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Immunity to varicella-zoster virus in immunocompromised patients

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Decreased immunity to varicella-zoster virus in giant cell arteritis

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ABSTRACT

Introduction

Herpes zoster, which can have a major impact on quality of life, results from reactivation of a latent varicella-zoster virus (VZV) infection. We hypothesized that giant cell arteritis (GCA) patients are at increased risk of herpes zoster because of treatment with high-dose glucocorticoids and advanced age. Aim of the study, therefore, was to determine cell-mediated and humoral immunity to VZV in patients with GCA, patients with closely related disease polymyalgia rheumatica (PMR; treated with lower doses of glucocorticoids) and healthy controls.

Methods

Cell-mediated immunity to VZV was determined by performing interferon- γ (IFN γ) enzyme-linked immunospot (ELISpot) and intracellular cytokine flow cytometry measurements in 11 GCA and 15 PMR patients, and in 26 age/sex-matched healthy controls. Immunoglobulin G antibodies to VZV glycoprotein (VZV-IgG) were measured in serum samples of 35 GCA and 26 PMR patients at different times of follow-up, and in 58 age and sex matched healthy controls by an enzyme-linked immunosorbent assay (ELISA).

Results

The number of VZV-specific IFN γ spot-forming cells was significantly lower in GCA patients on treatment, than in age-matched healthy controls ($p=0.029$), but was not different in PMR patients on treatment. Similar levels of VZV-IgG were found in GCA and PMR patients at baseline, compared to healthy controls.

Conclusion

The finding of a decreased cell-mediated immunity to VZV, known to be of great importance in defense to the virus, indicates an increased herpes zoster risk in GCA patients compared to an already at-risk elderly population. Herpes zoster vaccination is, therefore, of special importance in GCA patients, and would ideally be administered at time of diagnosis. Interestingly, as VZV was suggested to be the trigger in GCA pathogenesis, similar levels of VZV-IgG were found in GCA patients at time of diagnosis and age matched healthy controls, indicating that GCA patients did not experience herpes zoster substantially more often in the months preceding diagnosis than controls.

INTRODUCTION

Herpes zoster is caused by reactivation of a latent varicella-zoster virus (VZV) infection [1]. Almost the whole adult population of temperate countries has experienced a primary VZV infection in childhood (known as varicella or chickenpox) and is, therefore, at risk of herpes zoster [2]. Postherpetic neuralgia (pain lasting >90 days after onset of rash) is the most common complication of herpes zoster occurring in 8–27% of patients [3-6]. Impact on quality of life of herpes zoster and postherpetic neuralgia can be major [3,4].

With advancing age, a rise in the incidence of herpes zoster occurs concomitantly with a decline in VZV-specific cell-mediated immunity. The estimated incidence of HZ is about 3.4–4.82 per 1000 person years in the general population which increases to more than 11 per 1000 person years in those above 80 years of age [7].

Giant cell arteritis (GCA) and the closely related polymyalgia rheumatica (PMR) are inflammatory rheumatic disorders that occur almost exclusively in older adults [8]. In our hospital, GCA patients usually start on 40–60 mg of prednisolone daily, while PMR patients start at a lower dose of 15–20 mg prednisolone daily. In both patient groups, the prednisolone dose is slowly tapered. Use of glucocorticoids is associated with a higher herpes zoster risk [9-14]. Both because of advanced age and high-dose glucocorticoid use, especially GCA patients could be expected to have an increased herpes zoster risk.

A live attenuated herpes zoster vaccine was licensed in the US in 2006 for use in immunocompetent persons above 60 years of age [15]. In this group it was shown to be safe and effective in preventing herpes zoster and postherpetic neuralgia [6]. The Advisory Committee on Immunization Practices (ACIP) recommends the zoster vaccine for all persons aged ≥ 60 years [16]. Like immunocompetent older persons, GCA and PMR patients may benefit from zoster vaccination.

The ACIP in 2008, however, also stated that the vaccine should not be administered to persons on immunosuppressive therapy, including high-dose glucocorticoids (≥ 20 mg/day of prednisone or equivalent) lasting two or more weeks, although this recommendation was based on expert opinion only [16]. The 2010 European League Against Rheumatism (EULAR) recommendations for vaccination in patients with rheumatic diseases are inconclusive, stating that zoster vaccination may be considered in patients with rheumatic diseases [17].

