Brief Report

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Reversion of anergy signatures in clonal CD21^{low} B cells of mixed cryoglobulinemia after clearance of HCV viremia

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Key Points

- Anergic features of B cells of MC rapidly reverse after eradication of HCV with DAAs.
- Phenotypic and functional features of virus-specific B-cell exhaustion persist for several months after HCV eradication.

Hepatitis C virus (HCV) causes mixed cryoglobulinemia (MC) by driving clonal expansion of IgM$^+$CD27$^+$ B cells. These cells display both the features of anergy induced by continual engagement of the B-cell receptor (BCR), such as high expression of phosphorylated extracellular signal–regulated kinase (pERK) and reduced lifespan, and of virus-specific exhaustion, such as CD21^{low} phenotype and a defective response to ligation of BCR and Toll-like receptor 9 (TLR9). MC usually regresses after eradication of HCV with interferon, whose immunomodulatory activity might contribute to this effect. We investigated the phenotypic and functional changes in clonal B cells of MC patients with sustained virologic responses to direct-acting antivirals (DAAs), which lack immunomodulatory properties. We found that high pERK expression and accelerated apoptosis revert within 4 weeks after beginning therapy, whereas clonal B cells unresponsive to TLR9 stimulation persist for at least 24 weeks, although they may partially rescue normal CD21 expression. Thus, similar to mouse models, features of anergy in MC B cells rapidly revert after disengagement from HCV, whereas virus-specific exhaustion imparts a durable inhibitory imprint on cell function. Treatment of HCV$^+$ MC with DAAs provides a valuable tool for untangling the molecular mechanisms of anergy and exhaustion in human B cells. (*Blood*. 2017;130(1):35-38)

Introduction

Mixed cryoglobulinemia (MC) is a lymphoproliferative disorder caused by hepatitis C virus (HCV) and is characterized by clonal expansion of CD27$^+$IgM$^+$ B cells producing a rheumatoid factor often encoded by the V_H1-69 and V_k3-20 genes.[1,2] It is widely believed[3] that the clonal expansion of V_H1-69$^+$ B cells in MC and in HCV-associated non-Hodgkin lymphomas (NHLs) is triggered by the protracted stimulation by an HCV antigen that, however, remains elusive.[4]

Clonal B cells of patients with HCV$^+$ MC display peculiar phenotypic and functional features. In fact, they commonly express low levels of CD21 (CD21^{low} B cells), express an array of inhibitory and apoptosis-related genes and a distinctive pattern of homing receptors (including CD11c$^+$), fail to flux calcium upon B-cell receptor (BCR) triggering and proliferate in response to the stimulation of BCR or of Toll-like receptor 9 (TLR9), and are prone to die by apoptosis.[5-7] Identical CD21^{low} B cells are expanded in patients with common variable immunodeficiency (CVID)[8] and in HIV-infected individuals.[9] These CD21^{low} B cells have been defined "exhausted"[9] for their similarity with virus-specific exhausted T cells.[10] For some of their characteristics, human CD21^{low} B cells recall the murine "aged B cells" (ABCs) increased in aged mice,[11,12] which are CD21^{low}CD11c$^+$ B cells expressing the T-box expressed in T cells (T-bet) transcriptional factor and are important for the control of viral infections.[13] However, ABCs lack many signatures of human CD21^{low} exhausted B cells, such as the fact that they robustly respond to the stimulation of TLR7 or TLR9.[11-13]

CD21^{low} B cells of HCV$^+$ MC[7] and CVID patients[8] display high constitutive expression of the active phosphorylated form of extracellular signal–regulated kinase (pERK); this signature, together with reduced calcium flux and proneness to apoptosis, makes them closely resemble murine B cells made anergic by continual BCR engagement by antigen.[14] In murine anergic B cells, constitutive ERK signaling and reduced lifespan are reversed by dissociation of self antigen from BCR using hapten competition, indicating the need for continual BCR occupancy for maintaining anergy.[15] Here, we exploited the newly available direct-acting antivirals (DAAs), which rapidly suppress HCV viremia in HCV$^+$ MC patients[16] and lack the immunomodulatory properties of interferon, to untangle the effects of BCR disengagement in a human model of virus-driven anergy and exhaustion.

