Rheumatoid factor-producing CD21low anergic clonal B-cells in essential mixed cryoglobulinaemia: a model for autoantigen-driven pathogenesis of infectious and non-infectious cryoglobulinaemias

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ABSTRACT

Objective. Essential mixed cryoglobulinaemia (EMC) is a disorder of B-cells producing rheumatoid factor (RF), and is clinically and immunologically similar to mixed cryoglobulinaemia (MC) related to hepatitis C virus (HCV-MC). We report here the first comprehensive analysis of B-cell clonality, phenotype and function in EMC.

Methods. The study population included 16 patients with EMC and 24 patients with HCV-MC. Molecular analysis was done for the detection of circulating clonal B cells and for B cell receptor sequencing. B-cell phenotype, proliferative response, apoptosis and ERK signalling were analysed by flow cytometry.

Results. Molecular analysis of immunoglobulin genes rearrangements revealed circulating B-cell clones in about half of patients, on average of smaller size than those found in HCV-MC patients. Sequence analysis showed usage of the same stereotyped RF-encoding B-cell receptors frequently expressed in HCV-MC and in primary Sjögren's syndrome. B-cells with low expression of CD21 (CD21low) and unusual homing and inhibitory receptors were increased in EMC and in HCV-MC, but at a significantly lower extent in the former. The CD21low B-cells of EMC and HCV-MC patients shared functional features of exhaustion and anergy, namely reduced proliferation upon ligation of Toll-like receptor 9, high constitutive expression of phosphorylated ERK, and proneness to spontaneous apoptosis.

Conclusion. Our findings suggest a common pathogenetic mechanism in EMC, HCV-MC and primary Sjögren's syndrome, consisting of autoantigen-driven clonal expansion and exhaustion of selected RF-producing B-cells. The more massive clonal expansion in HCV-MC may be due to co-stimulatory signals provided by the virus.

Introduction

Mixed cryoglobulinaemia (MC) is a syndrome associated with the production of monoclonal (type II MC) or polyclonal (type III MC) rheumatoid factors (RF), which form with endogenous IgG cold-precipitable immune complexes that cause small-vessel vasculitis and multi-organ damage (1, 2). Chronic hepatitis C virus (HCV) infection is the aetiologic agent in about 90% of all cases of MC vasculitis (HCV-MC) (1), whereas infections by hepatitis B virus or more rarely by other infectious agents account for only a minority of cases (3). Among the patients with noninfectious MC vasculitis, about half of cases are associated with distinctive conditions such as lymphoproliferative disorders or connective tissue diseases, in particular primary Sjögren's syndrome (pSS), while the remaining cases are classified as idiopathic or essential MC (EMC) (4-6). EMC is generally characterised by recurrent episodes of palpable purpura along with visceral involvement (1, 2). In northwestern Spain, the incidence rate of biopsy-proven vasculitis secondary to EMC for people older than 20 years was 4.8 per million (7). Type II monoclonal mixed cryoglobulins are found in ~50% of EMC cases (4, 6), suggesting an underlying benign lymphoproliferative disorder similar to that driven in HCV-MC by protracted antigenic stimulation by the virus (8). Although the clinical characteristics of EMC patients have

been object of several studies, to our knowledge the B-cell pathophysiology of this disorder has not been thoroughly investigated so far.

In HCV-MC, circulating monoclonal B-cells bear stereotyped, or quasi-identical, B-cell receptors (BCRs), endowed with both RF and anti-HCV activities, which are often encoded by a VH1-69 heavy chain gene paired with a VK3-20 light chain variable gene (8, 9). These clonal B-cells have predominantly a CD27^{+} memory phenotype and low or absent expression of CD21 (CD-21^{low} B-cells); in addition, they express a peculiar array of inhibitory and homing receptors including high expression of CD11c, CD22, CD32b, CD72 and CD95, and low expression on CD62L (9-11). Interestingly, CD21^{low} B-cells are also expanded in HIV infection (12) and in some noninfectious immunological disorders such as common variable immunodeficiency (13, 14), systemic lupus erythematosus (15), rheumatoid arthritis (16) and pSS (17), as well as in aged female mice where they are called aged B-cells (18). The CD21^{low} B-cells of all of these noninfectious conditions are enriched in autoantibody-producing cells.

The CD21^{low} B-cells of patients with HCV-MC display functional features, recalling anergy and exhaustion (19), similar to those of CD21^{low} B-cells from HIV (12) and common variable immunodeficiency (14) patients. In fact, similar to virus-specific exhausted cells they fail to proliferate in response to the triggering of Toll-like Receptor 9 (TLR9) and are prone to spontaneous apoptosis, whereas, similar to B-cells made anergic by continual antigenic stimulation (20), they constitutively express high levels of phosphorylated extracellular signal-regulated kinase (pERK) (21).

