RHEUMATOLOGY

Original article

Epstein–Barr virus in bone marrow of rheumatoid arthritis patients predicts response to rituximab treatment

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Abstract

Objectives. Viruses may contribute to RA. This prompted us to monitor viral load and response to anti-CD20 therapy in RA patients.

Methods. Blood and bone marrow from 35 RA patients were analysed for CMV, EBV, HSV-1, HSV-2, parvovirus B19 and polyomavirus using real-time PCR before and 3 months after rituximab (RTX) treatment and related to the levels of autoantibodies and B-cell depletion. Clinical response to RTX was defined as decrease in the 28-joint disease activity score (DAS-28) >1.3 at 6 months.

Results. Before RTX treatment, EBV was identified in 15 out of 35 patients (EBV-positive group), of which 4 expressed parvovirus. Parvovirus was further detected in eight patients (parvo-positive group). Twelve patients were negative for the analysed viruses. Following RTX, EBV was cleared, whereas parvovirus was unaffected. Eighteen patients were responders, of which 12 were EBV positive. The decrease in the DAS-28 was significantly higher in EBV-positive group compared with parvo-positive group ($P = 0.002$) and virus-negative patients ($P = 0.04$). Most of EBV-negative patients that responded to RTX (75%) required retreatment within the following 11 months compared with only 8% of responding EBV-positive patients. A decrease of RF, Ig-producing cells and CD19^{+} B cells was observed following RTX but did not distinguish between viral infections. However, EBV-infected patients had significantly higher levels of Fas-expressing B cells at baseline as compared with EBV-negative groups.

Conclusions. EBV and parvovirus genomes are frequently found in bone marrow of RA patients. The presence of EBV genome was associated with a better clinical response to RTX. Thus, presence of EBV genome may predict clinical response to RTX.

Key words: Rheumatoid arthritis, Biological therapy, B-cell depletion, Viral infection, Epstein–Barr virus.

CLINICAL SCIENCE

Introduction

Several viral infections have been observed before onset of RA or flares in an already established RA [1]. Human parvovirus B19 is among the commonest [1], but other viruses belonging to the herpes family including EBV [2], CMV [3, 4] and HSV [5] have also been suggested to participate in the aetiopathogenesis of rheumatic diseases. The arthritogenic viral nucleic acid double-stranded RNA [6], which is expressed during replication by most viruses [7], is also found in the synovium of RA patients together with EBV, where it may contribute to accelerated inflammation and erosivity [8]. This is one way in which viral infections may contribute to arthritis.

EBV can persist in the bone marrow (mainly in B cells) [9] and parvovirus replicates predominantly in erythroid precursors in the bone marrow [10]. HSV-1, HSV-2 and polyomavirus also persists at haematopoetic sites including the bone marrow [11], whereas CMVs have a broad cell and tissue tropism including the bone marrow [12]. In RA, a direct traffic between the bone marrow and the synovium is possible due to penetration of the cortical

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barrier by inflammatory tissue [13]. Viruses present in the bone marrow could thus aggravate RA or precipitate flares by increasing the inflammatory drive in adjacent joints.

Several mechanisms have been proposed to explain the arthritogenicity of a viral infection. It has been suggested that sequence homology between gp110 of EBV and the shared epitope (SE) in the MHC Class II gene could be a link between RA and EBV. Tolerance to the SE DRB1*0401 HV3 peptide (amino acid sequence QKRAA) in people who express HLA-DRB1*0401 [14], could impair anti-gp110 immunity and thereby the ability of SE carriers to fight EBV infections. This could explain the altered EBV response in RA patients as compared with healthy individuals: EBV-infected RA patients have a diminished cytotoxic T-cell response to EBV as compared with non-RA controls [15, 16], increased number of EBV-infected B cells and high prevalence of antibodies to different EBV antigens [17]. Of these, antibodies to EBNA-1 cross-react with denatured collagen and keratin, two possible autoantigens in RA. Also, EBV infection may transform infected B cells into antibody-secreting plasma cells, thus increasing the likelihood of activating autoreactive B-cell clones [18].

Mechanistic theories of how other viruses may contribute to arthritis are numerous [1], but all evidence gathered so far is only circumstantial. A complicating factor in determining the role of viruses for the actual development of arthritis is the fact that today's RA patients often stand on immunosuppressive treatment that may expose them to an increased risk of viral infection [19]. Widely spread viruses may, therefore, become particularly important as a pathogen in immunocompromised patients. Infections acquired or elicited viral reactivation due to immunosuppressive treatment could thus confound aetiological agents and opportunistic infections. Also, immunosuppressive treatment that alters immune responses to pre-existing viral infections could theoretically influence response to treatment.

