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BRIEF DEFINITIVE REPORTS

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Long-lasting persistence of large B-cell clones in hepatitis C virus-cured patients with complete response of mixed cryoglobulinaemia vasculitis

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Abstract

Background & Aims: Hepatitis C virus (HCV)-related mixed cryoglobulinaemia vasculitis (MCV) is characterized by the expansion of rheumatoid factor-producing B-cell clones. The aim of this study was to assess whether B-cell clones may persist in these patients after the clearance of the virus with antiviral therapy, and whether their persistence influences clinical outcomes.

Methods: Forty-five HCV-cured MCV patients were followed up for a median of 18.5 (range 9-38) months after the clearance of HCV. Circulating B-cell clones were detected using flow cytometry either by the skewing of kappa/lambda ratio or by the expression of a V_H1-69-encoded idiotype.

Results: The clinical response of vasculitis was 78% complete, 18% partial and 4% null. However, cryoglobulins remained detectable in 42% of patients for more than 12 months. Circulating B-cell clones were detected in 18 of 45 patients, and in 17 of them persisted through the follow-up; nine of the latter patients cleared cryoglobulins and had complete response of vasculitis. Several months later, two of these patients had relapse of MCV.

Conclusions: B-cell clones persist in MCV patients long after HCV infection has been cleared but halt the production of pathogenic antibody. These 'dormant' cells may be reactivated by events that perturb B-cell homeostasis and can give rise to the relapse of cryoglobulinaemic vasculitis.

KEYWORDS

B-cell clone, direct-acting antivirals, Hepatitis C virus, mixed cryoglobulinaemia

Abbreviations: C4, complement fraction 4; DAAs, direct-acting antivirals; HCV, Hepatitis C virus; MCV, mixed cryoglobulinaemia vasculitis; NHLs, non-Hodgkin lymphomas.

1 | INTRODUCTION

Hepatitis C virus (HCV) causes monoclonal B-cell lymphoproliferative disorders including mixed cryoglobulinaemia vasculitis (MCV) and non-Hodgkin lymphomas (NHLs).[1] The B-cells clonally expanded in MCV produce monoclonal IgM rheumatoid factor (RF),[2] putatively cross-reactive with HCV and often encoded by the V_H1-69 variable gene,[3] which form cold-precipitable immune complexes responsible for vasculitis. These clonal B-cells display peculiar characteristics such as low expression of CD21 ($CD21^{low}$ B-cells) and features of functional exhaustion.[4-6] The eradication of HCV infection with direct-acting antivirals (DAAs) is associated in most cases with healing of MCV[7-9] and with regression of NHL,[10] but cryoglobulins persist in almost half of HCV-cured MC patients[8] and clinical relapses have been observed in some of them.[9,11]

We previously reported[12] that circulating B-cell clones may persist for at least some months after the eradication of HCV, suggesting that this might be the cause for HCV-independent persistence or relapse of MC. Here, we describe the long-term immunological and clinical outcomes of antiviral therapy with DAAs in patients with MCV with or without NHL, and correlate these outcomes with the presence and persistence of circulating B-cell clones.

Key Points

- Circulating B-cell clones persist in mixed cryoglobulinaemia patients long after HCV is cured.
- Cryoglobulins and vasculitis can disappear despite the persistence of large B-cell clones.
- B-cell clones may be activated by HCV-unrelated stimuli.

2 | METHODS

2.1 | Study population and assessment of clinical outcomes

We investigated 45 patients with HCV-associated MCV, 30 female and 15 male aged 40 to 84 years (median 69). Eight patients had MCV associated with indolent B-cell NHLs (MCV-NHL) and 18 patients had a clinical diagnosis of compensated cirrhosis or a Metavir score of F4.

Patients were treated with guideline-tailored DAA therapy and were followed up for a median of 18.5 (9-38) months after the clearance of HCV, which occurred in all cases after 1 month of therapy. The clinical response of vasculitis was defined as previously described[7] and detailed in Supplementary Methods. The immunological response was evaluated by changes in cryocrit and serum complement fraction 4 (C4) values.

Treatments were administered on-label and follow-up was done according to good clinical practice standard, and therefore this study did not require specific ethical approval. Laboratory studies beyond routine clinical care were approved by the Ethics Committee of Sapienza University of Rome and informed consent was obtained from patients.

2.2 | Detection of circulating B-cell clones by flow cytometry

Monoclonal B-cells were identified either by light chain restriction and distinctive immunophenotype, or by the expression of a V_H1-69-encoded idiotype as previously described.[3,12] Briefly, to identify V_H1-69-expressing B-cells, we used the G6 antibody recognizing an epitope of the V_H1-69-encoded protein (Figure S1). 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. Additional information is available in Supplementary Methods.

