Intravenous Immunoglobulin; SG-RC:

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Study group (SG) within retrospective screening cohort (RC); CG-RC: control group (CG) within retrospective screening cohort (RC); SG-PC: study group (SG) within prospective cohort (PC); CG-PC: control group (CG) within prospective cohort (PC); SG-PC-P: patients for PCR analysis of lesional skin within study group of prospective cohort (SG-PC); CG-PC-P: control group for SG-PC-P; MODCs: monocyte-derived dendritic cells; PASI: Psoriasis area and severity index

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INTRODUCTION

Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) are herpesviruses which – like all herpesviruses –persist life-long in a latent state in the host organism after primary infection(Stern et al., 2019, Thorley-Lawson, 2015). Worldwide, more than 90 % of individuals are affected by EBV(Dowd et al., 2013), and latent CMV infection affects 50-65 % of adults in Europe (Docke et al., 2003). Once the immune system is compromised by inflammation or immunosuppression, the viruses can be awaked from latency and cause EBV related lymphoproliferative disorders and malignancies(Andrei et al., 2019, Sangueza-Acosta and Sandoval-Romero, 2018) or active CMV infection such as pneumonia, ulcers and fever(Cook, 2007), respectively. But interestingly, also subclinical reactivation of latent infection has implications for general inflammatory processes. EBV has been associated with virus-disease independent autoimmune processes. It was shown that subclinical EBV primary infection and lytic reactivation induce Thyrotropin receptor autoantibodies(Tamoto et al., 2019). Moreover, EBV infection is regarded as an environmental risk factor for the development of lupus as the first lupus-specific autoantibodies may arise from antibodies directed against EBV(Harley and James, 2006). Also subclinical active CMV infection and more importantly the immune responses triggered by CMV reactivation can have important pathophysiological consequences which may lead to prolonged inflammation resulting in chronic allograft injury(Reinke et al., 1994). In line with this, CMV infected cells have been described to produce increased amounts of proinflammatory cytokines such as TNF-α (Alcendor et al., 2012) which in turn, have been shown to trigger CMV reactivation (Fietze et al., 1994, Forte et al., 2018).

Not only inflammatory cytokines and stress, also immunosuppressive treatments may induce reactivation of EBV and CMV: Glucocorticoids have been shown to induce reactivation of

EBV(Yang et al., 2010) and for CMV it was demonstrated that already low-dose glucocorticoid treatment was potent enough to reactivate CMV(Troselj-Vukic et al., 2007). Both chronic inflammation and immunosuppression to control inflammation are characteristics of the pathogenesis and treatment of chronic skin diseases, respectively. However, in contrast to patients receiving hematopoietic stem cell transplantation who are routinely monitored for viral loads(Lin and Liu, 2013, Stevens et al., 2001), the patient population suffering from chronic skin diseases under immunosuppression has not yet been in the focus of clinical and scientific interest. Thus, not much is known about the role of (sub)clinical reactivation of EBV and CMV infection in chronic skin diseases and chronic skin diseases requiring long-term immunosuppressive treatments such as inflammatory skin diseases, bullous autoimmune diseases and skin lymphoma.

We hypothesize that subclinical reactivation of EBV and/or CMV infection may be of particular relevance in patients with chronic skin inflammation. On the one hand, these patients fulfill two major prerequisites of EBV and CMV reactivation in the human organism, namely ongoing inflammation, and therapy-induced immunosuppression. On the other hand, cytokines released from virus-infected cells may also aggravate ongoing skin inflammation starting a vicious cycle of viral reactivation and inflammation. As both viruses are highly prevalent and at least for CMV infection antiviral therapies are available(Cook, 2007, Pagano et al., 2018), detection of EBV and CMV reactivation at an early stage may be clinically relevant to initiate antiviral treatment, to eventually control skin diseases and to reduce long-term hospitalization in dermatology departments.

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RESULTS

EBV but not CMV DNA was detected in the skin of immunocompromised patients suffering from chronic skin diseases

Latent infection with EBV is indicated by seroprevalence of anti-EBNA IgG(Hess, 2004) and/or detection of EBV-specific DNA in the skin. Latent infection with CMV is indicated by the seroprevalence of anti-CMV IgG (Docke et al., 2003) and/or detection of CMV specific DNA in the skin. To investigate whether patients with chronic skin conditions under immunosuppressive treatment would show increased of latent infection with EBV and/or CMV in lesional skin, we screened for viral DNA in skin biopsies in a cohort of 43 patients with chronic skin diseases under systemic immunosuppressive or immune-modulating therapies (SG-RC group, n=43) as well as in an age and sex matched group of patients suffering from chronic skin diseases without systemic immunosuppressive or immune-modulating therapies (CG-RC group, n=43). Examined skin conditions were chronic ulcers, autoimmune-mediated and chronic inflammatory skin diseases as well as skin lymphoma (details of the cohorts s. Table S1 and S2). However, for EBV analysis, skin lymphoma were excluded, as it could not be ruled out that EBV infection drove lymphoma pathogenesis in our patients(Novelli et al., 2009). We found that within SG-RC 5 out of 43 patients (11.6 %) were positive for EBV DNA in the skin (mean EBV load 0.87 mIU/cell ± 0.72 mIU/cell), whereas none of the CG-RC patients were positive for EBV DNA. To gain further insights into the cellular localization of EBV in the tissue EBER ISH was performed. In one of the EBV positive patients, Patient RC-1 with the diagnosis of pyoderma gangrenosum, a discrete signal within the lymphocytic infiltrate was detected (Figure 1 a). Moreover, this patient showed positivity for EBV early antigen EA-D as detected by immunohistochemistry (s. Figure

S1 a). Neither patients in the SG-RC nor patients in CG-RC groups were positive for CMV DNA.

Patients under immunosuppressive or immune-modulating therapy with treatment-refractory courses of chronic skin diseases show latent infection and reactivation of EBV and CMV infection

Patients suffering from chronic skin diseases not only being under immunosuppressive or immune-modulating therapies but also showing persistence of skin lesions despite intensive therapy might be particularly suspicious of viral infection. Therefore, we prospectively screened for both latent infection with EBV ad CMV and subclinical reactivation of latent viral infection with EBV and CMV in a cohort of patients fulfilling the criteria of both treatment with immunosuppressive/immune-modulating therapy and low response rate to therapy (SG-PC group, n= 30) and compared the data to an age and sex matched control group of patients suffering from chronic skin diseases who were not under immunosuppressive or immune-modulating therapy (CG-PC group, n = 90). Included skin conditions were chronic ulcers, autoimmune-mediated and chronic inflammatory skin diseases (s. Table S1-3). Reactivation of latent infection with EBV (CMV) was defined as positivity for anti-EBNA1 IgG (anti-CMV IgG) plus detection of anti-EBV-VCA-IgM levels (anti-CMV-IgM levels).

We found that frequencies of patients with anti-EBV-IgG antibodies did not differ between SG-PC and CG-PC cohorts (Figure 2 b), whereas titers of anti-EBNA1-IgG antibodies were significantly higher in the SG-PC than in the CG-PC group (Figure 2 c). 6.7 % of the patients in the SG-PC group showed both IgM and IgG antibodies to EBV, whereas no patient in the CG-PC group showed positivity for anti-EBV-VCA IgM antibodies (Figure 2 d). For CMV, prevalence

and titers of anti-CMV-IgG antibodies in SG-PC group were not significantly higher than in the CG-PC group (Figure 2 e, f). However, 5 out of 30 patients (= 16.7 %) in the SG-PC group showed both seroprevalence for IgG and IgM antibodies to CMV hinting at reactivation of CMV infection, whereas only 5 out of 90 patients (= 5.6 %) in the CG-PC group showed both IgG and IgM antibodies to CMV (Figure 2 g).

As patients did not improve under immunosuppressive or immune-modulating therapy, biopsies from lesional skin could be obtained in 14 out of 30 patients from SG-PC (SG-PC-P subgroup). We found that 4 out of 13 samples (= 30.8 %) in SG-PC-P were tested positive for EBV DNA, whereas none of the samples in the age and sex matched control group (CG-PC-P) was positive for EBV DNA (Figure 2 h). For CMV, in 3 out of 14 samples (= 21.4 %) CMV DNA could be detected, whereas no CMV DNA was detected in the CG-PC-P group (Figure 2 i). Taken together, a significant rate of both latent infection in the skin and reactivation of latent infection with CMV and EBV in therapy-resistant patients under immunosuppressive or immune-modulating therapies could be found.