Since the time of ACIP and EULAR recommendations, a large retrospective cohort study in patients with immune-mediated diseases, including patients using oral glucocorticoids and even biologicals, has shown that zoster vaccination is associated with a reduced incidence of herpes zoster, also within 42 days of vaccination (a safety concern, as the vaccine is life-attenuated) [10]. A randomized controlled trial in patients using glucocorticoids (with a limited number of included patients using more than 10 mg daily equivalent of prednisone), comparing live attenuated zoster vaccine to placebo vaccine, demonstrated that zoster vaccination was well tolerated [18].

When balancing pros and cons of zoster vaccination in GCA patients, information on the pre-existing cellular and humoral immunity to VZV is essential. The aim of this study, therefore, was to determine both cell-mediated immunity (most important in the defense to the virus) and humoral immunity to VZV in patients with GCA, the closely related PMR and age/sex-matched healthy controls.

MATERIALS AND METHODS

Study population and procedures

Prospectively collected serum samples and peripheral blood mononuclear cells (PBMCs) from patients with GCA and PMR, and matched healthy controls were used. Study procedures have been described previously [19,20].

GCA patients either had a positive temporal artery biopsy and/or positive ^{18}F -fluorodeoxyglucose-positron emission tomography-computed tomography (FDG PET-CT). PMR patients fulfilled the Chuang/Hunder criteria, or had a FDG PET-CT positive scan for PMR [21].

Serum was stored at -20°C , and peripheral blood mononuclear cells (PBMCs) were stored in liquid nitrogen until use. After evaluating cell viability by trypan blue staining, PBMCs were used in enzyme-linked immunospot (ELISpot) assays and flow cytometry analyses.

Samples for humoral analyses were randomly selected, and samples from patients at different times of follow-up were used: $T = 0$, $T \leq 2$ months, $T =$ approximately 6 months, $T =$ approximately 1 year and $T =$ approximately 2 years, or more. At $T = 0$, patients had not yet received treatment with prednisolone (or other immunosuppressive drugs). At $T \leq 2$ months, patients were within 2 months of follow-up and had all started prednisolone treatment.

Medical records from both patients and healthy controls were checked for a history of herpes zoster within 2 years before disease onset or sample collection. In The Netherlands, zoster vaccination is not advised and costs are not compensated in the national health care system. Since all patients were Dutch it could safely be presumed that none of the study subjects had been vaccinated against herpes zoster.

The study was approved by the institutional review board of the University Medical Centre Groningen (METc2012/375 for healthy controls and METc2010/222 for GCA and PMR patients), and written informed consent was obtained from all study participants.

Interferon- γ (IFN γ) ELISpot assay

Interferon- γ (IFN γ) ELISpot assay was performed as described previously [22]. PBMCs were stimulated for 48 hours with ultraviolet (UV)-inactivated varicella vaccine (Provarivax; Sanofi Pasteur). PBMCs stimulated with concanavalin A were used as controls. A negative control consisted of PBMCs in culture medium alone. After staining, spots were counted using an automated reader (AID ELISpot Reader; Autoimmun Diagnostika GmbH).

The mean number of spots in the negative control sample was subtracted from the mean number of spots in the VZV-stimulated wells. Results are referred to as the number of IFN γ spot-forming cells per 2×10^5 PBMCs.

Intracellular cytokine staining flow cytometry

Fluorescent T cell barcoding staining for tumor necrosis factor alpha (TNF α), interleukin 2 (IL-2) and IFN γ was performed as described previously [23] after stimulating PBMCs using UV-inactivated varicella vaccine. PBMCs stimulated with staphylococcal enterotoxin B (SEB; Sigma-Aldrich) were used as positive controls. A negative control consisted of PBMCs in medium alone. PBMCs were stimulated for 18 hours, of which the last 16 hours in the presence of 10 μ g/ml brefeldin A (Sigma-Aldrich). Kaluza software (Beckman Coulter) was used for analyses. Percentages of antigen-specific cells were expressed as the percentage of CD69+ cytokine-producing CD4+ or CD8+ T cells.