We therefore set out to determine: (i) the presence of parvovirus B19, EBV, polyomavirus, HSV-1 and HSV-2 and CMV before and 3 months after immunosuppressive treatment with rituximab (RTX); and (ii) the importance of specific viral infections for response to RTX. RTX is an mAb therapy that selectively depletes B cells by targeting the B-cell surface antigen CD20. B-cell depletion, in combination with MTX, has been shown to be a highly efficient therapy in patients with insufficient response to conventional DMARDs and non-responsiveness to anti-TNF-α therapy [20–23]. Upon treatment, CD20-expressing B cells are depleted [24], but the mechanism of B-cell depletion is not fully understood but may involve CD20-dependent pre-sensitization before Fas-mediated (CD95) apoptosis [25]. Intriguingly, there is no clear consensus regarding correlation between response to RTX and efficient depletion of B cells. Earlier reports have indicated both differences [26] and absence of differences [27] in B-cell depletion efficacy, and distinct repletion patterns of B-cell subpopulations in responders/non-responders [28, 29]. For example, unswitched memory B cells (IgD$^+$, CD27$^+$) were reported to repopulate the circulation faster after B-cell depletion in non-responders *vs* responders [29].

Our findings clearly show that RA patients having EBV replication at baseline, not only cleared their EBV load, but responded significantly better to the RTX treatment and presented a longer lasting response than patients who do not have EBV replication in the bone marrow.

Materials and methods

Patients and treatment

Thirty-five patients were included in the study (Table 1). All patients gave written informed consent to participate. The study was approved by the Regional Ethics Board in Gothenburg (Dnr 633–07). At baseline, 33 patients were on MTX, 1 on AZA and 1 on chlorambucil. Of the patients, 34 were earlier non-responders to anti-TNF-α treatment. All patients commenced anti-CD20 treatment by RTX (F Hoffman-La Roche Ltd, Basel, Switzerland) by infusion of 1000 mg followed by 1000 mg 2 weeks later.

The 28-joint disease activity score (DAS-28) [30] based on the ESR was performed at baseline, and at planned revisits at 3 and 6 months (32 of 35 patients fulfilled both revisits). Response was determined by the change in the DAS-28 and the European League Against Rheumatism response criteria, where an individual patient presenting a decrease in the DAS-28 > 1.3 is defined as a responder to the therapy [31]. Patient characteristics are described in Table 1.

Blood and bone marrow samples

Ten millilitres of heparinized bone marrow aspirates were collected from crista iliaca before the RTX infusion, whereafter mononuclear cells were isolated using a gradient separation on Lymphoprep (Axis-Shield PoC As, Oslo, Norway). Following separation, cells were kept on ice for a maximum of 2 h or immediately stained for flow cytometric analyses. Peripheral blood was obtained by venopuncture from the cubital vein directly in a sterile heparinized vacuum container.

Real-time PCR

Bone marrow and blood samples were evaluated for presence of CMV, EBV, HSV-1, HSV-2, parvovirus and polyomavirus at baseline and 3 months later using real-time PCR. Extraction of DNA from 200 μl of serum or from 200 μl aspirated bone marrow was done in a Magnapure LC instrument (Roche Applied Science, Mannheim, Germany), using the DNA Isolation Kit I (Roche). Real-time PCR, using primers and probes described in Table 2, was then performed on an ABI 7000 or 7900 instrument (Applied Biosystems, Foster City, CA, USA), with 10 μl of extracted sample in a reaction volume of 50 μl, including 25 μl of Universal Master Mix with Uracil *N*-glycosylase (Applied Biosystems). Quantification was obtained from a plot of C_t-values for a serial dilution of

TABLE 1 Clinical, demographic characteristics of patients with RA

Parameter	EBV positive (n = 15)[a]	ParvoB19 positive (n = 8)[a]	Virus negative (n = 12)	P-value[*]
Age, mean (range), years	61.1 (44–81)	57.3 (43–79)	54.2 (29–73)	0.18[Kr]
Gender, female/male	14/1	7/1	9/3	0.41[F]
Disease duration, mean (range), years	10.7 (3–31)	17.8 (3–33)	13.9 (3–43)	0.30[Kr]
RF, positive[b]				
At baseline, n (%)	13 (87)	8 (100)	8 (67)	0.16[F]
At 6 months follow-up, n (%)	9 (60)	2 (25)	5 (41)	0.28[F]
DAS-28				
At baseline, median (range)	5.90 (4.8–6.77)	5.50 (4.45–6.74)	6.21 (5.03–6.94)	0.23[AN]
At 6 months follow-up, median (range)	3.95 (2.16–5.97)	4.08 (2.76–5.52)	4.86 (2.7–6.37)	0.26[AN]
Change DAS28 > 1.3[c]	12 patients (80%)	1 patient (12.5%)	5 patient (42%)	0.007[F]
DMARD treatment				
MTX, mg/week	14 (17)	7 (19.6)	12 (16)	
Chlorambucil	1	0	0	
AZA	0	1	0	
HLA-DRB1 carriers,[d] n (%)	6/13 (46)	3/8 (37.5)	1/12 (8)	0.14[F]
Anti-CCP, median (range), U/ml	24058 (2236–400 000)	16 185 (1163–34 612)	15 689 (1000–400 000)	0.41[Kr]