Virus specific T cells may aggravate underlying chronic skin diseases in therapy-refractory patients

To elucidate if CMV or EBV antigen in the skin could trigger immune responses and possibly aggravate ongoing cutaneous inflammation, we were able to isolate and stimulate T cells from lesional skin of two patients (PC-1) and (PC-2) of the SG-PC group with EBV and CMV antigen (Figure 2, Table S3). Both PC-1 (Figure 2 a) and PC-2 (Figure 2 b) showed the profile of latent EBV infection and reactivation of CMV infection (Figure 2, Table 1). Indeed, a distinct population of EBV- and CMV-specific T cells in lesional skin was detected. Upon presentation

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of recombinant EBV proteins (BZLF1, a lytic infection-related immediate early antigen + EBNA3A, a expressed in all forms of latent EBV infection) by autologous human monocyte-derived dendritic cells (MODCs), lesional T cells proliferated *in vitro* at a 3.4 fold proliferation rate in PC-1 and at a 9.4 fold proliferation rate in PC-2 compared to non-stimulated MODCs (Figure 2 a and b). Upon presentation of CMV lysate T cells proliferated at a 14 fold proliferation rate in PC-1 and at a 6.5 fold proliferation rate in PC-2 compared to non-stimulated MODCs (Figure 2 a and b). With the exception of PC-1 who showed a higher percentage of CD8 positive than CD4 positive T cells within the fraction of proliferating CD3 positive T cells upon stimulation with EBV, the percentage of CD4-positive cells was overall higher compared to the CD8-positive T cells within the fraction of CD3 positive proliferating T cells hinting at a profound immunological bystander effect. To corroborate this assumption, we next analyzed cytokines in the supernatant of the virus-specific proliferating T cells. Although only a small proportion (range: 0.97–4.34 %) of all T cells in culture proliferated upon CMV or EBV antigen presentation, levels of cytokines produced by T cells in PC-1 and PC-2 were profoundly increased compared to control MODCs, in particular levels of cytokines related to Th1 and Th17 immunity such as CXCL-10 (EBV =38.2x; CMV = 43.3x), IFN-γ (EBV = 2.6x; CMV = 2.2x), IL-8 (EBV = 25.5x; CMV = 43.4x), and MCP-1 (EBV = 53.3x; CMV = 30.3x) (Figure 2 c).

Immunocompromised patients with treatment-refractory courses of disease and reactivation of CMV show improvement of skin lesions upon antiviral treatment

As (subclinical) reactivation of viral infection may play a clinically relevant and aggravating role for underlying skin conditions in patients who show therapy-refractory courses of disease, we sought to elucidate if those patients would benefit from anti-viral therapeutic intervention. In

total, four patients from the SG-PC group (PC-2, PC-3, PC-4 and PC-5) showing positivity for both anti-CMV-IgG and anti-CMV-IgM were treated with (val)ganciclovir (s. Table 1). One patient (PC-3) showed temporary and three patients (PC-2, PC-4 and PC-5) showed permanent improvement of skin lesions upon (val)ganciclovir treatment. Patients PC-2 and PC-5 will be presented in more detail in the following (Figures 3 and 4). 61-year-old male patient PC-5 (Figure 3) suffering from pyoderma gangrenosum showed rapid progression of skin disease despite topical wound care and intensive systemic treatment including prednisolone, infliximab, cyclosporine and IVIgs (Figure 3 a). Fulfilling the criteria of therapy-refractory chronic skin disease and immunosuppression, the patient was analyzed for EBV and CMV infection 9 weeks after the ulcer occurred for the first time. Indeed, both serology for CMV-IgG and CMV-IgM were positive and, moreover, PCR of lesional skin showed positivity for CMV DNA. Immunohistochemistry for CMV protein (CCH2 + DDG9 antibody) showed a weak positive nuclear signal (s. Figure S1 b). Ganciclovir treatment was initiated upon serological constellation of subclinical reactivation of CMV infection. Both CMV-IgG and CMV-IgM titers dropped during treatment (Figure 3 b) and full recovery of pyoderma gangrenosum was reached showing stable recovery until today (year 2). Patient PC-2 (Figure 4) was a 61-year-old woman suffering from psoriasis inversa et capitis and psoriatic arthritis. Despite intensive treatment with ixekizumab, UVB phototherapy and topical glucocorticoids, skin lesions did not improve. Serology showed the picture of subclinical reactivation of CMV infection. Initiation of treatment with valganciclovir was temporally correlated with improvement of skin lesions (Figure 4 a) and resulted in a decrease of CMV IgM antibodies (Figure 4 b).

Early detection of viral reactivation could reduce morbidity and health care costs

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The presented cases suggest that antiviral treatment for CMV reactivation is warranted in patients suffering from chronic skin diseases who show therapy-refractory courses of disease despite intensive treatment regimens. Prospective screening of patients at risk for CMV reactivation could not only reduce morbidity, but also reduce costs for the health care system. Therefore, we analyzed two representative inpatient wards of our department of dermatology regarding diagnoses, therapy regimens and courses of diseases. Within a period of eight weeks, 29 out of all 223 patients admitted (13.0 %) were treated with immunosuppressants and/or biologicals for at least 2 weeks prior to admission that have been implicated with CMV reactivation. Thereof, 8 patients (3.6 % of all 223 patients) showed prolonged exacerbation of the skin condition despite intense immunosuppressive treatment and are therefore at risk for CMV reactivation possibly benefiting from CMV screening. In our prospective study cohort PC, 5 out of 30 patients (= 16.7 %) at risk showed reactivation of CMV infection. Given the conservative assumption that reactivation of viral infection aggravates underlying skin diseases and antiviral treatment improves skin conditions in only 50 % of all cases receiving antiviral treatment, the absolute reduction of risk due to screening (and consequent therapy) for reactivation of CMV infection would be 8.3 %. This results in a number needed to screen(Rembold, 1998) of 12 which means that 12 patients at risk need to be screened to reduce CMV-induced morbidity in one patient.

DISCUSSION

The interaction between microbes and chronic skin diseases has been in the focus of research not only since modern sequencing technologies enabled extensive characterization of the human microbiome. One of the best analyzed examples is the increased colonization with

staphylococcus aureus in patients suffering from atopic eczema correlating with disease severity(Paller et al., 2019) or the association of acute guttate psoriasis with streptococcal infection of the throat (Leung et al., 1995). However, also the pathophysiological significance of viral infections as triggers and aggravators of chronic skin diseases has been increasingly recognized, not least because of the proinflammatory cytokines produced by infected and virus defending cells (Asadullah et al., 1999, Georgescu et al., 2019, Morar et al., 2010, Senger and Sinha, 2012). First evidence has been collected that not only the classically suspected viruses such as human immunodeficiency virus, herpes simplex virus and hepatitis C virus are of importance, but also the herpesviruses EBV and CMV may play a so far underestimated role as modulators for chronic skin diseases (Weitz et al., 2011),(Docke et al., 2003, Grimes et al., 1996) . To our knowledge previously unreported, we present a study to screen patients suffering from various chronic skin diseases under immunosuppression for latent and subclinical reactivation of EBV and/or CMV infection.

First, we found that in our retrospective study cohort (RC cohort) 11.6 % of immunocompromised patients suffering from chronic skin diseases were positive for EBV DNA in the skin. The tropism of EBV for lymphocytes and nasopharyngeal epithelial cells have been demonstrated (Kasahara and Yachie, 2002), whereas skin keratinocytes do not appear to be the physiological target cells for EBV infection *in vivo(Neuhierl et al., 2002).* Using in situ hybridization we corroborated that EBV was indeed localized in immune cells.