Study design

Twenty-four patients with HCV$^+$ MC were treated with guideline-tailored DAA therapy as described previously[16]; all patients had negative HCV viremia at the end of treatment and sustained virologic responses (SVRs) through a follow-up of 12 (SVR12) to 24 (SVR24) weeks after the end of treatment. All patients were investigated for phenotypic changes in clonal B-cell populations after the cure of

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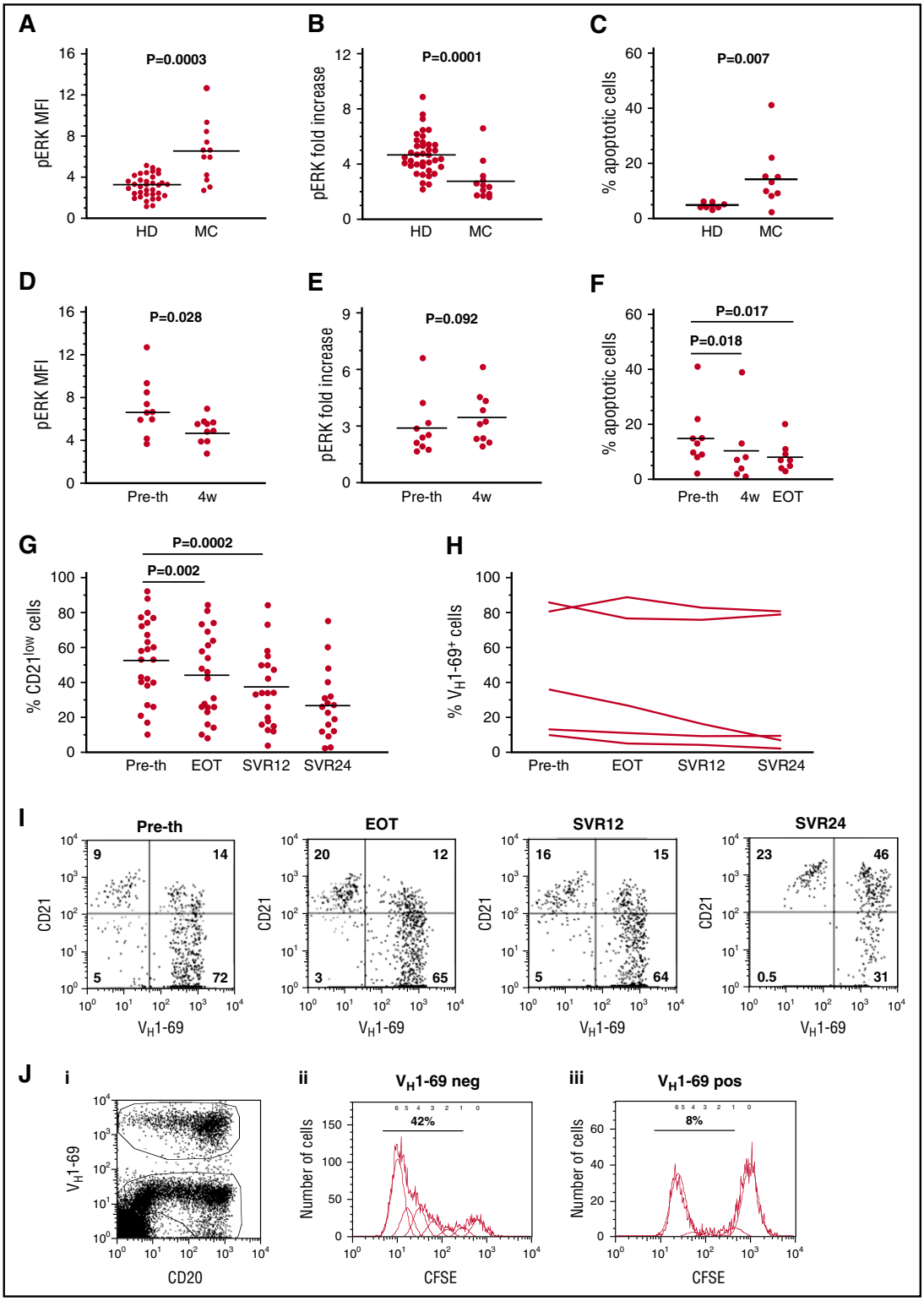