Antibody response to VZV

For quantitative detection of VZV-IgG antibodies, an in-house glycoprotein enzyme-linked immunosorbent assay (ELISA) was previously developed and validated [22]. VZV purified glycoproteins (EastCoastBio) were used as antigen, and pooled human serum with known levels of anti-glycoprotein VZV was used as standard.

Statistical analysis

Data were analyzed using SPSS 23 (IBM) and graphs were made using GraphPad Prism 5.0 (GraphPad Software). For correlations Spearman's rho was used. To compare differences between either the GCA or the PMR group to the healthy control group, Mann-Whitney U and Fisher's exact test were used as appropriate. Humoral immunity results at baseline were compared to the healthy control group separately for GCA and PMR patients using Mann-Whitney U tests. P values less than 0.05 (2-tailed) were considered significant.

RESULTS

Characteristics of patients and healthy controls

Characteristics of patients and controls are summarized in Table 1. Levels of VZV-IgG antibodies were measured in a larger number of samples, on different time-points of follow-up. Patient and control characteristics are, therefore, separately summarized in Supplementary Table 1. All patients started with prednisolone and some relapsing patients used methotrexate or leflunomide as glucocorticoid sparing drug. No other immunosuppressive medication was used.

Table 1. Characteristics of patients and healthy controls

	Healthy controls (n=26)	GCA patients (n=11)	PMR patients (n=15)
Sex, no. female/male	19/7	8/3	10/5
Age at sample date, median (interquartile range) years	73.2 (63.2-76.9)	73.2 (67.0-74.8)	71.3 (63.3-78.2)
GCA diagnosis: FDG PET-CT TAB FDG PET-CT+TAB	NR	6 3 2	NR
PMR diagnosis: FDG PET-CT Chuang FDG PET-CT+Chuang	NR	0 0 2	1 4 10
Time since diagnosis, median (interquartile range) months	NR	8.1 (4.9-9.1)	9.1 (8.0-10.0)
Cumulative prednisolone dosage, median (interquartile range) mg	NR	5843 (5028-8239)	3385 (2413-5320)
Use of immunosuppressants other than prednisolone, no. (%)	NR	2 (18) ^a	2 (13) ^b
No. of patients that experienced relapse since diagnosis (%)	NR	1 (9)	4 (27)
Leukocyte count, median (interquartile range) 10 ⁹ cells/L	5.50 (5.00-6.53)	9.80 (9.45-12.38) ***	8.30 (7.30-10.90) ***
Lymphocyte count, median (interquartile range) 10 ⁹ cells/L	1.75 (1.50-1.92)	1.68 (1.43-2.55)	1.37 (1.08-1.61) ***
CD3+ lymphocyte count, median (interquartile range) 10 ⁹ cells/L	1.24 (1.08-1.36)	1.30 (1.06-1.85)	1.06 (0.81-1.14) *
Haemoglobin, median (interquartile range) mmol/l	8.7 (8.2-9.2)	7.9 (6.6-8.3) **	8.3 (7.5-9.3)
ESR, median (interquartile range) mm/hr	9 (4-19)	24 (14-42) *	13 (8-38)
CRP, median (interquartile range) mg/L	<5 (<5-<5)	6 (<5-24) **	6.4 (<5-13) ***

GCA = giant cell arteritis; PMR = polymyalgia rheumatica; FDG-PET/CT = no. of patients with ¹⁸F-fluorodeoxyglucose-positron emission tomography-computed tomography supporting diagnosis; TAB = no. of patients with temporal artery biopsy positive for GCA; NR = not reported; Chuang: no. of patients fulfilling the Chuang and Hunder PMR criteria; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein.

^a Two patients received methotrexate (both 15 mg/week).

^b One patient received methotrexate (15 mg/week) and one patient received leflunomide (10 mg/day).

* P value <0.05; ** P value <0.01; *** P value <0.001 for comparison to healthy control group.