2.3 | Statistics

Data were analysed by unpaired t test and Mann-Whitney test using the GraphPad Prism software.

3 | RESULTS

3.1 | Clinical outcomes

Thirty-five of 45 patients (78%) were complete responders, eight (18%) partial responders and two (4%) non-responders. Similar to a previous report,[7] purpura, glomerulonephritis and skin ulcers tended to respond more rapidly and more completely than peripheral neuropathy or sicca syndrome. Eleven of the 18 patients with compensated cirrhosis showed an improvement of the F4 Metavir score when tested between months 9 and 12 of follow-up: one patient decreased to Metavir F3, four patients to Metavir F2 and six patients to Metavir F0-F1.

Cryocrit values decreased and C4 serum levels increased steadily after antiviral therapy (Figure 1A-B); however, between months 12 and 38 post-therapy 42% of patients still had detectable cryoglobulins (Figure 1A) and C4 levels were low in 31% (Figure 1B). Among these patients, 38% presented both normalization of C4 level and negative cryocrit, whereas 13% had still low C4 level and positive cryocrit. Baseline cryocrit was similar in patients with (7.3 ± 8.1%) or without (8.8 ± 11.7%) cirrhosis, and cryoglobulin persistence was unrelated to cirrhosis suggesting that reduced clearance of immune complexes was not responsible for it. Also, of 32 patients investigated at baseline for C4 levels those with cirrhosis had a similar prevalence of low C4 (5/12, 42%) as those without cirrhosis (10/20, 50%). No correlation between persistence of cryoglobulins and response of vasculitis was observed; in fact, among 24 patients with complete clinical response at months 12 to 38 (median 18.5), 10 (42%) still had detectable cryoglobulins.