[a]Four patients were positive for both EBV and parvoB19 viruses in the bone marrow and assigned to the EBV-positive group. [b]RF positivity is determined as >50 U/ml of RF of IgA, IgG or IgM isotype. [c]Mean change (95% CI) in DAS-28 is 1.72 (1.19, 2.25) (EBV positive), 1.24 (0.07, 2.4) (parvoB19 positive) and 1.34 (0.71, 1.96) (virus negative). [d]Thirty-three of the 35 patients were analysed for presence of HLA-DRB1. *Comparisons between all three groups. P-values inferior to 0.05 were regarded significant. AN: one-way ANOVA; F: Fisher's extended exact test; Kr: Kruskal–Wallis.

TABLE 2 Primers and probes used in real-time PCR

ID	Seq.
EBV_F[a]	GGAACCTGGTCATCCTTTGC
EBV_R[a]	ACGTGCATGGACCGGTTAAT
EBV_FAM[a]	CGCAGGCACTCGTACTGCTCGCT
CMV_F	TGATCACTGTTCTCAGCCACAAT
CMV_R	TCCTCTGATTCTCTGGTGTCACC
CMV_FAM	CCCGCACTATCCCTCTGTCCTCA
HSV1_F[b]	GCAGTTTACGTACAACCACATACAGC
HSV2_F[b]	TGCAGTTTACGTATAACCACATACAGC
HSV1 and 2_R[b]	AGCTTGCGGGCCTCGTT
HSV1_FAM[b]	CGGCCCAACATATCGTTGACATGGC
HSV2_FAM[b]	CGCCCCAGCATGTCGTTCACGT
ParvoB_F[c]	AAGCCGTGTGCACCCATT
ParvoBgt2_R	GGTACAGGAGGACGGTTGGTTA
ParvoBgt1 and 3_R[c]	TGGTACTGGTGGGCGTTTAGTTA
ParvoB_FAM[c]	ACACTCCCCACCGTGCCCTCA
Polyoma_F	GGAAAGTCTTTAGGGTCTTCTACCTTT
Polyoma_R	GGTGCCAACCTATGGAACAGA
Polyoma_FAM	TCATCACTGGCAAACATWTCTTCATGGCAAA

All probes were labelled with 5′ FAM (6-carboxyfluorescein) and 3′ TAMRA (6-carboxytetramethyl-rhodamine). The polyoma PCR detects both BK and JC viruses. As described by [a]Niesters et al. [43], [b]Namvar et al. [44] and [c]Knöll et al. [45].

quantification standards consisting of pUC57 plasmids with inserts of the targeted viral region. The real-time PCR assays have ranges of linear detection spanning from 200–400 to 10 million copies/ml as determined by testing of serial dilution of plasmids with viral inserts.

Determination of RF, anti-cyclic citrullinated peptides and Ig-secreting cells

Blood samples were evaluated for total levels of IgG, IgM and IgA using nephelometry and for RF of the IgG, IgM and IgA isotypes by an ELISA (Hycor Biomedical Ltd, Penicuit, UK) and for anti-cyclic citrullinated peptides (anti-CCP) antibodies of the IgG isotype by ELISA (Eurodiagnostica, Malmö, Sweden) following the manufacturer's instructions. The level of RF was calculated using serial dilutions of the standards provided in the kit and expressed in IU/ml. Levels of RF >50 IU/ml were considered positive. The number of IgG-, IgM- and IgA-secreting cells was evaluated in blood using the ELISpot technique.

Identification of SE carriers

The presence of HLA-DRB1 (DRB1*01) allele was evaluated using PCR amplification as described [32]. The genotyping was done at the Regional Blood Center, Sahlgrenska University Hospital, Göteborg.

Flow cytometry analysis

To determine alterations in B-cell phenotype, we used mouse anti-human mAbs in different fluorochromatic conjugations (phycoerythrin, FITC, allophycocyanin, peridinin–chlorophyll–protein or pacific blue) as well as in different combinations, as described by Rehnberg et al. [33]. The following antibodies were used: anti-CD27 (LI28), anti-CD95 (BD-Bioscience, Erembrogdem, Belgium), anti-CD19 (HIB19; eBioscience, San Diego, CA, USA) and anti-IgD F0059 (Dakocytomation, Glostrup,

Denmark). The mAbs were used at optimal concentrations.