Cellular immunity to VZV: IFN γ ELISpot assay

The number of IFN γ spot-forming cells was significantly lower among GCA patients on treatment as compared to healthy controls ($p=0.029$). PMR patients on treatment and healthy controls showed similar numbers of spot forming cells. The median number of IFN γ spot-forming cells per 2×10^5 PBMCs in response to VZV stimulation was 47.5 (range 0-255) among healthy controls, 7 (range 0-69) among GCA patients, and 83 (range 0-279) in PMR patients (Figure 1).

Cellular immunity to VZV: Flow cytometric analysis of cytokine production

Upon stimulation with VZV, patients with GCA and healthy controls showed similar frequencies of cytokine producing CD4 $^+$ T cells. Interestingly, higher frequencies of CD4 $^+$ T cells producing IFN γ and IL-2 were found in PMR patients compared to healthy control subjects ($p=0.019$ and $p=0.048$, respectively) (Figure 2A-C). Similar frequencies of cytokine-producing CD8 $^+$ T cells were observed in patient groups and controls following stimulation with VZV (Figure 3A-C).

Upon SEB stimulation as positive control for stimulation, patients with GCA, PMR and control subjects showed similar frequencies of IFN γ -, TNF α , and IL-2-producing CD4 $^+$ T cells (Figure 2D-F). For CD8 $^+$ T cell cytokine production upon stimulation with SEB there was a trend towards a lower frequency of CD8 $^+$ T cells producing TNF α in GCA patients compared to healthy controls ($p=0.077$) (Figure 3D-F).

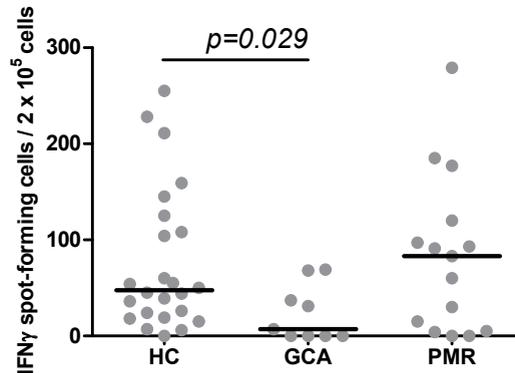


Figure 1. Levels of interferon- γ (IFN γ) spot-forming cells in response to varicella-zoster virus (VZV) stimulation. Numbers of IFN γ spot-forming cells per 2×10^5 peripheral blood mononuclear cells in 24 healthy control (HC) subjects, 9 giant cell arteritis (GCA) patients, 15 polymyalgia rheumatica (PMR) patients (B) are shown. Results are corrected for responses in non-stimulated cultures from the same sample. Bars show the median.