Figure 1. Phenotypic and functional changes in clonal B cells of HCV$^+$ MC patients after clearance of HCV infection by DAA therapy. (A) Constitutive pERK expression by whole B cells, expressed as mean fluorescence intensity (MFI), is higher in untreated MC patients than in healthy donors (HD), whereas (B) the relative increase of pERK induced by BCR ligation with anti-immunoglobuliln, expressed as fold increase compared with constitutive pERK MFI, is reduced in MC B cells. Bars denote the means. (C) MC B cells are more prone to spontaneous apoptosis than HD B cells. (D) pERK constitutive expression in MC B cells decreases significantly 4 weeks after beginning DAA compared with pretherapy (pre-th), whereas (E) the BCR-induced fold-increase of pERK remains unmodified at this time point. (F) Spontaneous apoptosis is significantly reduced in MC B cells 4 weeks after beginning DAA therapy and at the end of treatment (EOT). (G) Changes in the proportions of CD21^{low} B cells among circulating B cells in a cohort of 24 MC patients treated with DAA. (H) Changes in the proportions of V_H1-69$^+$ B cells in 5 MC patients treated with DAA. (I) In one MC patient, V_H1-69$^+$ B cell expansion persists unmodified after DAA therapy, but a large proportion of clonal B cells rescue a CD21^{high} phenotype at SVR24. (J) MC V_H1-69$^+$ B cells remain unable to proliferate efficiently in response to TLR9 ligation at SVR24. (Ji) Electronic gating of V_H1-69$^-$ and V_H1-69$^+$ B cells; the CD20^{dim} cells are plasmablasts. (Jii-Jiii) Analysis of the proliferative responses of (ii) V_H1-69$^-$ and (iii) V_H1-69$^+$ B cells. Percentages denote the number of cells that started dividing (precursor cohort); numbers denote cell divisions as calculated by the FlowJo software. CFSE, carboxyfluorescein diacetate succinimidyl ester.

infection; 10 patients also underwent functional studies of ERK signaling, apoptosis, and proliferative responses to stimuli. Patients, B-cell phenotyping, and functional assays are described in detail in supplemental Methods, available on the *Blood* Web site. This study was approved by the local institutional ethics committee, and informed consent was obtained from all patients.

Results and discussion

Clonal B cells of most patients with untreated HCV^+ MC had high basal levels of pERK (Figure 1A), while the relative increment (fold increase) of pERK upon BCR stimulation was reduced (Figure 1B); uncoupling of constitutive and BCR-induced ERK activation was previously observed in $CD21^{low}$ B cells of HCV^+ MC[7] and CVID[8] patients. Furthermore, patients' B cells showed increased apoptosis upon in vitro culture without stimuli (Figure 1C). In addition to these anergy-like features,[14,15] clonal B cells displayed the features of exhausted B cells,[5-7] as they were mostly $CD21^{low}IgM^+CD27^+CD11c^+FCRL4^+$ and failed to proliferate in response to BCR or TLR9 stimulation (not shown).

Four weeks after the beginning of DAA therapy, constitutive ERK phosphorylation was significantly reduced compared with pretherapy levels (Figure 1D), although BCR-induced ERK phosphorylation increased only slightly (Figure 1E). At this time point, spontaneous in vitro apoptosis was also significantly reduced (Figure 1F). The possibility that early normalization of constitutive ERK activation and apoptosis was due to replacement of clonal B cells by normal B cells contrasts with the fact that the proportions of $CD21^{low}$ B cells in these patients were unchanged at week 4 of therapy (54.9% ± 25% vs 53.8% ± 23% pretherapy).