In contrast to EBV, none of the patients from our retrospective cohort study was positive for CMV DNA in the skin. This finding might be explained by the higher prevalence rate of EBV infections compared to CMV infections in the general population(Docke et al., 2003, Dowd et al., 2013) and is in line with a previous study showing no significant difference between the

occurrence rates of CMV DNA in scleroderma patients and normal controls(Ohtsuka and Yamazaki, 2006). In contrast, however, Grimes et al. found CMV DNA in vitiligo and not in healthy controls (n=22)(Grimes et al., 1996). Interestingly, within the sub-cohort of these CMV positive vitiligo patients, the progression rate of disease, the rate of other autoimmune diseases as well as the antibody load was significantly higher indicating that particularly aggressive, non-improving skin conditions are at risk for CMV reactivation. We addressed this constellation in our second prospective cohort including immunocompromised patients suffering from chronic skin diseases that despite intensive therapy showed persistence or even progression of skin lesions (PC cohort).

We found that –frequencies of anti-EBV-IgM and/or anti-CMV-IgM were higher in the study group than in the control group implying reactivation of latent viral infection. Given the fact that immunosuppression may result in false negative seroprevalence, reactivation of viral infection may be even more present in the study cohort. Positivity for EBV and CMV DNA in the skin, respectively, was also higher in the prospective study group than in the control group hinting at a clear impact of immunosuppression leading to insufficient control of viral infection. As PCR for CMV in the skin was negative for control patients in both retrospective and prospective study cohort and moreover for immunocompromised patients suffering from inflammatory skin diseases in the retrospective cohort, the third condition – persistence or even progression of skin lesions despite intensive therapy – may be crucial for CMV positivity in the skin. Based on these findings, one might speculate that reactivation of CMV significantly aggravates ongoing chronic skin inflammation impeding improvement of skin lesions. Indeed, we found that T cells isolated from human lesional skin showed robust proliferation upon CMV antigen presentation. In transplant settings, monitoring of CMV-specific T cell responses is co-decisive for antiviral

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303 therapy and an adequate tool to decide on the appropriate level of immunosuppressive therapy in
304 transplant patients to avoid CMV-reactivation but also graft rejection(Korber et al., 2020). Our
305 data indicates that monitoring of EBV and CMV responses might be also of high relevance in the
306 immunocompromised dermatological patient population with treatment refractory courses of
307 disease. Proliferation of T cells upon viral proteins resulted in the release of pro-inflammatory
308 cytokines mainly of the CD8 as well as the Th1 and Th17 immune response pattern. These
309 cytokines have been widely accepted as disease driving cytokines in the pathogenesis of
310 psoriasis, pyoderma gangrenosum and other chronic skin diseases. Hence, not only CMV
311 infected cells themselves produce disease aggravating cytokines such as CXCL-8, CXCL-10, IL-
312 1beta, IL-6 and TNF-α which has been proven before (Alcendor et al., 2012, Cheeran et al.,
313 2003), but also virus defending cells may have a particularly amplifying effect and moreover, a
314 strong inflammatory environment in therapy-refractory patients might provoke reactivation of
315 CMV. This model of a vicious circle of cytokine release and CMV infection has first been
316 addressed in the context of graft rejection. Fietze et al. found a mutual relationship between graft
317 rejection and CMV infection in solid organ transplant recipients. Both acute rejection and the
318 following anti-rejection therapy with anti-T cell antibodies implicated TNF release resulting in
319 CMV reactivation in PBMCs. CMV infection was described to increase TNF-α serum levels and
320 TNF-α, in turn, has been shown to be involved in cytotoxic effects on the graft(Fietze et al.,
321 1994). The results of Reinke et al. support the view that subclinical active CMV infection and
322 more importantly the immune responses triggered by reactivation can have tremendous
323 pathophysiological consequences leading to prolonged inflammation that, for example, results in
324 renal allograft injury (Reinke et al., 1994).

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In dermatology, Döcke et al. proposed that CMV reactivation might be both consequence and trigger of exacerbation of atopic dermatitis as active CMV infection was associated with an inflammatory response and clearance of CMV antigenemia was shown during anti-eczematous treatment (Docke et al., 2003). In psoriasis, persistent CMV infection correlated with both disease activity and elevated TNF-α levels and a role for CMV specific T cells in psoriatic lesions has been suspected(Asadullah et al., 1999, Weitz et al., 2011) . Strong arguments for a disease modifying role of CMV activation deliver our case studies. Both patients with therapy-refractory courses of disease who were screened positive for subclinical CMV reactivation showed significant improvement of their underlying skin disease upon antiviral treatment. Although our cases only show a temporal correlation between antiviral treatment and improvement of skin lesions, they indicate that reactivation of latent CMV infection is a possible complication in therapy-refractory immunocompromised patients suffering from chronic skin diseases. Not only for the patients' sake but also for economic reasons, screening of immunocompromised patients in dermatological wards may be beneficial. As calculated by the number needed to screen, we found that only 12 patients would need to be tested to reduce one case of CMV induced morbidity. The costs for screening in serum and tissue are multiple times lower than the costs caused by repeated hospitalization and consumption of immunosuppressive medication. The shortcomings of this study are the relatively low number of patients in the prospective study cohort as well as the fact that the improvement of skin lesions seen in these patients upon ganciclovir may only temporally correlate to antiviral treatment but not be causally related to this therapeutic intervention. Further prospective randomized case-control studies will be needed to deliver a causal proof for the clinical benefit of ganciclovir treatment in patients showing the profile of subclinical CMV reactivation. However, previously reported cases

highlight the occurrence of CMV infection in the context of immunosuppressive treatment for pyoderma gangrenosum and corroborate our findings (Kikuchi et al., 2005, Tsutsumi et al., 2021).

To summarize, this study broadly addresses subclinical reactivation of EBV and CMV in the dermatological patient population. It highlights not only the importance of further research in this field, but also calls for increased vigilance when treating dermatological patients, particularly, as more and more systemically acting drugs, such as small molecules and biologicals, are administered and may induce reactivation of viral infection.

MATERIALS AND METHODS

Study cohort

The study followed the declaration of Helsinki and was approved by the local ethics committee (Klinikum Rechts der Isar, 514/17 S). Patient material - of which written informed patient consent was obtained - was taken from the The Biobank Biederstein which follows data protection rules and is approved by the local ethics committee (Klinikum Rechts der Isar, 5590/12).

Retrospective cohort (RC)

For virus specific screening in lesional skin of patients with chronic skin diseases, 43 patients were selected suffering from autoimmune or inflammatory skin diseases (n=21), chronic ulcers (n=15) or skin lymphomas (n=7) as reactivation of EBV and CMV has been associated with these disease categories(Asadullah et al., 1999, Docke et al., 2003, Grywalska and Rolinski,

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2015, Guo et al., 2015, Harley and James, 2006, Senger and Sinha, 2012, Weitz et al., 2011)(s. Table S1). Besides, patients had to receive immunosuppressant or immune modulating therapies in or outside the context of the underlying skin disease for at least 2 weeks prior to acquisition of biopsy to be included into the study group (SG-RC) (s. Table S2). As control group (CG-RC), non-lesional skin biopsies of age- and sex matched patients (n=43) who suffered from diseases within the same disease categories but were not under systemic treatment for at least six months prior to acquisition of biopsy were investigated. Absolute and relative lymphocyte counts of patients in the SG-RC were significantly lower than those of patients in the CG-RC (s. Figure S2 a-c).

Prospective cohort (PC)

For prospective screening of subclinical EBV and CMV reactivation, 30 patients were included into the study group (SG-PC). These patients suffered from autoimmune skin diseases (n=18), chronic ulcers (n=4) and chronic inflammatory skin diseases (n=8) (s. Table S1). Patients of the SG-PC were a) treated with an immunosuppressant and/or biological in or outside the context of the underlying skin disease at the time of blood withdrawal and acquisition of biopsy (s. Table S2) and b) their skin lesions did not improve or even worsen during a period of at least 8 weeks of intensive treatment which was evaluated by at least two experienced board-certified dermatologists. As control group for serology (CG-PC), sera of 90 age- and sex matched patients (n=90, matching 1:3) who suffered from diseases within the same disease categories (autoimmune or inflammatory skin diseases/chronic ulcers/lymphomas) but were not under immunosuppressive or immune-modulating treatment for at least six months prior to blood withdrawal were selected. Absolute and relative lymphocyte counts did not significantly differ between both groups; however, oral thrush was significantly more frequent in the study cohort

than in the control cohort (s. Figure S2 d-f). As control group for detection of viral DNA within lesional skin of patients from the SG-PC (n=14, SG-PC-P subgroup), non-lesional skin of age- and sex matched patients (n=14, CG-PC-P) who suffered from diseases within the same disease categories but were not under immunosuppressive or immune-modulating treatment was investigated (s. Table S1 and S2). A detailed overview of all patients in the SG-PC is given in Table S3.