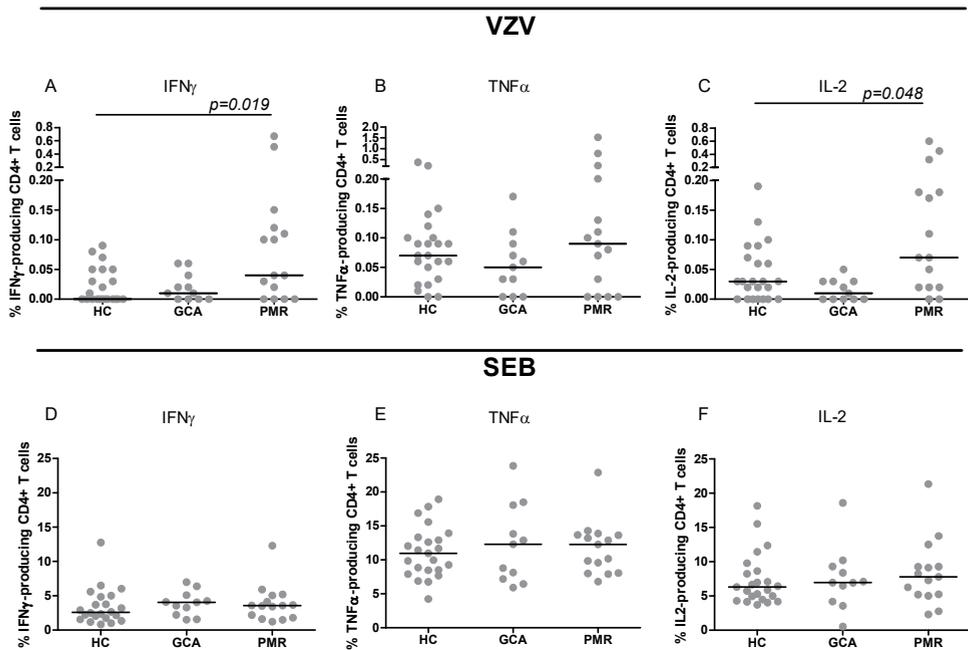


Figure 2. Frequencies of cytokine-producing CD4+ T cells upon stimulation with varicella-zoster virus (VZV) (A-B-C) and staphylococcal enterotoxin B (SEB, positive control) (D-E-F) in healthy control (HC) subjects (n=23), giant cell arteritis (GCA) patients (n=11) and polymyalgia rheumatica (PMR) patients (n=15). Results are corrected for responses in non-stimulated cultures. Bars show the median. IFN γ = interferon- γ ; TNF α = tumor necrosis factor α ; IL-2 = interleukin-2.

Humoral immunity to VZV

Median VZV-specific antibody levels of patients at baseline, before starting treatment, were similar in patient groups compared to healthy controls ($p=0.931$ for GCA and $p=0.777$ for PMR, Figure 4). Course of VZV-IgG antibody levels in GCA and PMR patients are shown in Figure 4.

Reactivations of VZV

In the group of patients and controls, in which VZV-specific IgG levels were determined (Figure 4), one patient was noted to have experienced herpes zoster about three months before GCA diagnosis. As can be expected, this patient showed a high VZV-IgG antibody level at time of diagnosis (7,100 mIU/ml). In another patient, a very high VZV-IgG antibody level of almost 40,000 mIU/ml was found, but medical records did not mention herpes zoster or symptoms suggesting this diagnosis. The high antibody level suggests that this patient is likely to have had a (subclinical) VZV reactivation, that escaped diagnosis.

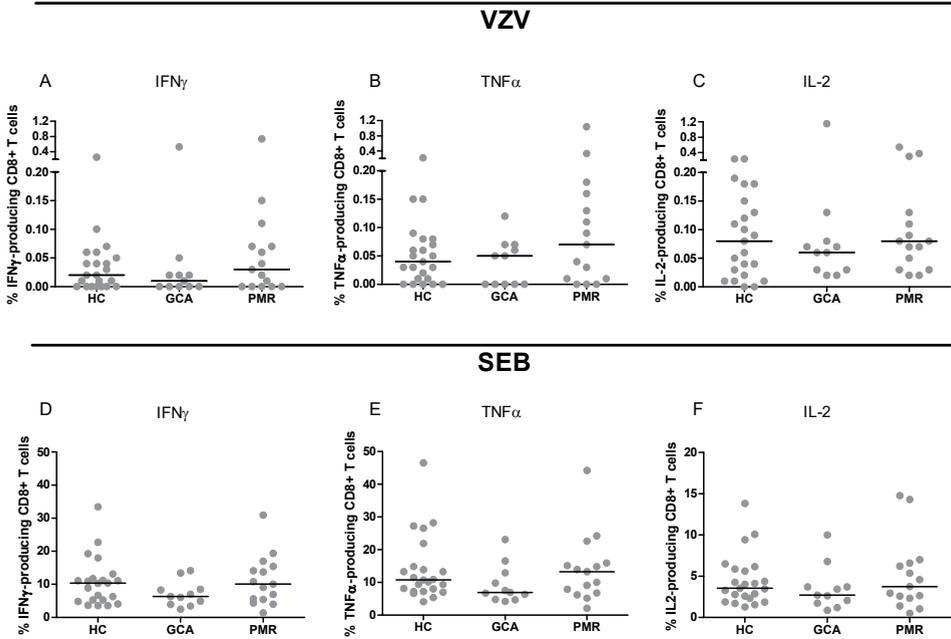


Figure 3. Frequencies of cytokine-producing CD8+ T cells upon stimulation with varicella-zoster virus (VZV) (A-B-C) and staphylococcal enterotoxin B (SEB, positive control) (D-E-F) in healthy control (HC) subjects (n=23), giant cell arteritis (GCA) patients (n=11) and polymyalgia rheumatica (PMR) patients (n=15). Results are corrected for responses in non-stimulated cultures. Bars show the median. IFN γ = interferon- γ ; TNF α = tumor necrosis factor α ; IL-2 = interleukin-2.