Here, we sought to compare the BCR repertoire, phenotype and function of B-cells in EMC and in HCV-MC patients. We found that EMC patients had smaller circulating clones of RF-producing B-cells and lower proportions of CD21^{low} B-cells than HCV-MC patients; however, the CD21^{low} B-cells of EMC and HCV-MC patients shared low proliferative responses to TLR9 li-

gation, accelerated apoptosis and high constitutive expression of pERK.

Patients and methods
Study subjects
The study population included 16 patients with EMC and 24 patients with HCV-MC attending the Referral Centre for Mixed Cryoglobulinaemia of the Sapienza University of Rome or the Rheumatology Clinic, Department of Medical Area, Azienda Ospedaliero-Universitaria S. Maria della Misericordia, Udine, Italy. Twenty healthy volunteers matched for age, sex and ethnicity were recruited as control population (Supplementary Table S1). Inclusion criteria for patients with EMC are detailed in supplementary methods. Laboratory assessment included the determination of serum C3 and C4 fraction of complement, cryoglobulin level, serum creatinine level, and a urinalysis to screen for haematuria and a 24-hour urine protein examination. Patients were screened for serological markers of infectious diseases (anti-HCV and HCV RNA, anti-HIV, HbsAg, HbsAb, HbcAb and HBV DNA) and autoantibodies (antinuclear antibodies, ANA; antibodies against extractable nuclear antigens, anti-ENA; anti-neutrophil cytoplasmic antibodies, ANCA; anti-double stranded DNA antibodies, anti-dsDNA). The cases of MC not associated with infectious or autoimmune diseases were defined as EMC according to established criteria (3-6).

This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Sapienza University of Rome (Prot.n. 678/18), and informed consent was obtained from patients.

Detection and phenotyping of circulating B cell clones
Peripheral blood mononuclear cells (PBMC) were obtained by density-gradient centrifugation. Immunophenotyping was done with combinations of fluorochrome-labelled monoclonal antibodies (Becton-Dickinson Biosciences) to CD19, CD20, CD11c, CD21, CD23, CD27, CD95, CD62L,

IgM, IgD, and kappa and lambda light chains. The G6 antibody, which recognises an epitope of the VH1-69-encoded protein (22), was kindly provided by R. Jefferis, Birmingham, UK.

Monoclonal B-cells were detected by the combined analysis of light chain restriction (>80% light chain-restricted B-cells), disease-associated immunophenotypes (CD5^{+}, CD23^{+}) and light scatter characteristics, or by the expression of a VH1-69-encoded idiotype (>6% G6-positive B-cells) as previously described (8, 11, 21, 23). Flow cytometric analyses were done with a FACSCalibur instrument (Becton-Dickinson Biosciences) using the CellQuest (Becton-Dickinson Biosciences) and FlowJo (Tree Star, Ashland, OR) software.

Molecular analysis of BCRs
Peripheral blood mononuclear cells (PBMC) were isolated through centrifugation on density gradient Ficoll-Histopaque® and genomic DNA (gDNA) extracted with the ArchivePure DNA Purification (5PRIME). To obtain cDNA, cells were lysed with TRIzol® Reagent and RNA was purified with Direct-zolTM RNA MicroPrep (ZymoResearch), then reverse transcription with GoScriptTMReverse Transcription System (Promega) was performed. Quality and quantity of DNA and RNA were tested through NanoDrop ND-1000 Spectrophotometer or Qubit 3.0 Fluorometer (Life Technologies).

BCR clonality for heavy and light chain rearrangements was assessed according to the "Biomed-2 concerted action for PCR-based clonality" guidelines (23). Sequence analysis of V(D)J functional rearrangements was done using the ImMunoGeneTics (IMGT) sequence alignment software and stereotyped CDR3 sequences were defined according to established criteria (25, 26). Additional information is available in Supplementary methods.

Proliferation assay
Cell proliferation was measured by the carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay. PBMC were labelled with CFSE (Invitrogen, Life Technologies) and

cultured at 2×10^5 cells per well in 96-well U-bottom plates in the absence or presence of the TLR9 ligand CpG (2.5 μg/mL; Sigma Genosys) and with F(ab′)2 anti-human Ig (4 μg/mL; Jackson ImmunoResearch Laboratories). Cell proliferation was measured at day 5 of culture by flow cytometry. The number of cells entering proliferation (percent of divided cells) was calculated using the FlowJo software (Tree Star). Before flow cytometric analysis, cells were permeabilised (Permeabilizing-Solution 2; Becton-Dickinson Biosciences) and counterstained with antibodies to CD20, IgM or with other antibodies according to the experimental design. Cell proliferation was measured in electronically gated whole CD20+ B cells or B cell subsets.

pERK expression

The intracellular pERK content was measured by the BD Phos-Flow system as per manufacturer's Protocol 1 (Becton-Dickinson Biosciences). Unstimulated PBMC (1.5×10^6 cells) suspended in 100 μL of RPMI 1640 containing 5% fetal bovine serum and fixed by the addition of 200 μL of prewarmed Phos-Flow Fix Buffer I for 10 min, washed twice in PhosFlow Perm/Wash Buffer I, split in two vials, and stained either with anti-pERK1/2-Alexa 488 or with mouse IgG-Alexa 488 as control. Samples were simultaneously stained with fluochrome-conjugated mAbs to CD20, CD21, CD27, IgM, or with other mAbs as requested by the experimental design. The pERK-specific mean fluorescence intensity (MFI) was calculated by subtracting the MFI values obtained with control mouse IgG from those obtained with anti-pERK antibody.