Statistical analysis

Categorical values (proportions in any number of different groups) were compared using an extended version of Fisher's exact test (Stata v11.0; StataCorp LP, College Station, TX, USA) to allow for small expected cell counts. Numerical parameters between two and three different groups were compared using Student's *t*-test and one-way ANOVA, respectively (Prism 5.04; GraphPad Software Inc., La Jolla, CA). When the assumptions of the *t*-test or ANOVA were not met, appropriate non-parametric alternatives were considered (Wilcoxon rank-sum test and Kruskal–Wallis). In Table 1, each given *P*-value is followed by an indication of the statistical test used.

Results

EBV and parvovirus B19 are frequently detected in bone marrow of RA patients

At baseline, 15 (43%) of 35 patients had EBV infection in bone marrow (Fig. 1 and Table 1). Of these 15 patients, 7 had detectable levels of EBV genome even in blood samples (Fig. 1). Four of the patients with EBV infection in the bone marrow also expressed parvovirus B19 genome in bone marrow. The parvovirus B19 genome was further detected in the bone marrow, but never in blood (data not shown), of eight EBV-negative patients (Table 1). Interestingly, all EBV-positive bone marrow and blood samples obtained 3 months following RTX treatment were converted to EBV negative (Fig. 1). In contrast, parvovirus genome was still preserved 3 months later in all bone marrow samples that were parvo-positive at baseline. One EBV-positive patient was positive for CMV in blood, whereas none had polyomavirus or HSV-1 or HSV-2 (data not shown). Patients that did not express any of the analysed viruses in bone marrow or blood constituted 34% (*n* = 12; Table 1).

Following baseline findings in bone marrow, the patients were divided into EBV-positive group (patients expressing EBV in bone marrow, *n* = 15), parvo-positive group (patients expressing parvovirus but not EBV in bone marrow, *n* = 8) and virus-negative group (*n* = 12; Table 1). The three groups showed no difference with respect to age, gender and disease duration or disease activity of RA at baseline (Table 1). For some comparisons, the parvo-positive group and virus-negative group were combined into an EBV-negative group. Also, the EBV genome load showed no correlation to the baseline DAS-28 levels (data not shown).

The existing sequence homology between the antigenic glycoprotein gp110 of EBV and the RA-associated SE in HLA-DR (amino acid sequence QKRAA), prompted us to compare the occurrence of SE (DRB1*01) and EBV infection. In all, 33% of the analysed patients expressed the SE (DRB1*01) epitope (Table 1). Among these, we observed a higher percentage of SE carriers among EBV-positive
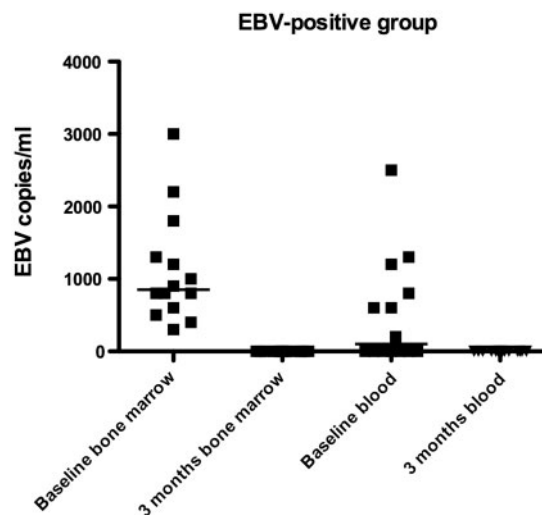
patients; 6 (46%) of 13 analysed EBV-positive patients expressed HLA-DRB1 allele, whereas among EBV-negative patients, only 5 (25%) of 20 analysed patients expressed this SE allele (non-significant, Table 1).

EBV positivity correlates to a better clinical response to RTX therapy

Clinical response to RTX treatment was established as a reduction of ⩾ 1.3 in the DAS-28 index. Six months after treatment, this criteria was fulfilled by 18 (51%) of 35 patients. Age, gender or disease duration did not predict response to therapy (data not shown). The majority of clinical responders [12 (67%) of 18] were accumulated in the EBV-positive group. Indeed, 80% of EBV-positive patients were responders, as compared with only 42% in the virus-negative group and 12.5% in the parvo-positive group (*P* = 0.007; Table 1). At baseline, the patient groups did not differ in the DAS-28 (Table 1), whereas at 6 months post-treatment, EBV-positive had lower DAS-28 values than patients in virus-negative group (Fig. 2A). Further analysis of the patients' response according to EBV positive *vs* non-EBV positive revealed a clear benefit of EBV. This was reflected in a significantly higher ΔDAS-28 in EBV-positive patients compared with EBV-negative patients (Fig. 2B) and an overall significantly higher frequency of responders (white bars, Fig. 2C).