ELISA

Virus-specific antibodies in serum were measured using the Novagnost Kits EBV-EBNA1/IgG (DiaSorin, #EBVG0580DB) and EBV-VCA/IgM (DiaSorin, #10445801) and the Enzygnost Kits Anti-CMV/IgG (Siemens, #OWBA15) and Anti-CMV/IgM (DiaSorin, #10446583). Experiments were carried out according to the manufacturers´ instructions. For CMV, samples were classified as negative [$\Delta A < 0.100$ (= cut-off)], borderline [$0.100 \leq \Delta A \leq 0.200$] or positive [$\Delta A > 0.200$]. For samples with an absorbance value higher than the cut-off, the lot-dependent constants α and β were used to calculate IgG titers with the α-method. For EBV, the sample was considered positive, if the absorbance value exceeded the cut-off by more than 15 % (> 11.5 NU). Samples with an absorbance value of 8.5 NU - 11.5 NU were classified as borderline. If the absorbance value was lower than 15 % below the cut-off (< 8.5 NU), samples were regarded as negative.

T cell proliferation assay

Monocytes isolated from PBMCs were differentiated to monocyte-derived dendritic cells (MODCs) by stimulation with 150 U/ml rhGM-CSF (Miltenyi, 130-093-864) and 150 U/ml

rhIL-4 (Miltenyi, 130-093-920) for 7 days. in HUDC medium containing 90 % RPMI 1640 (Gibco, 21875-091), 10 % FCS (GE Healthcare, SV30160.03), 1 % L-Glutamine (Life Technologies, 25030024) and 0,2 % Gentamycin (Life Technologies, 15710049) at 37 °C and 5 % CO_2. After 4 days, fresh HUDC medium supplemented with rhIL-4 and rhGM-CSF was carefully added.

Differentiated MODCs were then stimulated for 24 hours in HUDC medium with 10 µl rhEBV-proteins(Korber et al., 2020) (5 µl T-activated BZLF1 (Lophius, 12312001) + 5 µl T-activated EBNA3A (Lophius, 12312002)) or 10 µg/ml human CMV lysate (The Native Antigen Company, CMV-CL-100). For a positive control, MODCs were stimulated with 10 µg/ml rhTetanus-Toxoid (Enzo Life Sciences, ALX-630-108-C100). For MODC – T cell co-culture, primary human T cells isolate from fresh lesional skin biopsies were labeled with 1 µM cell proliferation dye CFSE using Cell Trace™ CFSE Cell Proliferation Kit (ThermoFisher, C34554) according to the manufacturer's protocol. Then, 5 x 10^5 CFSE-labeled lesional T cells were co-cultured with 2.5 x 10^4 antigen-stimulated MODCs in T cell proliferation medium for 10 days at 37 °C and 5 % CO_2. After 6 days 20 U IL-2 (Proleukin, Novartis) was added to co-culture.

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. To compare control group and study group, 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

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corrected chi-square was computed (continuity correction). Fisher´s exact test was applied 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-tail 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 labelled as follows: $p \geq 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

See Supplementary Information for further details on material and methods.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and its supplementary files.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ACKNOWLEDGEMENTS

SE and NGS were supported by a grant of the Helmholtz Center Munich (Preclinical Transfer Fund) to perform this study. We thank Jana Sänger and Kerstin Weber for excellent technical support.

AUTHOR CONTRIBUTIONS STATEMENT

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Conceptualization: NGS, SE. Data curation: NGS, SE, PS, MJ. Formal analysis: PS, NGS, MJ. Funding Acquisition: NGS, SE. Investigation: SP, MJ, NGS, SE, PS, KS, TB, CS. Supervision: NGS, SE. Visualization: NGS, MJ. Writing - Original Draft: NGS. Writing – Review and Editing: PS, MJ, SE, UP, CSW, TB.

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588 **TABLE**

589

Patient ID	Diagnosis	CMV IgG	CMV IgM	CMV PCR [blood]	CMV PCR [lesional skin]
PC-1	SLE	positive	borderline	negative	negative
PC-2	Psoriasis inversa et capitis	positive	positive	negative	negative
PC-3	Drug-induced exanthema	positive	positive	positive	positive
PC-4	Pemphigus vulgaris	positive	borderline	positive	not assessed
PC-5	Pyoderma gangrenosum	positive	positive	negative	positive

590 Table 1: CMV profile of patients PC 1-5.

591

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FIGURE LEGENDS

Figure 1: Latent infection and viral reactivation in chronic skin disease patients under immunosuppressive or immune-modulating therapy

EBER ISH for EBV in the skin of patient RC-1 (pyoderma gangrenosum) of the retrospective cohort tested positive for EBV DNA on PCR-level (a). Serum IgG and IgM frequencies and levels for EBV (b-d) and CMV (e-g) as well as PCR for EBV (h) and CMV (i) DNA in the skin of patients of the prospective cohort, SG-PC (n=30)/SG-PC-P subgroup (n=14), suffering from chronic skin diseases under immunosuppressive treatment regimens in comparison to age and sex matched control patients of the prospective cohort, CG-PC (n=90)/CG-PC-P (n=14). Scale bar: 100 µm.

Figure 2: Virus-specific T cells may aggravate underlying chronic skin diseases in therapy-refractory patients

Proliferation activity of T cells isolated from lesional skin and co-cultured with MODCs of patients PC-1 and PC-2 from the SG-PC cohort upon stimulation with EBV or CMV proteins. T cell proliferation upon stimulation with both EBV and CMV proteins in patient PC-1 (a) and patient PC-2 (b) with Tetanus-Toxoid (TT) as a positive control. Dominance of Th1 and Th17 cytokines detected in cell free supernatants derived from T cell – MODC-antigen co-cultures of PC-1 and PC-2 (c).

Figure 3: Patient PC-5 with pyoderma gangrenosum

PC-5 had been suffering from painful pyoderma gangrenosum for 3 weeks prior to admission at the right malleolus which showed fast progression despite topical wound care and oral

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prednisolone 200 mg daily (w0=admission) (a). Since w2 therapy was stepwise intensified (s. regimen w6) as the ulcer rapidly enlarged in size and began uncovering the Achilles tendon. Despite intensified treatment, the ulcer increased in size and depth (w6). Showing the pattern of (sub)clinical reactivation of CMV infection (b), intravenous ganciclovir was initiated (800 mg/d for 14 d, w6-8) leading to pain relief and steadily improving lesions. Medication was slowly tapered off (w24 and w90). Until year 2 after first occurrence (y2) the lesion shows stable remission. Consistently, anti-CMV-IgM levels declined after ganciclovir treatment and were negative the first time 86 weeks after initiation of antiviral treatment (b). (d = day, w = week, y = year).

Figure 4: Patient PC-2 with psoriasis inversa et capitis and psoriatic arthritis.

In PC-2 (a), different systemic therapies (fumaric acid ester, etanercept, infliximab, methotrexate, and adalimumab) had been discontinued over the years because of adverse effects or secondary loss of efficacy. During treatment with Ixekizumab started 7 months prior to admission lesions improved (PASI 2-4). However, 5 w prior to admission skin lesions worsened (PASI of 8.3) (w0=admission). Phototherapy with UVB (311 nm) was initiated at w0 and treatment with ixekizumab and topical glucocorticoids was continued. However, the lesions worsened with massive burning and itching and oral thrush was diagnosed at w4. PC-2 was tested positive for anti-CMV-IgG and anti-CMV-IgM and oral valganciclovir was initiated (1800 mg/d for 21 days and 900 mg/d for another 7 d). During antiviral treatment (w4-w8) skin lesions markedly improved and itching decreased (a). At w8, skin lesions were almost completely under remission and anti-CMV IgM levels decreased (b). (day = day, w = week, y = year).