Apoptosis

A total of 10^6 PBMC in 1 mL of RPMI containing 10% fetal calf serum were incubated at 37°C for 16 hours in 96-well U-bottom plates (2×10^5 cells/well), and then washed, resuspended in phosphate-buffered saline with 10% fetal calf serum, and stained with annexin V–FITC (Molecular Probes, Life Technologies), 7-amino-actinomycin (7AAD) (Sigma-Aldrich), anti-CD19 APC and, in some experiments, G6 counterstained with

PE-conjugated goat anti-mouse IgG. After electronic gating of CD19+ B-cells, early apoptotic B cells were identified as annexin V+/7AAD-, and late apoptotic cells as annexin V+/7AAD+ cells; the values reported in the Results and Discussion section refer to total apoptotic B cells (early plus late).

Statistical analysis

Data were analysed by the Mann-Whitney U-test for unpaired two groups, by paired or unpaired *t* test, or by Wilcoxon signed rank test for paired two groups. Data were analysed using Graph-Pad Prism v. 7 (La Jolla, CA, USA).

Results

Patients' clinical and immunologic presentation

The demographic, clinical and immunologic characteristics of the patients with EMC or HCV-MC are summarised in Table I. The two groups did not differ significantly for age and sex distribution, duration of the disease, and clinical presentation of cryoglobulinaemic vasculitis at the time of enrolment. Fifty percent of EMC patients and 54% of HCV-MC patients had type II cryoglobulins. Cryocrit values tended to be lower in EMC patients (3.8±4.1% *vs.* 7.6±8.7% in HCV-MC), although not reaching statistical significance, and C4 levels were similar. Notably, the number of circulating total B-cells was

significantly higher in HCV-MC patients than in EMC patients (Table I); intriguingly, EMC patients had significantly lower numbers of circulating B-cells than age-matched healthy donors (191±232 *vs.* 248±108; *p*=0.0126).

EMC patients harbour small RF-producing B-cell clones

We exploited flow cytometry to investigate the presence of circulating B-cell clones in 13 patients with EMC and in 24 patients with HCV-MC. None of the EMC patients had B-cell clones detectable either by light chain restriction or by VH1-69 expression, although eight of them had type II MC with an IgM monoclonal component. By contrast, we detected B-cell clones in 13 out of 24 patients with HCV-MC, six of which had VH1-69+ clones (data not shown). Since small B-cell clones may pass undetected through flow cytometry, we further assessed clonality by molecular analysis of BCR rearrangements. PCR amplification of the heavy chain VH-FR1 and VH-FR2 regions revealed a B-cell clone in the peripheral blood or in the bone marrow of seven out of 12 EMC patients. However, the PCR amplification bands were very faint in most EMC patients compared to those usually obtained in HCV-MC patients (Fig. 1A), suggesting that B-cell clones were on average of smaller size in the former patients. PCR amplification of

Table I. Demographic and clinical characteristics of patients with EMC and HCV-MC.

Variables	EMC	HCV-MC	*p*-value
Number of patients	16	24	-
Age (y)*	74 (56-83)	68 (40-84)	ns
Female sex	8 (50%)	16 (67%)	ns
Disease duration (mo)*	24 (6-240)	60 (9-288)	ns
Clinical manifestations			
Purpura	13 (81%)	20 (83%)	ns
Ulcers	3 (19%)	2 (8%)	ns
Arthralgia	6 (38%)	11 (46%)	ns
Neuropathy	8 (50%)	18 (75%)	ns
Nephropathy	5 (31%)	5 (21%)	ns
Sicca Syndrome	8 (50%)	8 (33%)	ns
Cryocrit %**	3.8 ± 4.1	7.6 ± 8.7	ns
Type II cryoglobulins	8 (50%)	13 (54%)	ns
Positive RF	12 (75%)	19 (79%)	ns
C4 level (mg/dL)**	8.3 ± 8	12.2 ± 9.2	ns
B cells /μL**	191 ± 232	529 ± 795	0.0303