Additionally, only 1 of 12 responders in EBV-positive group was retreated with RTX within 11 months, whereas four of the six responders among non-EBV patients (virus-negative group and parvo-positive group) were retreated within 11 months (depicted as proportion of responders by black bars in Fig. 2C). Taken together, the presence of EBV genome in bone marrow before

FIG. 2 Patients expressing EBV at baseline have a better clinical outcome to RTX therapy than EBV-negative patients. (**A**) DAS scores [median and interquartile range (IQR)] at baseline, 3 and 6 months post-initiation of RTX in patients expressing EBV (EBV positive), expressing only parvovirus (parvo positive) and expressing none of the analysed viruses (virus negative) in bone marrow (*EBV positive *vs* virus negative, $P = 0.038$). (**B**) Median reduction (and IQR) in DAS at 6 months in EBV-positive *vs* EBV-negative patients (*$P = 0.049$). (**C**) Per cent responders (defined as a decrease in DAS-28 > 1.3 at 6 months after initiation of RTX therapy) and proportion of responders receiving retreatment with RTX within 11 months from baseline in EBV-positive and EBV-negative patients. There is significantly more responders (height of the bars) in the EBV-positive group as compared with the EBV-negative group (**$P = 0.0034$) and the proportion of responders (the height of the black space in relation to the height of the bar) receiving retreatment within 11 months is significantly higher in the EBV-negative group (*$P = 0.020$).
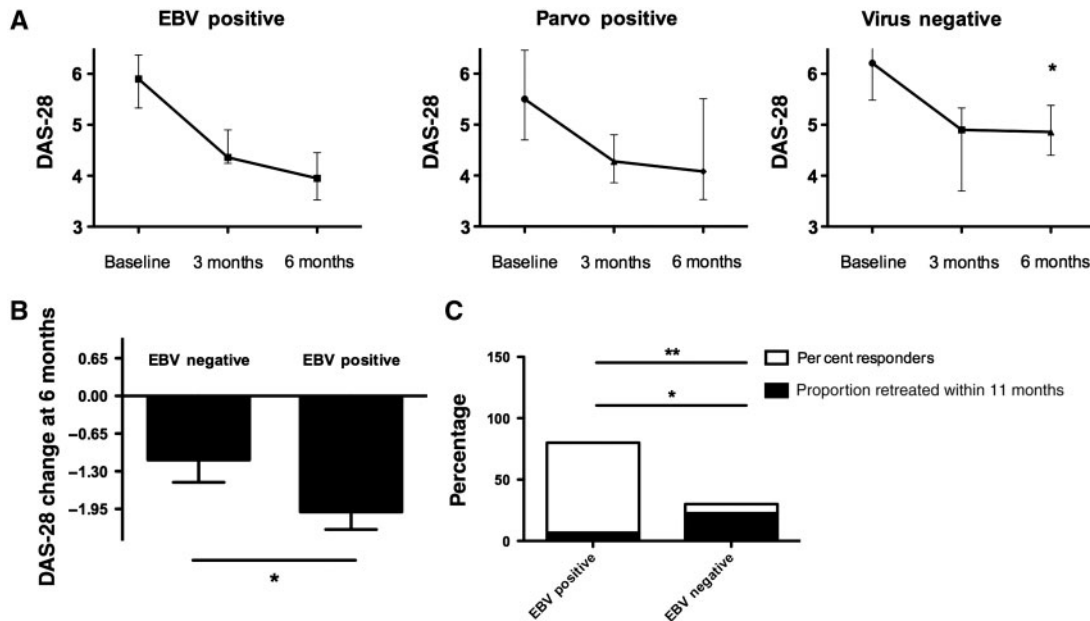
treatment correlated clearly to a better clinical response to RTX treatment. It may also be associated with a longer lasting effect of RTX.

The carriers of HLA-DRB1 comprised 46% of EBV-positive group (see above). Given the overall better response in this group, we thus analysed if the presence of the HLA-DRB1 allele was associated with clinical response to RTX treatment. Of the 10 SE carriers, 6 (60%) were clinical responders, thus comparable with the overall response of 54%. Five (83%) of six SE carriers in EBV-positive group were responders but only one (25%) of four SE carriers in the EBV-negative groups was a responder, indicating that clinical response was not directly related to the presence of HLA-DRB1.