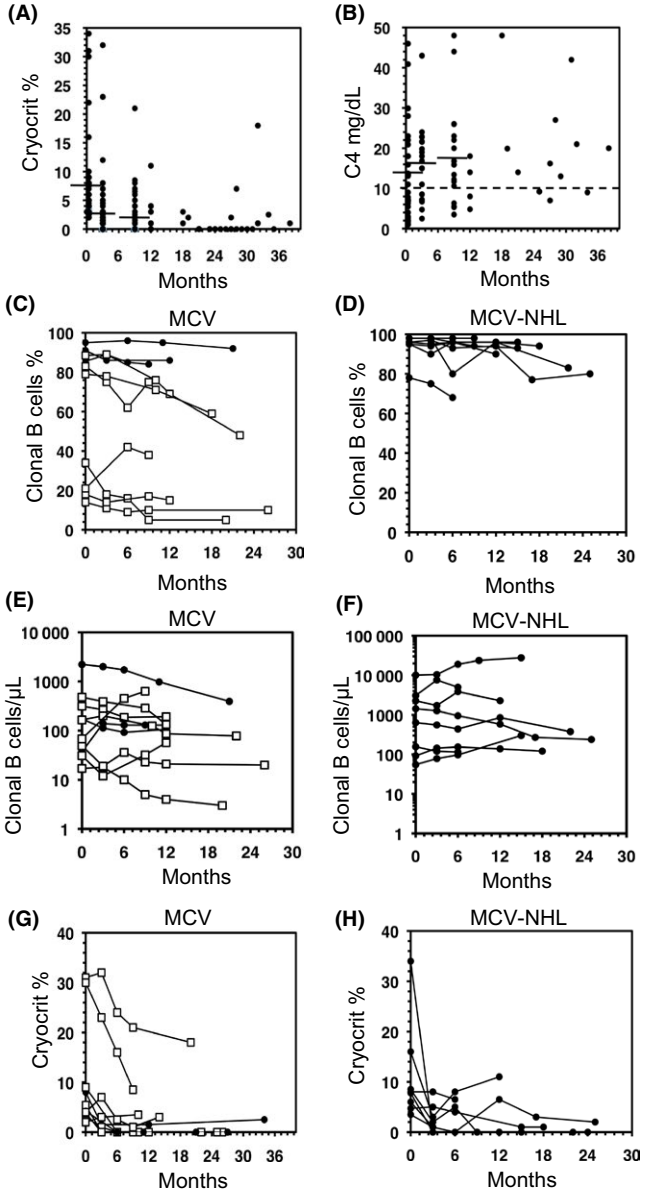

FIGURE 1 Immunological response and circulating B-cell clones in hepatitis C virus-cured patients. (A) Cryocrit values and (B) serum C4 levels in 45 patients with mixed cryoglobulinaemia vasculitis (MCV) or MCV-NHL. Bars denote the means, and the dashed line denotes the lower normal limit of serum C4. (C) Sizes of circulating B-cell clones detected in 10 patients with MCV and (D) in eight patients with MCV-NHL. Clonal B-cells were identified by skewed kappa/lambda ratio (closed circles) or by the expression of a V_H1-69-encoded idiotype (open squares); the clone size was calculated as the percentage of clonal B-cells among total B-cells. (E) Absolute numbers of circulating clonal B-cells in patients with MCV and (F) with MCV-NHL. (G) Cryocrit values in the patients with MCV and (H) with MCV-NHL who had detectable B-cell clones

3.2 | Detection and characterization of circulating B-cell clones

Circulating B-cell clones could be detected in 18 of 45 (40%) patients, eight of which had MCV-NHL (Figure 1C,D). At baseline, clones constituted 14% to 95% (median 81%) of circulating B-cells in MCV patients, and 78% to 98% (median 96%) in MCV-NHL patients; the absolute number of clonal B-cells ranged between 17 to 2214 (median 110) cells/µL in MCV patients, and 55 to 10080 (median 1029) cells/µL in MCV-NHL patients (Figure 1E,F). The clonal B-cells of 7 of 10 patients with MCV (Figure 1C), and of none of the patients with MCV-NHL (Figure 1D), expressed a V_H1-69-encoded idiotype. The higher frequency of $V_H1\text{-}69^+$ clones in MCV than in MCV-NHL is puzzling since HCV-associated NHLs often express this idiotype,[13] and can be due in part to the fact that MCV patients often have small-size clones that, when V_H1-69-negative, pass undetected through light chain restriction analysis.

The relative size of circulating B-cell clones, expressed as the percentage of clonal B-cells among total B-cells, remained substantially unchanged through the follow-up in 9 of 10 MCV patients and in all of eight MCV-NHL patients (Figure 1C,D); only in one patient with MCV, the size of the $V_H1\text{-}69^+$ clone decreased from 34% at baseline to 5% at month 9, a proportion below the 6% upper percentage of circulating $V_H1\text{-}69^+$ B-cells found in healthy subjects.[3] The absolute number of circulating monoclonal B-cells decreased only in two of 10 MCV patients (Figure 1E) and in one of eight MCV-NHL patients (Figure 1F).

At baseline, most of the clonal B-cells of MCV and of MCV-NHL patients were $CD21^{low}$; as previously reported in a small series of patients,[12] the percentage of $CD21^{low}$ clonal B-cells decreased steadily after the clearance of HCV (Figure S2). Further characterization revealed that, either before or after HCV cure, the $CD21^{low}$ but not the $CD21^{high}$ clonal B-cells displayed a peculiar $CD19^{high}CD11c^{pos}CD95^{pos}CD62L^{low/neg}$ phenotype (Figure S3) previously described in $CD21^{low}$ B-cells of MCV patients[4-6] and of patients with other immunological disorders.[14,15]

3.3 | Correlation of the persistence of B-cell clones with clinical outcomes

Surprisingly, we found no correlation between the persistence of B-cell clones and that of serum cryoglobulins or of vasculitis. In fact, nine patients (five with MCV and four with MCV-NHL) cleared cryoglobulins (Figure 1G,H) and had a complete clinical response of vasculitis despite the persistence of large clones. By contrast, one patient had clearance of the B-cell clone by month 9 but high-titre (18%) cryoglobulins were detected up to month 20 and he had an only partial clinical response. Among the remaining eight patients who had persistence of B-cell clones and failed to clear cryoglobulins, five had a complete and one a partial response, while two were non-responders. One of the latter patients had HCV-NHL, and had no improvement of kidney disease and of peripheral neuropathy and persistence of cryoglobulins for 18 months after the clearance of HCV. The other non-responder had MCV with mild sensory neuropathy and was diagnosed with lung cancer shortly after the start of antiviral therapy; after the clearance of HCV she had persistence of a large B-cell clone and of high-titre cryoglobulins, and died 9 months later of respiratory failure because of dramatic progression of motor neuropathy.