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Diagnosis	SG-RC (n=43)	CG-RC (n=43)	SG-PC (n=30)	CG-PC (n=90)	SG-PC-P (n=14)	CG-PC-P (n=14)
Autoimmune skin diseases	**20 (46.5)**		**18 (60.0)**	**2 (2.2)**	**6 (42.9)**	
Bullous pemphigoid, no. (%)	12 (27.9)		2 (6.7)	1 (1.1)	1 (7.1)	
Dermatomyositis, no. (%)	1 (2.3)		2 (6.7)		1 (7.1)	
Epidermolysis bullosa acquisita, no. (%)	1 (2.3)		1 (3.3)			
Linear IgA dermatosis, no. (%)	1 (2.3)		1 (3.3)			
Pemphigus vulgaris, no. (%)	4 (9.3)		5 (16.7)	1 (1.)	1 (7.1)	
Systemic lupus erythromatosus, no. (%)			5 (16.7)		3 (21.4)	
Systemic sclerosis, no. (%)	1 (2.3)		2 (6.7)			
Chronic ulcers	**15 (34.9)**		**4 (13.3)**	**2 (2.2)**	**3 (21.4)**	
Pyoderma gangrenosum, no. (%)	8 (18.6)		4 (13.3)		3 (21.4)	
Ulcus cruris, no. (%)	7 (16.3)			2 (2.2)		
Inflammatory skin diseases	**1 (2.3)**	**41 (95.3)**	**8 (26.7)**	**86 (95.6)**	**5 (35.7)**	**14 (100.0)**
Urticaria, Urticarial vasculitis, no. (%)		1(2.3)		10 (11.1)		
Granuloma anulare, no. (%)		1 (2.3)				
Lichen sclerosus, Lichen ruber, no. (%)		1 (2.3)	1 (3.3)	1 (1.1)		2 (14.3)
Autoinflammatory (Pityriasis rubra pilaris, Still's disease), no. (%)		2 (4.6)				
Dermatitis (e.g. atopic/nummular/prurigoform), prurigo, no. (%)		14 (32.6)		61 (67.8)		8 (57.1)
Erythroderma			1 (3.3)		1 (7.1)	

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Drug-induced exanthema			2 (6.7)	6 (6.7)	1 (7.1)	
Psoriasis inversa/vulgaris (et arthropathica), no. (%)	1 (2.3)	22 (51.2)	4 (13.3)	8 (8.9)	3 (21.4)	3 (21.4)
Other inflammatory skin diseases		2 (4.6)				1 (7.1)
Skin lymphomas	**7 (16.3)**	**2 (4.7)**				
Mycosis fungoides, no. (%)	3 (7.0)					
Sézary syndrome, no. (%)	2 (4.7)					
Other skin lymphomas, no. (%)	2 (4.7)					
Age in years, median (range)	72 (23-90)	71 (24-89)	60.5 (40-97)	60.5 (40-99)	56.5 (40-88)	55.5 (40-88)
Female sex, no. (%)	23 (53.5)	23 (53.5)	22 (73.3)	66 (73.3)	10 (71.4)	10 (71.4)

Table S1: Characteristics of study group (SG-RC) and control group (CG-RC) in the retrospective study cohort (RC) and in the prospective cohort (PC), respectively. In the prospective cohort, lesional skin could be obtained and investigated in a total number of 14 study patients (SG-PC-P). The results were compared to 14 age- and sex- matched control patients (CG-PC-P).

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Medication	**SG-RC (n=43)**	**SG-PC (n=30)**	**SG-PC-P (n=14)**
Biologicals		**12 (40.0)**	**5 (35.7)**
Adalimumab, no. (%)		1 (3.3)	1 (7.1)
Belimumab, no. (%)		3 (10.0)	1(7.1)
Infliximab, no. (%)	2 (4.7)	4 (13.3)	2 (14.3)
Ixekizumab, no. (%)		1 (3.3)	1 (7.1)
Rituximab, no. (%)	1 (2.3)	3 (10.0)	
Chemotherapy, no. (%)	**3 (7.0)**	**0 (0.0)**	**0 (0.0)**
Classical immunosuppressants			
Azathioprine, no. (%)	5 (11.6)	4 (13.3)	2 (14.3)
Corticosteroids, no. (%)	36 (83.7)	23 (76.7)	11 (78.6)
Cyclophosphamide, no. (%)	1 (2.3)		-
Cyclosporine, no. (%)	2 (4.7)	2 (6.7)	1 (7.1)
MTX, no. (%)	2 (4.7)	3 (10.0)	1 (7.1)
Mycophenolate mofetil, no. (%)	3 (7.0)	3 (10.0)	
≥ 2 immunosuppressive regimens, no. (%)	**12 (27.9)**	**15 (50.0)**	**6 (42.9)**

Table S2: Medication administered in study patients of the retrospective cohort (SG-RC), in study patients of the prospective cohort (SG-PC) and in the subgroup of study patients of the prospective cohort from whom skin tissue was analyzed for virus DNA (SG-PC-P) .

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Name	Sex	Diagnosis	Age	Immunosuppression (at time of admission)	Oral thrush	%LYMPH (%)	#LMYPH (x 10^3/µl)	CMV IgG	CMV IgM	EBNA1 IgG	EBV-VCA IgM	CMV PCR [lesional skin]	EBV PCR [lesional skin]	CMV PCR (EDTA blood)	EBV PCR (EDTA blood)
PC-1	female	systemic lupus erythematosus	42	prednisolone	yes	14.9	0.80	positive	borderline	positive (VCA IgG)	negative	negative	positive	negative	negative
PC-2	female	psoriasis inversa et capitis (et arthropathica)	61	ixekizumab	yes	38.0	3.12	positive	positive	positive	negative	negative	negative	negative	negative
PC-3	female	drug-induced exanthema	66	prednisolone, IVIg	yes	22.8	0.48	positive	positive	positive	negative	positive	not determined	positive	weakly positive
PC-4	male	pemphigus vulgaris (mucocutaneous type)	45	prednisolone, azathioprine, rituximab	no	7.5	0.90	positive	borderline	positive	negative	not determined	not determined	positive	not determined
PC-5	male	pyoderma gangrenosum	62 61	prednisolone, cyclosporine, infliximab, IVIg	no	18.0	1.57	positive	positive	positive	negative	positive	negative	negative	negative
PC-6	female	bullous pemphigoid	88	prednisolone, azathioprine	no	19.0	1.60	positive	negative	positive	negative	positive	positive	negative	negative
PC-7	female	pyoderma gangrenosum	43	prednisolone, adalimumab, IVIg	no	10.9	1.10	positive	negative	positive	negative	negative	negative	negative	negative
PC-8	female	pyoderma gangrenosum	40	prednisolone, cyclosporine, IVIg	no	16.5	2.00	positive	negative	positive	positive	negative	positive	negative	negative
PC-9	male	dermatomyositis	75	prednisolone, IVIg	no	8.5	0.60	positive	negative	positive	negative	not determined	not determined	negative	negative
PC-10	female	pemphigus vulgaris	52	prednisolone, IVIg	no	34.2	1.60	positive	negative	positive	negative	negative	negative	negative	negative
PC-11	male	bullous pemphigoid	97	prednisolone, mycophenolate sodium	no	14.0	2.00	negative	negative	positive	negative	not determined	not determined	negative	negative
PC-12	female	systemic lupus erythematosus	42	belimumab	yes	22.6	2.70	positive	negative	positive	negative	not determined	not determined	negative	negative
PC-13	female	psoriasis vulgaris et arthropathica	71	prednisolone	no	20.4	2.10	negative	negative	negative	negative	negative	positive	negative	negative
PC-14	female	linear IgA dermatosis	75	rituximab, IVIg	no	22.4	1.30	positive	negative	positive	negative	not determined	not determined	negative	negative
PC-15	female	pemphigus vulgaris	67	prednisolone, azathioprine, IVIg	no	32.0	2.40	positive	negative	positive	negative	not determined	not determined	negative	negative