DISCUSSION

Confirming our hypothesis, a reduced cellular immunity to VZV in GCA patients was demonstrated using IFN γ ELISpot, even when compared to an already at risk age-matched healthy control group. Results in patients with PMR were found to be similar compared to age-matched controls. VZV-specific IgG levels were found to be similar when comparing the patient groups to the healthy control group.

It is widely accepted that cell-mediated immunity is an important measure of susceptibility to VZV [24-26]. Since healthy controls and GCA patients were shown to have similar total numbers of CD3+ T lymphocytes, which probably are the main IFN γ producing cells among PBMCs [27], we can conclude that our results reflect a decreased cell-mediated immunity to VZV. Accordingly, GCA patients are even more prone to herpes zoster than the already at-risk healthy elderly population.

To date only one, for an epidemiological study relatively small, retrospective study investigated the incidence of herpes zoster in GCA patients (with 21 and 38 cases of herpes zoster in 204 GCA patients and 407 controls, respectively). In this study GCA patients did not seem to be at higher risk of herpes zoster than age matched controls.

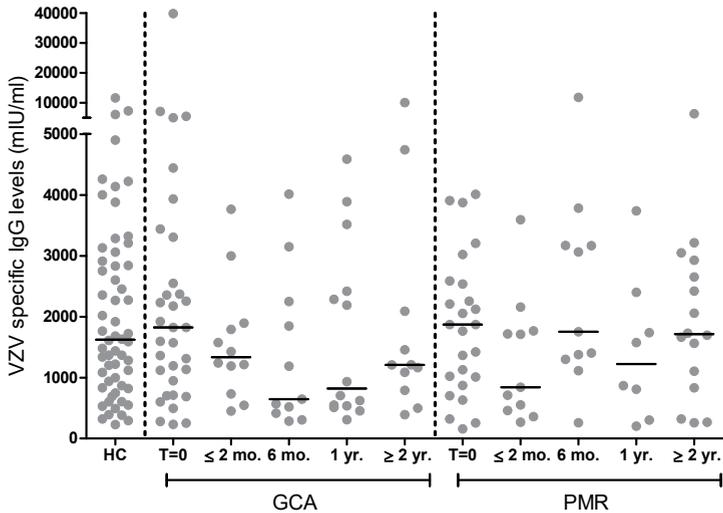


Figure 4. Levels of IgG antibodies against varicella-zoster virus (VZV) among 35 giant cell arteritis (GCA) patients and 26 polymyalgia rheumatica (PMR) patients at different time points of follow-up, and 58 healthy control (HC) subjects. Bars show the median. T=0 = before start of treatment with prednisolone; ≤ 2 mo. = within 2 months after start of treatment; 6 mo. = at app. 6 months of follow-up; 1 yr. = at app. 1 year of follow-up; ≥ 2 yr. = at app. 2 years of follow-up, or more.

However, with an incidence of over 11 per 1000 person years, the herpes zoster risk in GCA patients was found to be high [28]. Interestingly, several large studies reported an increased risk of herpes zoster in persons (mostly diagnosed with rheumatoid arthritis) receiving glucocorticoid therapy [9-11,14,29,30]. In our hospital, GCA patients usually start on 40-60 mg of prednisolone daily, while PMR patients start at a lower dose of 15-20 mg prednisolone daily. In both patient groups, the prednisolone dose is slowly tapered.

Because of the high risk, herpes zoster vaccination needs to be considered in GCA patients. Ideally, vaccination would be administered at time of diagnosis, before patients reach a high cumulative dose of glucocorticoids. Herpes zoster vaccination has been shown to be safe in subjects receiving glucocorticoids, although most subjects studied received relatively low doses [31]. Efficacy of herpes zoster vaccination in glucocorticoid treated patients is a topic that needs to be studied further, as only humoral immune responses were evaluated in these patients previously [31].