TABLE 1 Features of hepatitis C virus-cured patients with relapse of mixed cryoglobulinaemia vasculitis

Pt. n.	Age/sex	Baseline			Month of relapse	Concomitant event	At relapse		
		Symptoms	Cryocrit	B-cell clone[a]			Symptoms	Cryocrit	B-cell clone[a]
1	73/F[b]	Purpura, skin ulcers, neuropathy	8	91	15	None	Purpura, progressive neuropathy	2	86
2	76/F	Purpura, neuropathy, nephropathy	7.5	0	19	Respiratory infection	Diffuse purpura	5	0
3	60/F	Purpura, neuropathy	3	88	25	None	Purpura	1	48

[a]Percent of circulating B-cells.
[b]Patient with partial clinical response.

Three patients had relapse of MCV several months after the cure of HCV infection (Table 1). Two patients had cleared cryoglobulins and were complete clinical responders, while one healed from purpura and skin ulcers but had stable neuropathy and persistence of traces of cryoglobulins and was therefore classified as partial responder. Relapses were characterized by the recurrence of purpura, which was unusually diffuse in one case and was associated with dramatic progression of peripheral neuropathy in another. Two of the patients had large B-cell clones that persisted through the follow-up while one apparently lacked clonal B-cells in peripheral blood; in the latter patient relapse occurred in concomitance with an acute upper respiratory tract infection.

4 | DISCUSSION

We observed that circulating B-cell clones persisted in most patients with MCV or with MCV associated with indolent NHL for up to 2 years after the cure of HCV infection. Only one MCV patient showed disappearance of the circulating B-cell clone, retaining, however, high titres of cryoglobulins. It might be possible that clonal B-cells responsible for cryoglobulin production persisted confined in the lymphoid tissue, bone marrow or liver.[16] This scenario might be similar to what observed in the majority of our MCV patients with cryoglobulins and apparently absence of circulating B-cell clones.

Intriguingly, several MCV or MCV-NHL patients cleared serum cryoglobulins and all clinical signs of vasculitis despite the persistence of large B-cell clones, suggesting that the clonal B-cells could have switched to a 'dormant' state in which the production of pathogenic antibody was suppressed. The hypothesis of a switch to a non-pathogenic state is supported by the phenotypic and functional changes that take place in clonal B-cells once HCV is cleared. In fact, we provide evidence that after antiviral therapy the predominantly $CD21^{low}$ clonal B-cell population is gradually substituted by a clonal population mostly made of $CD21^{high}$ cells lacking the peculiar array of homing and inhibitory receptors typical of $CD21^{low}$ B-cells. In addition, it has been shown[12] that after the clearance of HCV, clonal B-cells undergo functional changes in B-cell receptor signalling through the extracellular signal-regulated kinase pathway and in their susceptibility to spontaneous apoptosis.

How B-cell clones can survive for such a long time in the absence of the HCV trigger, and whether they may be reactivated by stimuli other than HCV are puzzling issues. An appealing possibility is that the survival of B-cell clones after the clearance of HCV is related to the poly-(auto)reactive nature of their BCRs that, besides the still elusive[13] anti-HCV specificity, are endowed with RF activity.[2] Thus, IgG, especially in the form of immune complexes, could be the autoantigenic trigger responsible for the survival and reactivation of B-cell clones beyond HCV. In this regard, it is of interest that relapses of MCV in HCV-cured patients have been observed in concomitance with respiratory infections or the occurrence of lung cancer,[11] or shortly after influenza vaccination.[17] In the cohort reported here, one complete responder had recurrence of purpura and of cryoglobulinaemia

in concomitance with an acute respiratory infection; also notably, a lung cancer was diagnosed during antiviral therapy in a patient who had dramatic progression of vasculitis despite the clearance of HCV. Altogether, these cases suggest that abundant immune complexes produced during infections, vaccination or lung cancer[18] might re-activate B-cell clones leading to the relapse of MCV. Experiments in transgenic mice support the concept that immune complexes can induce pre-activated, but not naïve, RF-producing B-cells to proliferate and secrete antibody.[19,20] Similar in vitro experiments with RF-producing clonal B-cells of patients may help to clarify a role for immune complexes in the pathogenesis of MCV.

CONFLICT OF INTEREST

LG and AL.Z. have received consulting or lecturing fees from Gilead Sciences, Bristol Myers Squibb, Merck, AbbVie and Janssen-Cilag. All other authors have declared no conflicts of interest.