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PC-16	male	pyoderma gangrenosum	49	prednisolone, IVIg	no	17.0	2.86	positive	negative	positive	negative	not determined	not determined	negative	negative
PC-17	female	systemic sclerosis	60	prednisolone, rituximab	no	18.8	1.80	positive	negative	positive	negative	not determined	not determined	negative	negative
PC-18	male	psoriasis vulgaris et arthropathica	70	infliximab	no	17.8	2.40	negative	negative	negative	negative	negative	negative	negative	negative
PC-19	female	pemphigus vulgaris	51	prednisolone, azathioprine, IVIg	no	13.3	1.50	negative	negative	positive	positive	not determined	not determined	negative	negative
PC-20	female	epidermolysis bullosa acquisita	74	prednisolone, IVIg	no	9.0	0.27	positive	negative	positive	negative	not determined	not determined	negative	negative
PC-21	female	systemic sclerosis	78	prednisolone, mycophenolate mofetil	no	12.0	0.78	positive	positive	negative	negative	not determined	not determined	negative	negative
PC-22	female	systemic lupus erythematosus	48	prednisolone	no	24.0	2.96	negative	negative	positive	negative	negative	negative	negative	negative
PC-23	female	pemphigus vulgaris	50	prednisolone, IVIg	no	15.8	2.10	positive	negative	positive	borderline	not determined	not determined	negative	small amount
PC-24	female	PAPASH syndrome	47	infliximab, methotrexate	no	35.5	2.50	negative	negative	positive	negative	not determined	not determined	negative	positive
PC-25	female	systemic lupus erythematosus	47	prednisolone, belimumab	no	28.2	1.10	negative	negative	positive	negative	not determined	not determined	negative	negative
PC-26	female	lichen sclerosus et atrophicus	70	methotrexate, IVIg	yes	24.5	2.40	positive	positive	positive	negative	not determined	not determined	negative	negative
PC-27	male	dermatomyositis	64	prednisolone, mycophenolate mofetil, IVIg prednisolone, azathioprine	no	13.1	1.10	positive	negative	positive	negative	negative	negative	negative	negative
PC-28	female	erythroderma	44	prednisolone, infliximab	no	12.0 9.9	1.42 1.60	negative	negative	positive	negative	negative	negative	negative	negative
PC-29	male	systemic lupus erythematosus	44	belimumab, methotrexate	no	24.0	1.24	negative	negative	positive	negative	negative	negative	negative	negative
PC-30	female	drug-induced exanthema	61	pulsed prednisolone	no	16.0	1.11	positive	negative	positive	negative			negative	negative

Table S3: Detailed characteristics of patients in the study group of the prospective cohort (SG-PC).

Figure 1 Speth et al.

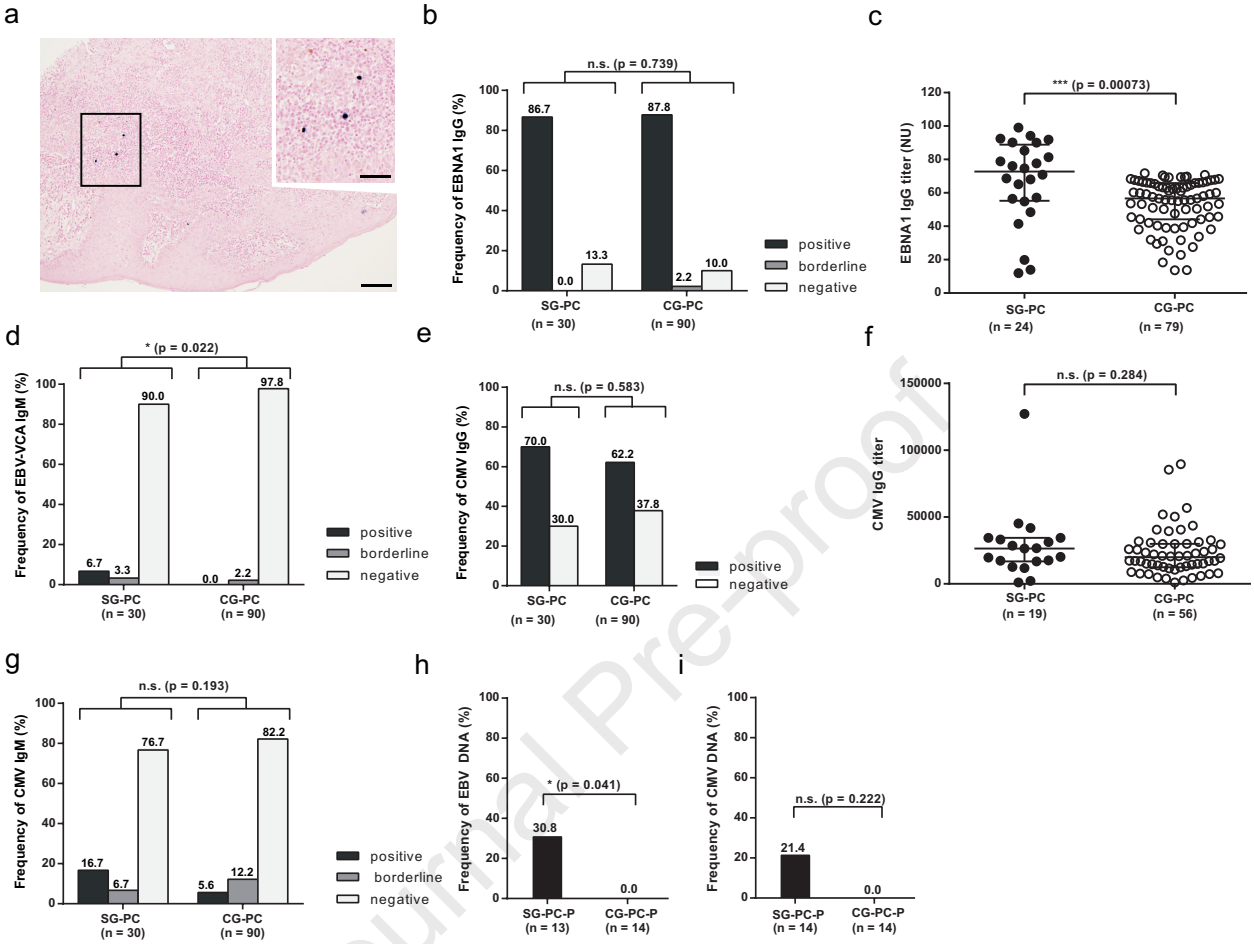

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Figure 2 Speth et al.