Due to the limited number of patients included, no firm epidemiologic conclusions can be drawn on the incidence of clinically overt herpes zoster in GCA or PMR patients in the present report. Furthermore, herpes zoster could have been missed before diagnosis and during follow-up, as it does not necessarily come to attention of the treating rheumatology specialist, especially during more stable disease periods in which outpatient clinic visits are less frequent. As mentioned, for one patient notes on a herpes zoster

period shortly before GCA disease onset were found in the medical records. In hindsight, another patient possibly had a VZV reactivation at time of GCA diagnosis.

No difference was found in the percentages of cytokine producing CD4+ and CD8+ T cells in response to VZV stimulation between GCA patients and controls. When evaluating low-level cell-mediated immune responses, ELISpot is considered to be more sensitive than intracellular flow cytometric analysis while cytokine flow cytometry allows for phenotypic discrimination of responding cells [25,27]. The effector function of T cells upon VZV-specific activation is more clearly seen during active secretion of the cytokines, while flow cytometry shows the potential to react to VZV.

PMR patients were shown to have a higher percentage of IL-2 and IFN γ producing CD4+ T cells in response to VZV stimulation using flow cytometry. As the lymphocyte count in PMR patients was lower than in the healthy control group, the difference in absolute numbers of CD4+ T cells producing cytokines in response to stimulation with VZV between these groups is probably less pronounced than suggested by the relative values.

Of note, as it is known that VZV-specific IgG levels only slowly decline after herpes zoster [32-34], the finding of similar VZV-specific IgG levels in patients at time of diagnosis and healthy controls, indicates that herpes zoster did not occur substantially more often in GCA and PMR patients in the months before onset of disease.

In recent years, several studies have investigated the link between VZV and GCA with the finding of VZV antigen in temporal artery biopsies of a majority of GCA patients as the pivotal evidence claiming an etiologic role for VZV in GCA [35-37]. Other research groups, however, did not detect VZV antigen nor VZV-DNA in temporal arteries of GCA patients [38-40]. A recent paper by Rhee et al., describing a large retrospective study using an electronic database, reported an only modest association between antecedent infections and the incidence of GCA. GCA patients were more likely to have had a prior herpes zoster infection (median time from zoster to GCA of 6 years) than control subjects, but also had significantly more other infections e.g. respiratory and urinary tract infections. No higher occurrence of clinically overt herpes zoster infections at time of diagnosis was found [41]. Our results, demonstrating similar humoral immunity in GCA and PMR patients when compared to healthy controls, are in line with these results.

To the best of our knowledge, this is the first prospective study investigating both humoral and cellular immunity to VZV in GCA and PMR patients. Concluding with our most important result, a decreased cell-mediated immunity to VZV indicates an even higher risk to herpes zoster in GCA patients on glucocorticoid treatment than in age matched healthy controls. Herpes zoster vaccination therefore needs to be considered in patients with GCA, and should ideally be administered at time of diagnosis, before reaching high cumulative doses of glucocorticoids.

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SUPPLEMENTARY DATA

Supplementary Table 1. Characteristics of patients and healthy controls whose samples were used for determination