EBV positivity and laboratory changes following RTX treatment

RTX selectively depletes CD20-expressing B cells leaving pre-B cells and plasma cells that do not express CD20 [24] untouched. Given the beneficial effect of EBV on response to RTX, we therefore analysed the impact of RTX on B cells and B-cell products in EBV-positive and EBV-negative patients. We could not detect any difference in total Ig or RF of isotype IgA, IgM or IgG between EBV-positive and EBV-negative patients before, at 3 or 6 months post-treatment, which was also true for

responders *vs* non-responders (data not shown). Furthermore, there was no significant difference in levels of anti-CCP antibodies or total RF between responders and non-responders (data not shown) or EBV-positive and EBV-negative patients (Fig. 3), although a tendency towards higher baseline titre was observed in EBV-positive patients. In accordance, 60% of EBV-positive patients remained RF-positive, whereas only 35% of EBV-negative patients (parvo-positive and virus-negative combined) remained RF positive; Table 1).

The number of B cells, defined as CD19^{+}, was totally depleted in circulation and efficiently but not entirely in bone marrow (Fig. 4 and [33]) 3 months following RTX therapy in all patients, irrespectively of EBV status (Fig. 4). As the rate of repopulation of B cells of different maturity after RTX-mediated depletion has been reported to differ between responders and non-responders [29], we determined the proportions of B cells of different maturity in EBV-positive (of which 80% were clinical responders, Fig. 2C) and EBV-negative patients (of which 30% were clinical responders, Fig. 2C). At baseline and at 3 months after treatment, there were no significant differences in the proportions of bone marrow immature (CD19^{+}, CD27^{−}, IgD^{−}), naïve (CD19^{+}, CD27^{−}, IgD^{+}), unswitched memory (CD19^{+}, CD27^{+}, IgD^{+}) and switched memory B cells (CD19^{+}, CD27^{+}, IgD^{−}) between EBV-positive and

Fig. 3 No significant difference in RF or anti-CCP between EBV-positive and EBV-negative patients. Levels of total RF (**A**) and anti-CCP (**B**) antibodies at baseline in EBV-positive and EBV-negative group. Individual values are represented by dots and the median by the horizontal line.
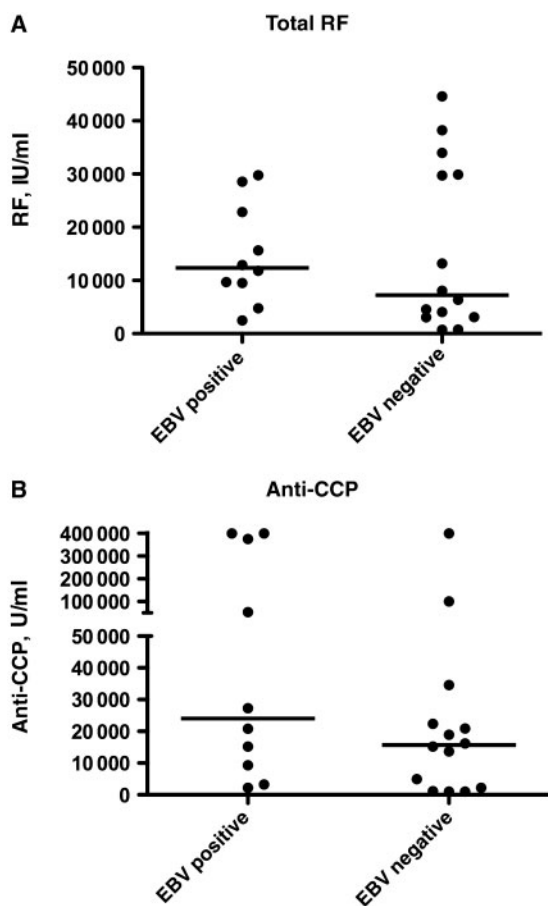
EBV-negative patients (see supplementary figure 1, available as supplementary data at *Rheumatology* Online). In contrast, a population of CD19$^+$CD95$^+$ (Fas) expressing B cells in the bone marrow was significantly higher at baseline in EBV-positive than EBV-negative patients (Fig. 5). As the total B-cell population (Fig. 4), this CD19$^+$CD95$^+$ population was also depleted by RTX, which thus quenched the difference between EBV-positive and EBV-negative patients (data not shown).

Discussion

Viruses may be part of the pathogenesis in RA. Also, susceptibility to viral infection may in fact increase in RA patients as a result of immunosuppressive therapy. This prompted us to relate viral load in RA patients to clinical response during the course of immunosuppressive (B-cell depletion by RTX) therapy and immunological parameters related to disease activity. Circulating CMV, EBV, HSV-1, HSV-2, parvovirus B19 and polyomavirus virus was

determined in blood and bone marrow, where many viruses can persist.