EMC: essential mixed cryoglobulinaemia; HCV-MC: hepatitis C virus-mixed cryoglobulinaemia; RF: rheumatoid factor.
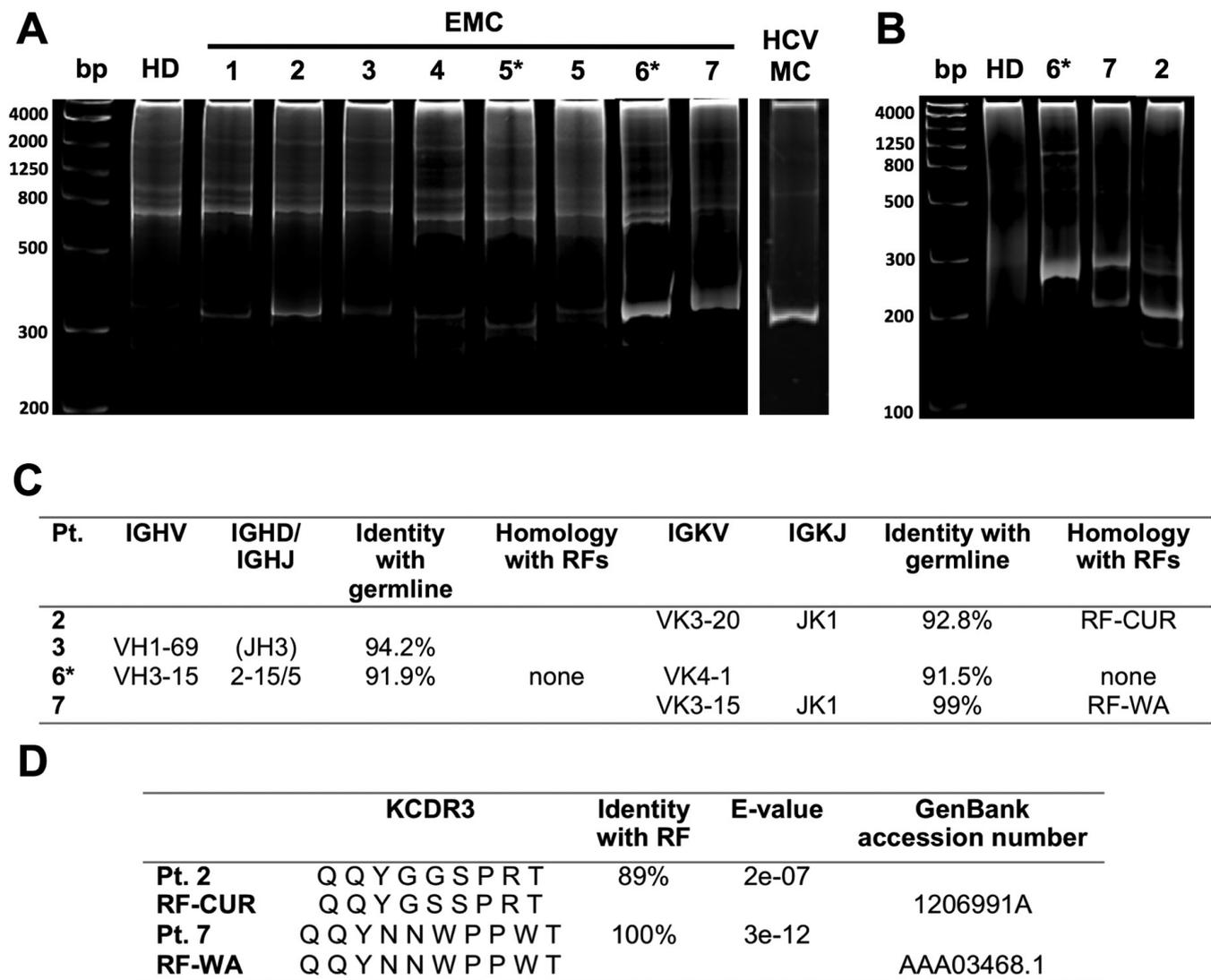*Median (range); **Mean ± Standard deviation (SD).

Fig. 1. Molecular analysis of B-cell clones in EMC. (**A**) Immunoglobulin variable heavy-chain clonal rearrangements of VH framework region 1 in seven patients with EMC and in one representative patient with HCV-MC; *denotes bone marrow samples. (**B**) Immunoglobulin variable kappa light-chain clonal rearrangements in three EMC patients with relatively large B-cell clones. (**C**) BCR heavy and light chain V(D)J gene usage, identity with germline sequences and CDR3 sequence homology with RFs in four patients with EMC; due to the paucity of the amplification products only partial heavy and/or light chain sequences could be obtained. (**D**) Homology of light chain KCDR3 sequences of two EMC patients with the KCDR3 sequences of RFs from GenBank.

light chain genes was successful in three patients and in all revealed a single clonal rearrangement (Fig. 1B).