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REFERENCES

1. Monti G, Pioltelli P, Saccardo F, et al. Incidence and characteristics of non-Hodgkin lymphomas in a multicenter case file of patients with hepatitis C virus-related symptomatic mixed cryoglobulinemias. *Arch Intern Med*. 2005;165:101-105.
2. Charles ED, Orloff MI, Nishiuchi E. Somatic hypermutations confer rheumatoid factor activity in hepatitis C virus-associated mixed cryoglobulinemia. *Arthritis Rheum*. 2013;65:2430-2440.
3. Carbonari M, Caprini E, Tedesco T, et al. Hepatitis C virus drives the unconstrained monoclonal expansion of VH1-69-expressing memory B-cells in type II cryoglobulinemia: a model of infection-driven lymphomagenesis. *J Immunol*. 2005;174:6532-6539.
4. Charles ED, Brunetti C, Marukian S, et al. Clonal B-cells in patients with hepatitis C virus-associated mixed cryoglobulinemia contain an expanded anergic CD21low B-cell subset. *Blood*. 2011;117:5425-5437.
5. Terrier B, Joly F, Vazquez T, et al. Expansion of functionally anergic CD21-/low marginal zone-like B-cell clones in hepatitis C virus infection-related autoimmunity. *J Immunol*. 2011;187:6550-6563.
6. Visentini M, Cagliuso M, Conti V, et al. Clonal B-cells of HCV-associated mixed cryoglobulinemia patients contain exhausted marginal zone-like and CD21 low cells overexpressing Stra13. *Eur J Immunol*. 2012;42:1468-1476.
7. Gragnani L, Visentini M, Fognani E, et al. Prospective study of guideline-tailored therapy with direct-acting antivirals for hepatitis C virus-associated mixed cryoglobulinemia. *Hepatology*. 2016;64:1473-1482.
8. Cacoub P, Si Ahmed SN, Ferfar Y, et al. Long-term efficacy of interferon-free antiviral treatment regimens in patients with hepatitis C virus-associated cryoglobulinemia vasculitis. *Clin Gastroenterol Hepatol*. 2019;17:518–526.
9. Bonacci M, Lens S, Mariño Z, et al. Long-term outcomes of patients with HCV-associated cryoglobulinemic vasculitis after virologic cure. *Gastroenterology*. 2018;155:311-315.
10. Arcaini L, Besson C, Frigeni M, et al. Interferon-free antiviral treatment in B-cell lymphoproliferative disorders associated with hepatitis C virus infection. *Blood*. 2016;128:2527-2532.
11. Visentini M, Quartuccio L, Del Padre M, et al. Late relapses of hepatitis C virus-cured mixed cryoglobulinaemia associated with infection or cancer. *Rheumatology (Oxford)*. 2018;57:1870-1871.
12. Del Padre M, Todi L, Mitrevski M, et al. Reversion of anergy signatures in clonal CD21low B-cells of mixed cryoglobulinemia after clearance of HCV viremia. *Blood*. 2017;130:35-38.
13. Ng PP, Kuo CC, Wang S, et al. B-cell receptors expressed by lymphomas of hepatitis C virus (HCV)-infected patients rarely react with the viral proteins. *Blood*. 2014;123:1512-1515.
14. Rakhmanov M, Keller B, Gutenberger S, et al. Circulating CD21low B-cells in common variable immunodeficiency resemble tissue homing, innate-like B-cells. *Proc Natl Acad Sci USA*. 2009;106:13451-13456.
15. Moir S, Ho J, Malaspina A, et al. Evidence for HIV- associated B-cell exhaustion in a dysfunctional memory B-cell compartment in HIV-infected viremic individuals. *J Exp Med*. 2008;205:1797-1805.
16. Vallat L, Benhamou Y, Gutierrez M, et al. Clonal B cell populations in the blood and liver of patients with chronic hepatitis C virus infection. *Arthritis Rheum*. 2004;50:3668-3678.
17. Sollima S, Milazzo L, Vassalini P, Antinori S, Galli M. Recurrence of mixed cryoglobulinaemia vasculitis following influenza vaccination despite clearance of hepatitis C virus infection. *Clin Exp Rheumatol*. 2018;36:161-162.
18. Ohyama K, Yoshimi H, Aibara N, et al. Immune complexome analysis reveals the specific and frequent presence of immune complex antigens in lung cancer patients: A pilot study. *Int J Cancer*. 2017;140:370-380.
19. Rifkin IR, Leadbetter EA, Beaudette BC, et al. Immune complexes present in the sera of autoimmune mice activate rheumatoid factor B-cells. *J Immunol*. 2000;165:1626-1633.
20. Sang A, Niu H, Cullen J, et al. Activation of rheumatoid factor-specific B-cells is antigen dependent and occurs preferentially outside of germinal centers in the lupus-prone NZM2410 mouse model. *J Immunol*. 2014;193:1609-1621.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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