Unambiguously, B-cell depletion eradicated all traces of EBV infection in blood and bone marrow at 3 months post-RTX treatment, which is in line with the EBV that mainly resides in B cells [9] and are thus removed concomitantly with B-cell removal. Although depletion of B cells in the bone marrow was not complete, no genomic EBV-DNA could be detected, indicating that EBV mainly resides in RTX-sensitive B cells. MTX can contribute to lower levels of EBV-DNA in circulation [34], but does not, as RTX totally eradicates all traces of EBV. No other detected viral infection was removed by RTX treatment. Of note, in the group of patients expressing EBV at baseline, the great majority (12 of 15) were responders (ΔDAS-28 > 1.3 at 6 months), whereas most non-responders were found in the parvo-positive and virus-negative group (14 of 17). Importantly, the DAS-28 values at baseline did not differ between patient groups, thus confirming that the lower DAS values in virus-negative patients at 6 months were not due to an overall lower disease activity in EBV-positive patients. In fact, it has been earlier reported that high EBV load correlates to higher disease activity [34]. We could, however, not find any correlation between disease activity and EBV load. The reason for this discrepancy is not known, but could be due to the lower number of patients included in this study.

Our data clearly showed that patients that were carriers of EBV had a significantly better clinical response to B-cell depletion therapy than did patients without EBV infection (Fig. 2). RA is a multifactorial disease and response to various therapeutics show a great individual variation, emphasizing that RA is a heterogeneous disease. The identification of RTX therapy as selectively better therapy in EBV-positive *vs* EBV-negative patients (Fig. 2C) indicates that EBV may be an aetiopathogenic agent in a subpopulation of RA patients.

EBV is widespread in the population but RA patients clearly have an altered response to EBV as compared with healthy controls. For instance, RA patients have much higher levels of EBV-DNA [35], EBV-specific antibodies [17] and a higher number of EBV-infected cells [36] than healthy controls, indicating that RA patients have a less efficient anti-EBV response than EBV-infected controls. Our identification of EBV-infected RA patients as better responders to RTX therapy, thus, indicates that this RA-associated, altered EBV response may represent a fraction of RA patients with an aetiopathogenesis possibly different from non-EBV RA patients. This fraction is characterized by a better and more long-lasting response to RTX therapy. EBV may contribute to RA by eliciting anti-EBV antibody responses with putative cross-reactivity to autoantigens (e.g. anti-EBNA1 can cross-react with collagen or keratin) and EBV is unique among viruses in that it may also transform B cells (irrespectively of antigen specificity in the B cell) to become antibody-secreting plasma cells [18, 37]. This can occur in the absence of the cognate antigen and secondary signals

Fig. 4 No significant difference in RTX-mediated B-cell depletion efficiency between EBV-positive and EBV-negative patients. Per cent of B cells (of white blood cells) defined as CD19+ cells [(**A**) in blood and (**B**) in bone marrow] in EBV-positive and EBV-negative group at baseline and 1–3 months after RTX treatment. The line in each box represents the median, whereas the box represents the IQR with whiskers at ±1.5 IQR. Outliers are indicated.
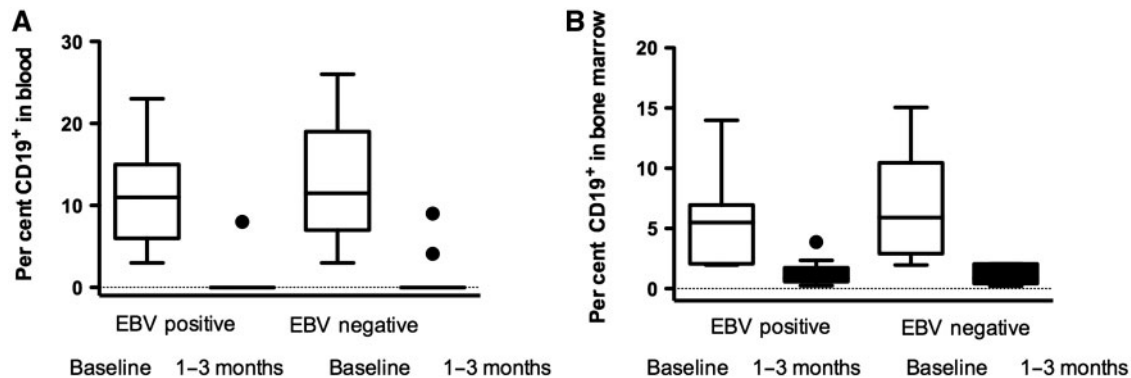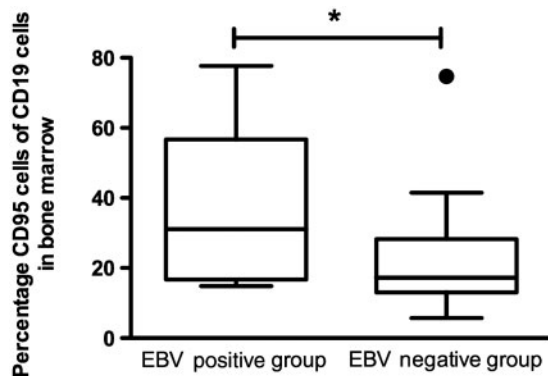