Due to the paucity of the amplification products obtained from most patients, we could obtain partial heavy chain and/or light chain sequences only in four cases (Fig. 1C). Heavy chain rearrangements were obtained in two patients and involved the VH1-69 and the VH3-15 genes; the HCDR3 sequence (AAEAAHCTGGSYCT) obtained in one case (n. 6* in Fig. 1) had no homology with RFs reported in GenBank. Light chain rearrangements obtained in three patients involved the VK3-15,

VK3-20 and VK4-1 genes, which are frequently used by RF-producing B-cell clones in HCV-MC (27) and in other RF-producing lymphoproliferative disorders (28). The KCDR3 sequences obtained in 2 of these 3 patients (Fig. 1D) were homologous to those of stereotyped BCRs, namely RF-WA and RF-CUR, often expressed by RF-producing clonal B-cells of HCV-MC (29, 30) and pSS (31, 32) patients.

EMC patients have expanded populations of circulating CD21^{low} B-cells

We investigated whether EMC patients

had, similarly to HCV-MC patients, expanded populations of circulating CD21^{low} B-cells. We found that EMC patients had percentages of CD21^{low} B-cells significantly higher than healthy donors, but significantly lower than HCV-MC patients (Fig. 2A and Suppl. Table S1). The absolute number of circulating CD21^{low} B-cells of EMC patients was also significantly lower in EMC than in HCV-MC patients, and in EMC was close to that of healthy donors (Fig. 2B).

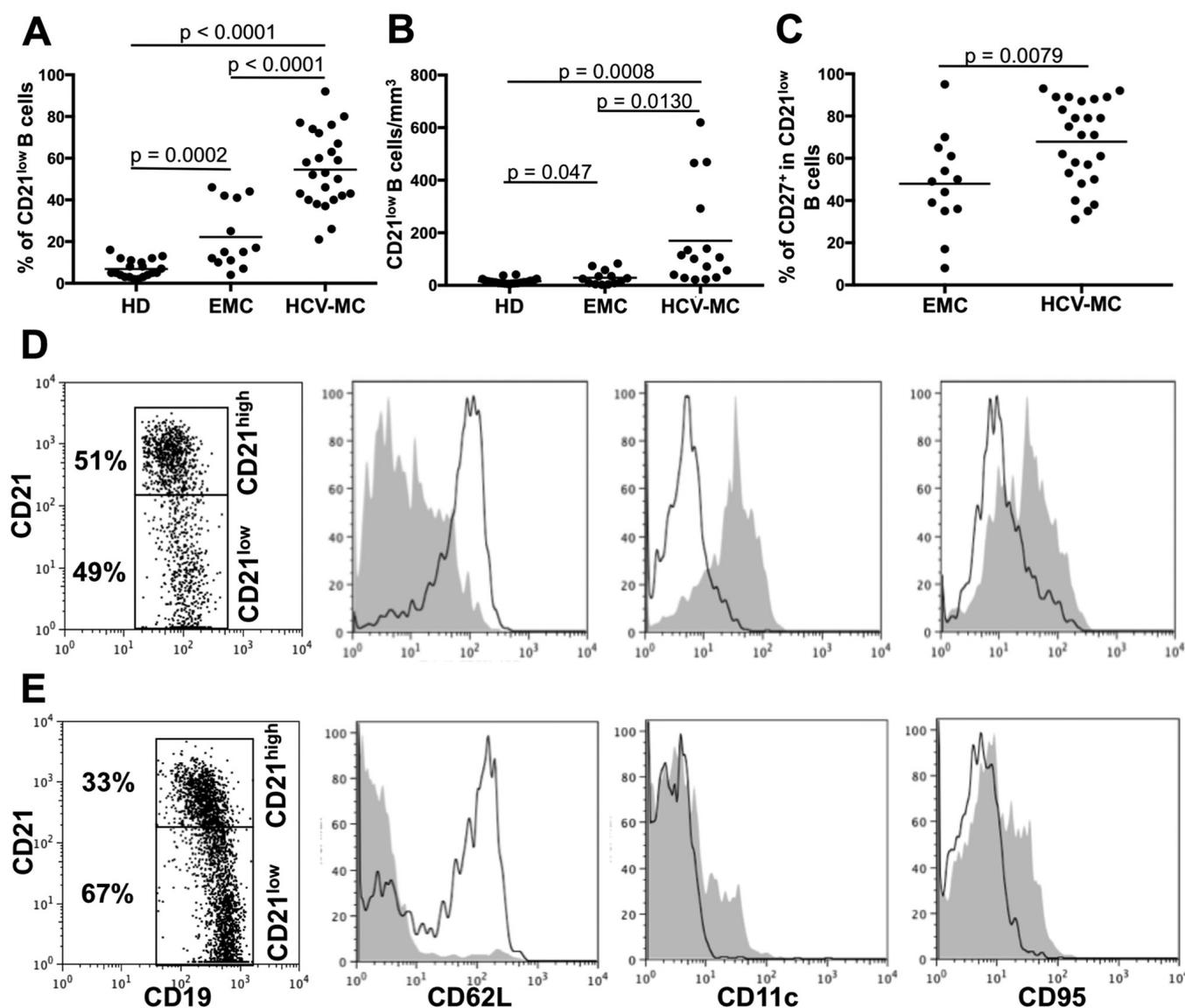The CD21^{low} B-cells of EMC and of HCV-MC patients shared a peculiar CD19^{high} CD62L$^{low/neg}$CD11c^{pos}CD95^{pos}