Our results support the idea that, as in the case of antigen-induced reversible B-cell anergy in mice,[15] reduction in the lifespan of B cells of HCV^+ MC patients depended on chronic stimulation and could be reversed by disengagement of the BCR. Although it is believed that reduced lifespan of murine anergic B cells is largely due to unfavorable competition for B-cell–activating factor,[17] the rapid increase of lifespan after BCR disengagement suggests that chronic BCR signaling is sufficient to initiate apoptosis.[15] Thus, we investigated whether reduced lifespan of MC B cells was related to ERK signaling; treating MC B cells with the MEK/ERK inhibitor U0126, as previously described,[8] failed to reduce spontaneous in vitro apoptosis (not shown), suggesting that ERK signaling is not directly involved in their proapoptotic pathway.

The proportions of $CD21^{low}$ B cells declined steadily up to SVR24 (Figure 1G), although they remained on average higher than in healthy donors (mean ± SD, 5.8% ± 2.5%). However, the proportions of V_H1-69-expressing B cells remained stable up to SVR24 in 3 out of 5 patients (Figure 1H), suggesting that phenotypic changes occurred in clonal B cells after eradication of HCV, as previously observed in HCV^+ MC patients treated with interferon.[18] Indeed, in these 5 patients, the proportions of $V_H1\text{-}69^+CD21^{low}$ cells decreased from 84.8% ± 11.1% pretherapy to 36.4% ± 11.8% at SVR24 ($P = .043$) (supplemental Results), indicating a reversion of the $CD21^{low}$ phenotype. Figure 1I shows representative cytograms from 1 patient. Despite phenotypic changes, long-lived clonal B cells failed to restore their capacity to proliferate in response to TLR9 stimulation. Indeed, the number of $V_H1\text{-}69^+$ cells entering division (precursor cohort) in 3 patients (supplemental Results) was 2.8 ± 1.2 pretherapy and 5.3 ± 2.4 at SVR24, whereas it was 35.6 ± 6.5 and 32.6 ± 8.5, respectively, in autologous $V_H1\text{-}69^-$ cells. Figure 1J shows representative cytograms from 1 patient. This indicates that HCV-driven B-cell exhaustion makes use of durable reprogramming mechanisms irrespective of the $CD21^{low}$ phenotype, as suggested by previous studies.[18,19]

Recently, $T\text{-}bet^+CD21^{low}CD11c^+$ B cells, similar to murine ABCs, were found increased in patients with chronic hepatitis C or cirrhosis without MC, and, importantly, eradication of HCV with DAAs led to their decrease, indicating dependence on infection.[20] Although we did not investigate and cannot exclude T-bet expression in clonal B cells of MC patients, their $CD27^+IgD^+IgM^+$ B cells[1,2] are phenotypically distinct from the $T\text{-}bet^+$ B cells of HCV-infected patients without MC, which are mostly $CD27^-IgD^-IgM^-IgG^+$ class-switched cells.[20] Interestingly in this regard, while the $T\text{-}bet^+CD27^-IgG^+$ B cells of HCV-infected patients decrease after antiviral therapy, the few $T\text{-}bet^+IgD^+IgM^+$ B cells appear to increase.[20]

In summary, we show that clonal B cells of HCV^+ MC display signatures of anergy induced by continual BCR occupancy and of exhaustion driven by chronic viral infection. Anergy features (pERK overexpression and accelerated apoptosis) rapidly revert after disengagement from HCV, while phenotypic and functional features of exhaustion persist for several months. The rapid clearance of HCV viremia with DAAs, which unlike interferon do not act as immunological modulators, offers a unique model for untangling the interplay of virus-driven anergy and exhaustion in human B cells.

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M.D.P. and S.C. are PhD candidates at Sapienza University of Rome, and this work is submitted in partial fulfillment of the requirement for the PhD.

Authorship

Contribution: M.D.P., M.V., and M.F. designed the research; M.D.P., M.M., R.M., and L.T. performed experiments; M.C., S.C., and M.V. recruited and followed up the patients; M.D.P. and M.V. analyzed results; and M.D.P., M.C., M.V., and M.F. wrote the paper.