	HC (n=58)	GCA patients (n=35)		
Sex, no. female/male	41/17	25/10		
Age at time of first sample, median (interquartile range) years	67.7 (62.2-74.9)	73.2 (63.8-79.0)		
GCA diagnosis at baseline:				
FDG PET-CT TAB FDG PET-CT+TAB	NA	17 9 9		
PMR diagnosis at baseline:				
FDG PET-CT Chuang FDG PET-CT+Chuang	NA	3 0 6		
		T=0 (n=33)	≤2 mo. (n=12)	6 mo. (n=11)
Time since diagnosis, median (interquartile range) months	NA	NA	0.5 (0.4-0.7)	7.1 (6.4-8.6)
Cumulative prednisone dosage, median (interquartile range) mg	NA	NA†	840 (615-1185)	5655 (5600-6233)
Use of methotrexate, no. (%) – dose range in mg/week	NA	NA	0 (0)	1 (9) – 15
Use of leflunomide, no. (%) – dose range in mg/day	NA	NA	0 (0)	0 (0)
No. of patients that experienced relapse since diagnosis (%)	NA	NA	1 (9)	0 (0)
Leukocyte count, median (interquartile range) 10 ⁹ cells/L	5.5 (4.9-6.4)	9.1 (8.1-11.5) ***	17.2 (9.2-23.6) ***	11 (9.6-12.2) ***
Haemoglobin, median (interquartile range) mmol/l	8.7 (8.2-9.2)	7.1 (6.6-7.5) ***	7.8 (7.3-8.0) ***	8.1 (7.5-8.5) **
ESR, median (interquartile range) mm/hr	9 (4-13)	99 (59-104) ***	11 (5-45)	24 (16-34)***
CRP, median (interquartile range) mg/L	<5 (<5-<5)	54 (25-96) ***	5 (<5-6)***	7 (<5-12)***

HC = healthy controls; GCA = giant cell arteritis; PMR = polymyalgia rheumatica; T=0 = before start of treatment with prednisone; ≤ 2 mo. = within 2 months after start of treatment; 6 mo. = at approximately 6 months of follow-up; 1 yr. = at approximately 1 year of follow-up; 2 yr. = at approximately 2 years of follow-up, or more; FDG PET-CT = no. of patients with ¹⁸F-fluorodeoxyglucose-positron emission tomography/computed tomography supporting diagnosis; TAB = no. of patients with temporal artery biopsy positive for GCA; NA = not applicable; Chuang: no. of patients fulfilling the Chuang and Hunder PMR criteria; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein. † One patient did not fulfil Chuang and Hunder criteria or had FDG PET-CT proven PMR, but fulfilled the 2012 European League Against Rheumatism/American College of Rheumatology criteria for PMR. ‡ One patient received prednisone treatment because of PMR until approximately 5 months before GCA diagnosis. * P value <0.05; ** P value <0.01; *** P value <0.001 for comparison to healthy control group.

on of VZV-IgG levels (Figure 4).

		PMR patients (n=26)				
		17/9 73.7 (64.7-78.9) *				
		NA				
		3 5 17†				
1 yr. (n=14)	≥2 yr. (n=11)	T=0 (n=25)	≤2 mo. (n=12)	6 mo. (n=11)	1 yr. (n=8)	≥2 yr. (n=16)
11.6 (10.2-13.6)	32.6 (21.3-35.8)	NA	0.7 (0.5-0.8)	7.5 (6.8-8.4)	9.8 (9.4-10.5)	24.9 (23.0-27.8)
6561 (5600-8175)	10808 (7446-12186)	NA	360 (320-1670)	2760 (1895-3090)	3375 (3198-4301)	5949 (3377-7600)
2 (14) – 15-15	3 (27) – 15-25	NA	0 (0)	0 (0)	1 (13) - 15	1 (6) - 10
1 (7) - 10	0 (0)	NA	0 (0)	1 (9) - 10	0 (0)	1 (6) - 10
6 (43)	6 (55)	NA	0 (0)	4 (36)	2 (25)	9 (56)
9.5 (7.2-12.2) ***	7.9 (7.0-9.8) ***	8.7 (7.1-11.1) ***	11.4 (9.3-13.5) ***	9.1 (6.2-12.3) ***	8.3 (7.5-9.3) ***	7.4 (5.5-9.7) **
8.1 (6.6-8.8) **	7.7 (6.8-8.3) ***	7.5 (7.3-8.4) ***	8.3 (7.6-8.7)*	8.1 (7.8-9.2)	8.4 (7.8-9.2)*	8.2 (7.1-9.0)
28 (14-37)***	28 (15-40)***	51 (39-74)***	12 (6-30)	22 (9-30)**	13 (3-44)	15 (5-36)
6 (<5-13) ***	10 (<5-15) ***	44 (27-73) ***	<5 (<5-7) **	7 (<5-11)***	10 (<5-26) ***	<5 (<5-6) **