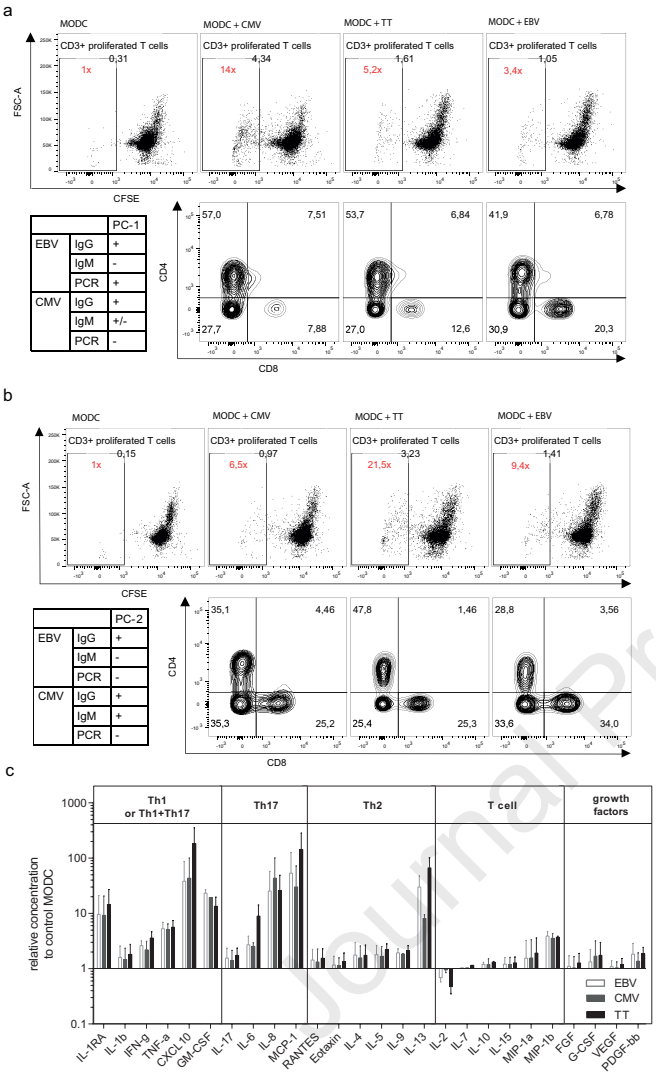

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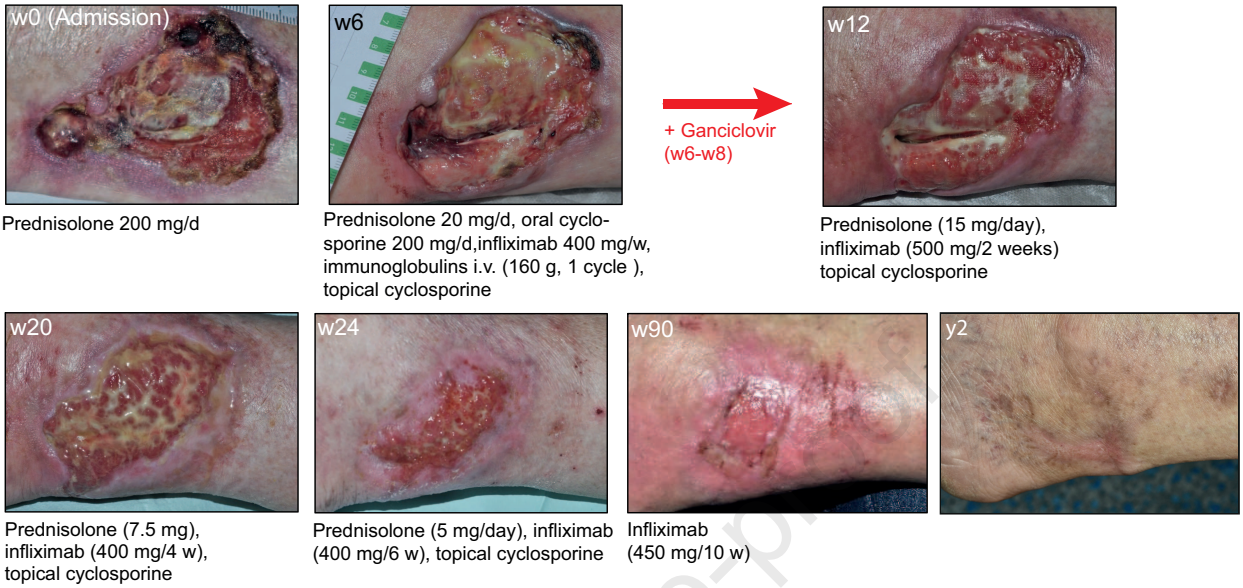
Figure 3 Speth et al.

a



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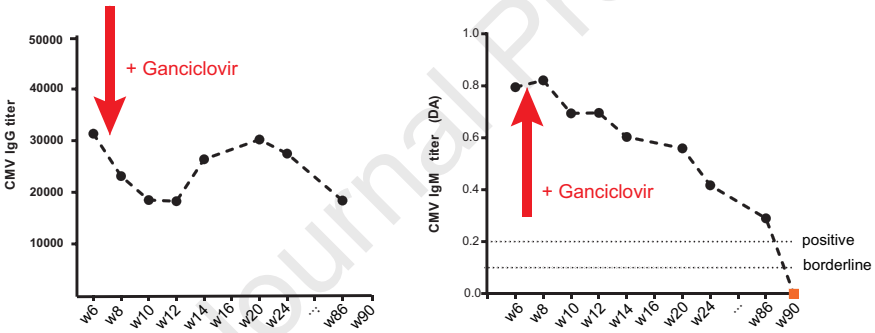

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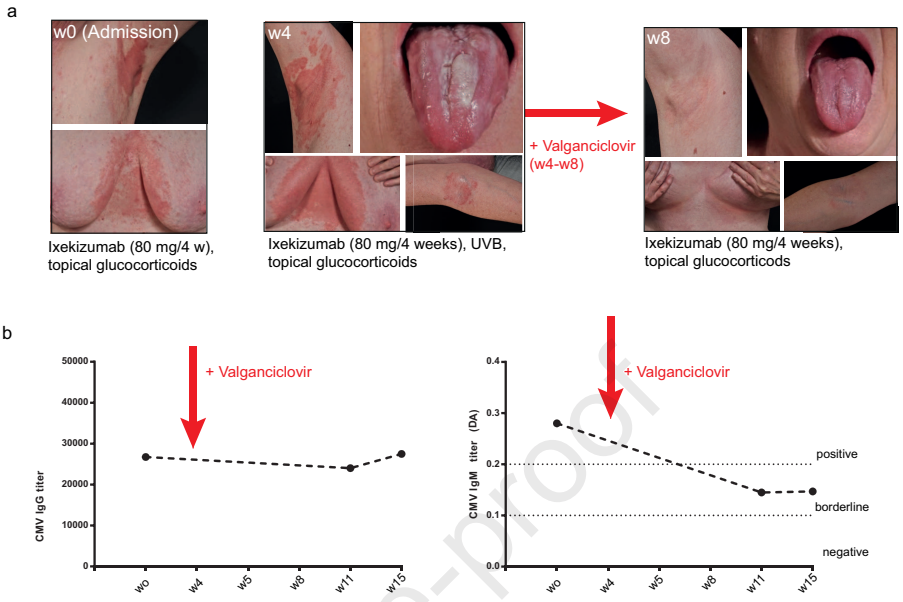
Figure 4 Speth et al.



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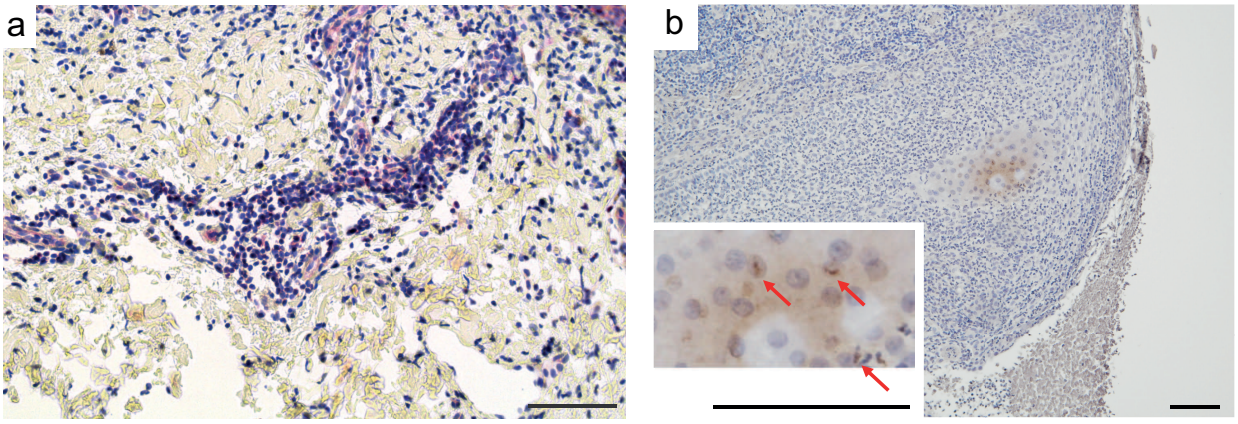