Fig. 2. Frequency and phenotype of CD21^low B cells in EMC and in HCV-MC.
(**A**) Percentages and (**B**) absolute numbers of circulating CD21^low B-cells in EMC and HCV-MC patients and in healthy donors (HD). (**C**) Percentages of CD27^+ CD21^low B-cells in EMC and HCV-MC patients. Bars denote the means. (**D, E**) Expression of CD62L, CD11c and CD95 by electronically gated CD-21^low (shadowed histograms) or CD21^high (open histograms) B-cells of (**D**) one representative EMC patient and of (**E**) one representative HCV-MC patient.

phenotype (Fig. 2 D-E). This B-cell phenotype is associated, in HCV-MC and in other immunological disorders (9-14, 16, 17), with functional features of anergy and exhaustion. However, the CD21^low B-cells of EMC patients differed from those of HCV-MC patients since naïve-like CD21^lowCD27^- cells were significantly more abundant in the former patients (Fig. 2C). Interestingly, an unusual population of CD27^-IgM^- switched B-cells represented 14.9±11.2% of CD21^low B-cells in EMC and 4.83±2.48% (*p*=0.0475) in HCV-MC patients (Suppl. Fig. S1). Expansion of predominantly naïve-like

CD21^lowCD27^- B-cells was previously reported in CVID (13, 14) and in pSS (17), but in the latter disorder these cells could be assigned to the memory compartment due to the fact that their Ig genes were somatically hypermutated (17). Although we could obtain Ig gene sequences in only few patients, we found that clonal B-cells were mutated, *i.e*. their IGH or IGK sequences had <98% identity with germline (33), in 3 of 4 EMC cases (Fig. 1C). Thus, our data suggest that, although mostly CD27^-, the CD21^low B-cells of EMC patients are predominantly memory cells that have undergone antigen-driv-

en selection and in part have undergone class switch recombination.

The CD21^low B-cells of EMC and HCV-MC patients share functional signatures of anergy and exhaustion
Since CD21^low B-cells display functional features of anergy and exhaustion irrespective of the underlying disorder (9-14, 16, 17), we investigated the functional characteristics of the CD21^low B-cells of EMC patients. We found that circulating B-cells had defective proliferative responses to ligation of TLR9 with CpG both in EMC patients and in HCV-MC patients (Fig.
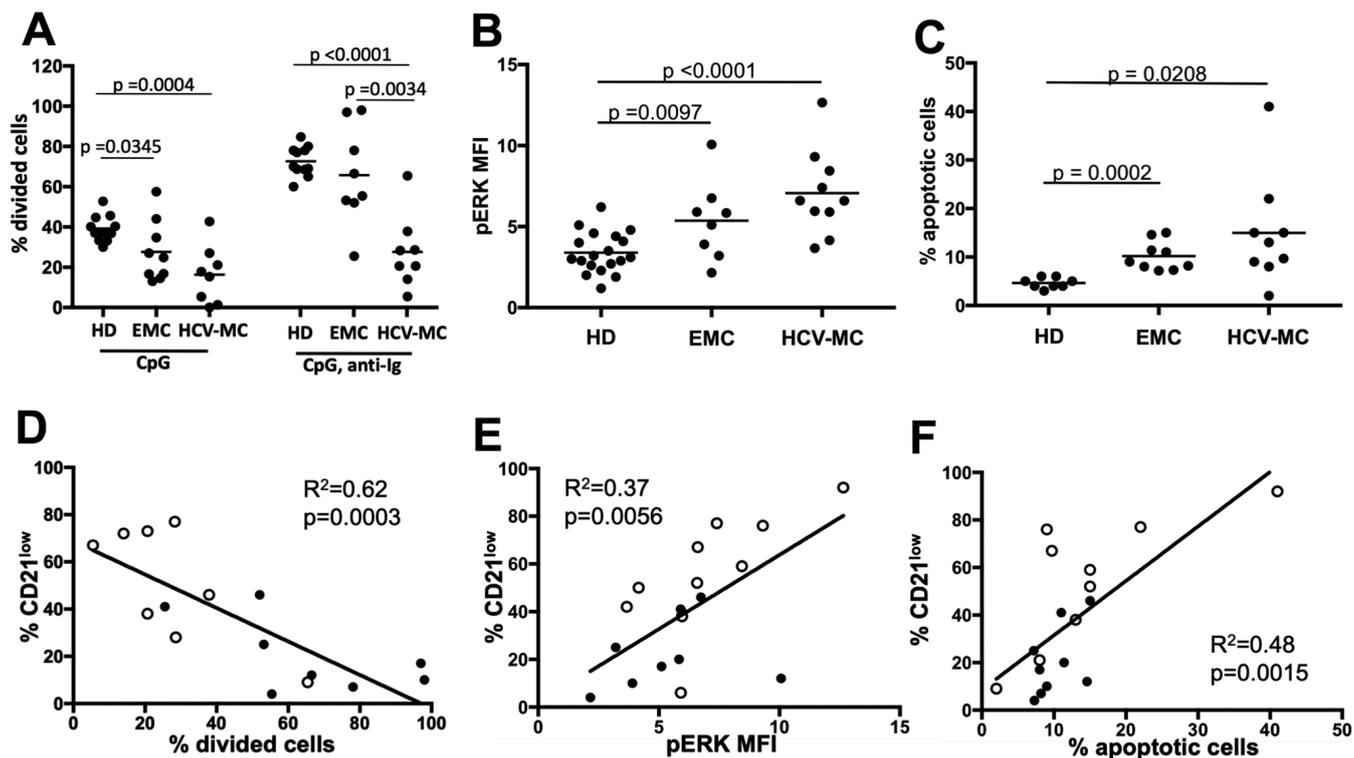
Fig. 3. Features of anergy and exhaustion in CD21^{low} B cells of EMC patients. (**A**) Proliferative responses to stimulation with CpG or CpG plus anti-Ig, (**B**) constitutive ERK phosphorylation, and (**C**) frequency of spontaneous apoptosis in circulating B-cells from EMC and HCV-MC patients or from healthy donors (HD); bars denote the means. (**D, E, F**) Correlations of the frequency of CD21^{low} B cells with (**D**) the frequency of B-cells dividing after stimulation with CpG plus anti-Ig, (**E**) the levels of constitutive pERK expression, and (**F**) the frequency of B-cells undergoing spontaneous apoptosis; closed circles denote EMC patients and open circles HCV-MC patients.