Fig. 5 Higher baseline frequency of CD95-expressing B cells in EBV-positive than EBV-negative patients. Per cent CD95-expressing B cells in bone marrow of all B cells at baseline in EBV-positive and EBV-negative group. The line in each box represents the median, whereas the box represents the IQR with whiskers at ±1.5 IQR. Outliers are indicated. *$P = 0.043$.

provided by dendritic cells and T cells that are normally required for B-cell activation. EBV-transformed B cells may also be a source of antigen-presenting cells [38]. These properties of EBV may contribute to the development of autoantibodies and, under certain circumstances, autoimmune disease.

Another possible link between RA and EBV is the accumulated expression among RA patients of the SE, which is homologous to EBV gp110 glycoprotein [39]. A larger proportion of the EBV-positive patients (46%) expressed HLA-DRB1 than the EBV-negative patients (20%), although this was not statistically significant. There was, however, no correlation between presence of HLA-DRB1 and disease activity or response to treatment, indicating that response to RTX was primarily associated with the presence of the EBV genome.

RTX is a humanized mAb directed against the CD20 antigen that is selectively expressed by B cells. RTX therapy in RA patients of this study selectively depleted B cells from blood and bone marrow compartments. The efficacy of depletion did not differ between EBV-positive and EBV-negative patients (Fig. 4) (and not between responders and non-responders; data not shown) indicating that efficacy of B-cell depletion *per se* does not reflect clinical response. This has not only been reported earlier [27], but also challenged after development of a highly sensitive assay [26]. Also, Roll *et al*. [29] identified distinct repopulation patterns of circulating B cells in responders *vs* non-responders. In all their patients, B cells started to repopulate the circulation within 5.6–10.2 months, but the unswitched memory B-cell subset (IgD+ CD27+) repopulated faster in patients non-responsive to RTX. We therefore analysed the proportions of different maturational stages of B cells in the bone marrow (where no complete depletion occurs [33]) at baseline and 3 months after RTX. We could, however, not detect any significant differences regarding the proportions of B cells of different maturity, in EBV *vs* non-EBV infected patients (Fig. 5) or in responders *vs* non-responders [33]. Thus, the accelerated repopulation of memory B cells to the circulation in non-responders is not reflected in the proportion of residual, non-depleted B cells in the bone marrow.

One mechanism of B-cell depletion by RTX is Fas-mediated apoptosis [25]. In this way, RTX sensitizes Fas-expressing CD20+ B cells to subsequent Fas-mediated killing [40]. At baseline, we identified significantly higher proportions of B cells expressing Fas (CD95) in EBV-positive than EBV-negative patients (Fig. 5). Higher expression of CD95 on B cells in EBV-infected patients could be the result of EBV-mediated up-regulation of CD95 [41]. It is thus possible that EBV could predispose B cells to apoptosis by RTX by up-regulation of Fas. In general, the apoptosis machinery is perturbed in RA,

including Fas-mediated apoptosis due to elevated levels of soluble FasL [42]. EBV may overcome high levels of soluble FasL by up-regulating the receptor (Fas), thus rendering cells more susceptible to apoptosis. However, this mechanism is not sufficient to explain why EBV-infected patients responded better to therapy than EBV-negative patients expressing lower levels of CD95, since B-cell depletion was equally efficient regardless of EBV status. Although the pathophysiology of CD95-expressing B cells is unclear, high expression of CD95 on B cells together with EBV infection could be a biomarker for response to RTX.

In conclusion, the significantly better effect of RTX therapy in EBV-positive patients as opposed to non-EBV patients indicates that B cells are part of the RA pathogenesis in RA patients infected with EBV, but less so in the pathogenesis of uninfected RA patients. What defines these B cells, apart from being infected with EBV, remains to be determined.

> ### Rheumatology key messages
> - EBV in bone marrow of RA patients predicts response to RTX treatment.
> - RTX treatment efficiently clears EBV from the bone marrow.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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