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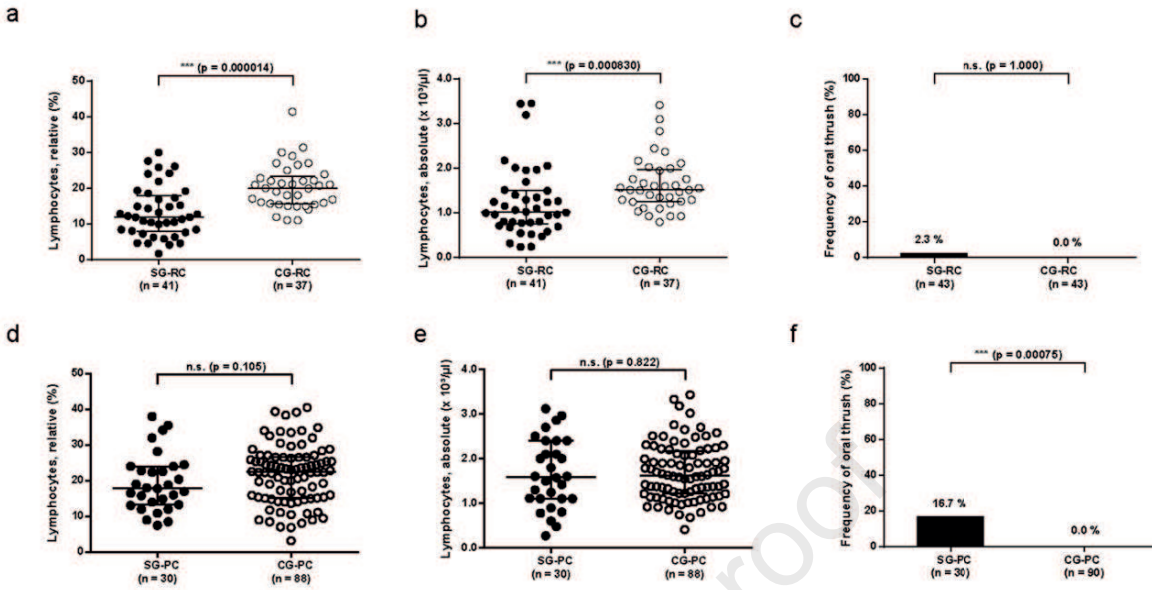

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Supplementary Text

Immunocompromised patients with therapy-refractory chronic skin diseases show reactivation of latent EBV and CMV infection

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Supplementary methods

Isolation of DNA and real time PCR

DNA was isolated from FFPE-embedded tissue or RNAlater (Qiagen, 1018087) preserved tissue coming from freshly taken punch biopsies. DNA was prepared using the QIAamp DNA FFPE Tissue Kit (Qiagen, 56404) for FFPE-embedded tissue, QIAamp DNA Micro Kit (Qiagen, 56304) for RNAlater preserved tissue according to manufacturer's protocol. Virus-specific DNA was detected and quantified using probe-based RealStar CMV PCR Kit (altona Diagnostics, 021013) or RealStar EBV PCR Kit (altona Diagnostics, 131013) on Applied Biosystems ViiA7 Real-Time PCR system (Thermo Fisher Scientific) according to the manufacturer's protocol. Primers and probes are listed below.

TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, 4444557) for PDH-PCR

Primer/probe			**Company (head office)**
Target (human)	**Direction**	**Sequence (5´-3´)**	
PDH-primer	for	TCG ATC GGG ACT GCT TTC C	Metabion (Planegg, Germany)
PDH-primer	rev	CCC ACA ACC TAG CAC CAA AAG A	Metabion (Planegg, Germany)

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PDH-probe	-	6-FAM CAT CTC CTT TTG CTT GGC AAA TCT GAT CC-TAMRA	Metabion (Planegg, Germany)

Isolation of PBMC and CD14$^+$ monocytes

PBMCs were isolated from peripheral blood using LymphoPrep^{TM} (Progen Biotek, 1114547) according to manufacturer's protocol. For isolation of monocytes, PBMCs were magnetically separated for CD14$^+$cells using human CD14 MicroBeads (Miltenyi, 130-050201) with the autoMACS^{TM} Pro Separator (Miltenyi, 130-092-545) according to manufactures instructions. Until usage, monocytes were frozen in liquid nitrogen in freezing medium consisting of 90 % FCS (GE Healthcare, SV30160.03) and 10 % DMSO (Applichem, A3672.0250).

Isolation of lesional T cells

Primary human T cells were isolated from fresh lesional skin biopsies by emigration towards an IL-2 gradient followed by expansion with α-CD3/α-CD28 stimulation as described previously (Garzorz-Stark et al., 2018, Lauffer et al., 2018). T cells were cultured in T cell proliferation medium consisting of RPMI 1640 medium (Gibco, 21875-091) supplemented with 5 % human serum (Sigma, H4522), 0.1 mM NEAA (Gibco, 11140-035), 2 mM L-Glutamine (Gibco, 25030-024), 1 mM sodium pyruvate (Gibco, 11360-039) and 100 U/ml penicillin/streptomycin (Gibco, 15140-130) at 37 °C, 5 % CO_2. Until further use, T cells were frozen in liquid nitrogen in freezing medium containing 50 % RPMI 1640 (Gibco, 21875-091), 40 % FCS (GE Healthcare, SV30160.03) and 10 % DMSO (Applichem, A3672.0250).

Flowcytometry

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To determine the number of dead cells, cells were stained with Aqua (Invitrogen, L34957) in PBS for 30 minutes. Additionally, cells were stained for CD3-BV711 (Biolegend, 317327, clone Okt3, 1:100), CD4-AF700 (Biolegend, 300526, clone RPA-T4, 1:500) and CD8-APC-H7 (BD Bioscience, 561423, clone SK1, 1:50). Stained samples were acquired with a LSR Fortessa flowcytometer and analyzed with FlowJo_V10 (www.flowjo.com).

Bioplex analysis

Cell free supernatants derived from T cell – MODC co-cultures were analysed for 27 cytokines, chemokines and growth factors using the Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad Laboratories, Hercules, Calif) according to the manufacturer's recommendation. IL12p70 was not detectable and is therefore not shown in Figure 2 c.

Epstein-Barr encoding region (*EBER*) in situ hybridization

In situ hybridization was performed on the fully-automated BenchMark Ultra IHC/ISH system (Roche Diagnostics) according to the guidelines from the supplier using the Ventana ISH/VIEW Detection kit (Ventana Medical Systems, 800-092) and the INFOFRM EBER Probe (Ventana Medical Systems, 800-2842) to detect Epstein-Barr encoded RNA.

Immunohistochemistry

Skin samples were fixed in 10% formalin and embedded in paraffin. Four micrometer sections were cut and dewaxed. Staining for EBV was performed with an automated BOND system, according to the manufacturer's instructions: after rehydration and antigen retrieval in a pH 6 citrate buffer–based epitope retrieval solution (Leica, Wetzlar, Germany), sections

were incubated with the Anti-EBV EA-D-p52/50 antibody, clone R3 (Merck, Darmstadt, Germany) at a dilution of 1:2000. Secondary polymeric alkaline phosphatase–linked anti-rabbit antibody and horseradish peroxidase (HRP)–linked anti-mouse antibody (Zytomed Systems) were applied, and the complex was visualized with the substrate chromogen Fast Red or 3,39-diaminobenzidine. Eventually, slides were counterstained with hematoxylin. For staining with CMV sections were processed accordingly but incubated with the Anti-Cytomegalovirus antibody CCH2 + DDG9 (Dako, Jena) at a dilution of 1:30 and visualized with the OptiView DAB IHC detection kit (Ventana).

Figure legend

Figure S1: Immunohistochemical staining for EBV and CMV

Immunohistochemical staining for Anti-EBV EA-D-p52/50 antibody shows positivity within the infiltrate of pyoderma gangrenosum patient RC-1 (a). Immunohistochemical staining for Anti-Cytomegalovirus antibody CCH2 + DDG9 shows weak nuclear staining in patient PC-5 (b). Scale bar: 100 µm.

Figure S2: Characteristics of RC and PC

In the retrospective cohort (RC), absolute (a) and relative (b) lymphocyte counts of study patients (SG-RC) were significantly lower than those of control patients (CG-RC). In contrast, no significant difference was observed for the frequency oral thrush between both groups (c). In the prospective cohort (PC), absolute (d) and relative (e) lymphocyte counts did not differ significantly between both groups; however, oral thrush was significantly more frequent in the study group (SG-PC) than in the control group (CG-PC) (f).

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Literature

Garzorz-Stark N, Lauffer F, Krause L, Thomas J, Atenhan A, Franz R, et al. Toll-like receptor 7/8 agonists stimulate plasmacytoid dendritic cells to initiate TH17-deviated acute contact dermatitis in human subjects. The Journal of allergy and clinical immunology 2018;141(4):1320-33 e11.

Lauffer F, Jargosch M, Krause L, Garzorz-Stark N, Franz R, Roenneberg S, et al. Type I immune response induces keratinocyte necroptosis and is associated with interface dermatitis. The Journal of investigative dermatology 2018.

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