3A and Suppl. Table S1). However, the contemporaneous ligation of BCR and of TLR9 restored the proliferative capacity of whole B-cells from EMC but not HCV-MC patients (Fig. 3A). A possible explanation for this finding is that EMC patients, having less CD21^{low} B-cells, had larger pools of circulating normal naïve B-cells that could be recruited by BCR stimulation to express TLR9 and thus to respond to CpG (34). Consistently with this possibility, we observed a significant inverse correlation between the percentage of CD21^{low} B-cells and that of B-cells induced to proliferate by BCR/TLR9 co-stimulation (Fig. 3D). Nevertheless, an intrinsic difference in the functional properties of CD21^{low} B-cells from EMC or HCV-MC patients cannot be ruled out. We found that the B-cells of EMC patients had high constitutive expression of pERK, a trait of anergy induced by continual antigenic stimulation (20), although less markedly than those of HCV-MC patients (Fig. 3B and Suppl. Table S1). Accelerated B-cell apop-

tosis, another signature of antigen-induced anergy (20), was also observed in EMC as well as in HCV-MC patients (Fig. 3C and Suppl. Table S1). Increased constitutive pERK expression was a characteristic of CD21^{low} B-cells, as indicated by a direct correlation between the frequency of CD21^{low} B-cells and the levels of pERK expression (Fig. 3E) and by co-staining of B-cells with anti-pERK and anti-CD21 antibodies (Suppl. Fig. 2). Also, the direct correlation between the frequency of CD21^{low} B-cells in the sample before culture and the frequency of apoptotic B-cells after culture (Fig. 3F), as well as the direct staining of apoptotic B-cells with CD21 (Suppl. Fig. 3), indicate that accelerated apoptosis was mostly restricted to CD21^{low} B-cells although it cannot be excluded that phenotypic changes during the culture period could somewhat confound the identification of original CD21^{low} B-cells by direct staining.

Discussion
The immunopathogenesis of EMC

has been scarcely investigated and, to our knowledge, this is the first study providing a genetic, phenotypic and functional landscape of B-cells in this disorder. The clinical presentation of our patients did not differ substantially from that described in large cohorts of EMC patients (3-6). Although molecular data in our study are limited, they provide evidence that patients with EMC harbor small B-cell clones that make biased usage of the same Ig variable genes characteristic of RF-producing clones in HCV-MC (27), chronic lymphocytic leukaemia (28) and pSS (31, 32). Usage of the same restricted set of variable genes, which are rarely used by RF-producing B-cells of RA patients and healthy donors (35), suggests that autoantigen, *i.e.* IgG immune complexes, is the primary driver for clonal expansion of peculiar RF-producing B-cell populations in all of the former disorders. We also found that EMC patients have expanded populations of CD21^{low} B-cells sharing unique phenotypic and functional features of

anergy and exhaustion with those of HCV-MC (21) and pSS (17) patients. The more intense lymphoproliferation in HCV-MC than in EMC or pSS may be due to the additional contribution of the costimulatory signal imparted by HCV through the interaction of the E2 protein with CD81 (36).