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Comment on Del Padre et al, page 35

Persistence of exhaustion in cured hep C

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In this issue of *Blood,* Del Padre et al[1] evaluated the impact of eliminating chronic antigen exposure on B-cell exhaustion in the human therapeutic setting of direct-acting antiviral therapy for chronic hepatitis C virus infection (HCV) complicated by mixed cryoglobulinemia (MC). The phenotype of hepatitis C–related MC B cells has been previously demonstrated by this group and others[2] to include low-level CD21 expression ($CD21^{low}$) and hypoproliferation in response to stimulation either through the B-cell receptor or Toll-like receptor 9, imperfectly termed "exhausted" or "anergic." At baseline, $CD21^{low}$ B cells from HCV-infected patients expressed markers of chronic activation, with elevated and near-maximal basal levels of phosphorylated extracellular signal–regulated kinase, and were also predisposed to apoptosis. During oral direct-acting antiviral therapy, the authors observed that by 4 weeks (at which point, most patients have no circulating HCV RNA), these basal abnormalities at least partially normalized. During longitudinal follow-up after patients were documented as cured, the overall frequency of $CD21^{low}$ cells progressively declined from 50% to 30% of circulating B cells. However, the frequency of $V_H1\text{-}69^+$ B cells specific for HCV-related MC generally remained stable, although some regained CD21 expression. Despite partial resolution of the exhaustion phenotype, surviving $V_H1\text{-}69^+$ B cells remained hypoproliferative upon Toll-like receptor 9 ligation, suggesting that the B-cell exhaustion phenotype is durably programmed into antigen-specific B cells during long-term extracellular antigen exposure.

Mixed cryoglobulinemia is one of several B-cell proliferative disorders that may result from chronic hepatitis C infection, and it is thought to be driven by expansion of HCV envelope-specific B cells that preferentially use immunoglobulin gene segments $V_H1\text{-}69$ and $V_k3\text{-}20$,[3] and to evolve rheumatoid factor activity through somatic hypermutation.[4] Although MC is an infrequent complication of chronic hepatitis C infection, the technological ability to identify and isolate HCV-specific B cells via an anti-idiotype $V_H1\text{-}69$–specific antibody (G6) has made this condition an important model for studying humoral immune dysfunction during human chronic viral infection. Prior studies have identified that $V_H1\text{-}69^+$ B cells manifest genetic signatures of enhanced interferon-mediated responsiveness, apoptosis, and B-cell anergy; are phenotypically most commonly $CD27^+/IgM^+/CD11c^+/CD21^{low}$; are prone to apoptosis; and are hyporesponsive to B-cell receptor crosslinking.[2,5]

B-cell anergy, an adaptive response to chronic antigen exposure for autoreactive B cells, has been postulated to be a vulnerability exploited by pathogens to evade humoral sterilization and enhance permissiveness for chronic bloodborne infections. Recent data suggest that the T-box expressed in T cells (T-bet) transcription factor, critical for inducing sterilizing humoral immunity in acute infections,[6] may also regulate the exhausted state in both autoreactive[7] and antigen-induced anergic B cells.[8,9] No data to date specifically link the anergy properties observed in cryoglobulin-producing B cells and T-bet, but there are phenotypic similarities ($CD11c^+/CD21^{low}$) that suggest a possible relationship that should be explored; rapid upregulation of T-bet in convalescent $CD21^{low}$ B cells upon reexposure to autologous HCV strains ex vivo has been observed,[8] suggesting a link between B-cell receptor ligation, T-bet, and the $CD21^{low}$ exhaustion phenotype.

How are these data clinically relevant? The persistence of $V_H1\text{-}69$ B cells after sustained virological response clearly explains the persistence of symptomatic cryoglobulinemic vasculitis observed in some HCV-cured patients.[10] However, these data may have broader implications on the early and late immunopathogenesis of chronic infection. That antigen-specific B-cell anergy is durably programmed suggests that B-cell exhaustion might rapidly redevelop in the setting of reinfection, potentially contributing to the lack of protective immunity observed in many resolved chronic infections. The current study creates the context for further exploration of the mechanisms associated with induction of B-cell exhaustion in vivo. Once the regulation of B-cell anergy in chronic infection is better understood, therapeutic manipulation could significantly affect the outcomes not only of chronic hepatitis C, but also of other chronic viral and parasitic infections.

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