Among the noninfectious conditions in which anergic CD21^{low} B-cells are expanded, common variable immunodeficiency, rheumatoid arthritis and pSS have been investigated in greater detail. The CD21^{low} B-cells of common variable immunodeficiency patients are predominantly CD27$^-$ unmutated naïve B-cells (16), whereas in rheumatoid arthritis they include naïve, memory and CD27$^-$ switched B cells (16, 18). The RF-producing CD21^{low} B-cells of pSS patients are mostly CD27$^-$ but carry mutated immunoglobulin genes, and therefore belong to the memory compartment (17); similarly, the CD21^{low-}CD27$^-$ B-cells found at low frequency in normal individuals are mostly memory cells (37). The RF-producing CD-21^{low} B-cells of HCV-MC patients are, instead, predominantly CD27$^+$ memory B-cells with mutated immunoglobulin genes (27). In this regard, the CD21^{low} B-cells of EMC patients resemble more closely the CD27$^-$CD21^{low} memory B-cells of pSS patients rather than the CD27$^+$CD21^{low} B-cells of HCV-MC patients.

The CD21^{low} B-cells of pSS patients are similar to those found in EMC and in HCV-MC with regard to dysregulated BCR signalling, defective proliferation and accelerated apoptosis (27). However, unlike in HCV-MC and EMC, in pSS the CD21^{low} B-cells are able to proliferate, although to a somewhat reduced extent, in response to TLR9 ligation (27). These differences might be related to distinctive pathogenetic mechanisms or to the preferential homing in pSS of distinct functional subsets of CD21^{low} B-cells to the salivary gland tissue; indeed, FCRL4$^+$ CD21^{low} B-cells are not found in the peripheral blood of pSS patients (17) while they are enriched in their salivary glands (38).

Type II cryoglobulinaemia and cryoglobulinaemic vasculitis characterise subgroups of patients suffering from HCV infection or pSS, which are very different conditions from an aetiological point of view but share several clinical manifestations, *i.e.* sicca syndrome, widespread chronic pain, and fatigue (39-43). Our findings that EMC shares with these disorders biased BCR repertoires as well as B-cell phenotypic and functional features, suggest a common pathogenic mechanism primarily involving antigen-driven clonal expansion and anergy of RF-producing B-cells carrying distinctive BCRs. Interestingly, nearly identical RF BCRs are expressed in a chronic lymphocytic leukaemia subset and allow leukemic B-cells cells to proliferate when stimulated by immune complexes (28).

An important difference between EMC and pSS on one side and HCV-MC on the other is the smaller size of B-cells clones (44) and the significantly lower number of circulating CD21^{low} B-cells (17) in the former disorders compared to the latter (23). The expansion of CD-21^{low} B-cells driven by viruses, such as HCV (23), HIV (12) or HBV (45) is, in general, much more massive than that found in noninfectious disorders such as pSS (16), rheumatoid arthritis (16) or systemic lupus erythematosus (15). It is possible that these chronic viral infections are able to impart to target B-cells strong stimuli driving robust clonal expansion whereas autoantigens, the putative triggers of noninfectious CD-21^{low} B-cell expansions, provide weaker stimuli.

A hypothetical scenario for the activation and clonal expansion of RF-producing B-cells in pSS delineates, as first step, the formation of immune complexes with Ro and La autoantigens in ectopic salivary germinal centres. These immune complexes would stimulate polyclonal RF-producing B-cells within the marginal zone eventually leading to their oligoclonal and, subsequently, monoclonal expansion (46). In EMC, the putative driving autoantigen would also be represented by immune complexes, which are formed at low amounts even in normal conditions and more abundantly under several circumstances. Besides overtly pathologic conditions such as infections or cancer (47), it is possible that subclinical changes in the infectome (the array of microbial interactions of the host) may disturb immunologic homeostasis, as suggested with regard to the pathogenesis of some autoimmune disorders (48), leading to increased formation of immune complexes and to the activation of RF-producing B-cells. It has been shown that immune complexes, in combination with TLR9 or TLR7 ligands of microbial origin, can break "clonal ignorance" and activate RF-expressing B-cells in autoimmunity-prone mouse strains but not in normal mice (49). Thus, it is possible that human subjects who develop EMC have a permissive genetic background, analogous to that of autoimmunity-prone mice, which allows naïve RF-producing B-cells to be activated under appropriate circumstances. The recently reported findings in HCV-cured patients with HCV-MC of long lasting persistence of large B-cell clones (23) and of very late recurrences of cryoglobulinemic vasculitis due to concomitant infections or cancer (47) also supports this model. Thus, HCV might act as a co-stimulator of B-cell activation rather than the primary aetiologic agent of HCV-MC, and mixed cryoglobulinaemias might be considered as primary autoimmune disorders irrespective of their infectious or non-infectious context. Although limited by the low number of enrolled patients due to the rarity of both HCV-MC and EMC, our study proposes a novel pathogenetic landscape that might prompt more effective therapeutic strategies for lymphoproliferative disorders of RF-producing B-cells.

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