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Decline of cellular activation in non-B cells after rituximab treatment in hepatitis C-associated mixed cryoglobulinemia vasculitis

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Summary

Mixed cryoglobulinemic vasculitis is associated with the monoclonal expansion of pathognomonic B cells in chronic hepatitis C. Recently, treatment with B-cell depletion, including rituximab, a CD20 monoclonal antibody, has been successful in achieving remission from the active disease. We investigated whether B-cell depletion therapy has an impact on activation of non-B cells in the periphery. Results demonstrated that B-cell depletion therapy is associated with a statistically significant decline in activated T cells, from pretherapy to follow-up while on rituximab therapy: CD4+ CD38+ HLA-DR+ (DR+), CD8+ CD38, CD8+ CD38+ DR+, and CD8+ DR+. Birmingham Vasculitis Activity Score and cryoglobulin had a strong correlation coefficient (R) of 0.72 ($P=.0005$), while cryoglobulin showed moderate correlation with CD8+ DR+ ($R=.61$), CD3+ CD38+ DR+ ($R=.57$), CD3+ DR+ ($R=.50$), CD4+ CD38+ DR+ ($R=.53$), CD4+ DR+ ($R=.52$), and CD8+ CD38+ DR+ ($R=.67$). These results suggest B-cell expansion has a direct and indirect effect on the pathogenesis of Hepatitis C-associated mixed cryoglobulinemic vasculitis.

Keywords

chronic hepatitis C; hepatitis C virus; mixed cryoglobulinemia; rituximab; vasculitis

1 | INTRODUCTION

The prevalence of chronic hepatitis C (CHC) in the United States (USA) is estimated at 2.7–3.9 million individuals.^{1,2} The natural history of untreated CHC is well-characterized, with a gradual progression to fibrosis and cirrhosis, hepatocellular carcinoma, liver failure and

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DISCLAIMER

This study does not represent the views of the Department of Health and Human Services.

CONFLICT OF INTEREST

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death;³ it is the leading cause of chronic liver disease, hepatocellular carcinoma and liver transplantation in the USA.^{4,5} Today, oral direct-acting antiviral (DAA) drugs have a high efficacy to cure hepatitis C virus (HCV) in patients by achieving sustained virologic response (SVR).⁶ Extra-hepatic manifestations (EHM) occur at least once in 40%–74% of HCV-infected patients.^{7,8} EHM includes mixed cryoglobulinemia (MC) vasculitis, lymphomas, rheumatic disorders, autoimmune thyroiditis, hypothyroidism, papillary thyroid cancer and type 2 diabetes;⁸ some are closely and others are provisionally associated with CHC.⁹ Type II MC vasculitis is strongly associated with CHC,^{7–10} as 70%–86% of patients have HCV viraemia.^{11–13} HCV-associated MC vasculitis is characterized by preferential expansion of monoclonal B cells, likely triggered by HCV antigens or epitopes, to produce soluble IgM with rheumatoid factor activity that develops into immune complexes and small-vessel vasculitis.^{9,13} Clinical manifestations include cutaneous vasculitis, arthralgia/arthritis, peripheral neuropathy and membranoproliferative glomerulonephritis.¹⁴ The pathogenesis of immunological abnormalities associated with MC vasculitis, the nature of HCV infection leading to MC vasculitis and the role of B cells are not completely known. Clinical trials have shown HCV-MC vasculitis patients can tolerate and be effectively treated with rituximab, an anti-CD20 monoclonal antibody.^{10,15} Response to rituximab therapy results in a clinical response to active vasculitis indicated by a lowered Birmingham Vasculitis Activity Score (BVAS). Rituximab has also been shown to normalize peripheral B- and T-lymphocyte homeostasis including improving regulation, activation, and Th1/Th2 imbalances,¹⁶ and increase in CD4+.¹⁷ Among healthy donors, B cells exposed to rituximab in vitro showed rapid and robust loss of measured CD19+ cells representing a loss of surface protein in a complement-independent manner.¹⁸ In other diseases, treatment with rituximab has shown a change in both B and T cells. Patients with rheumatoid arthritis treated with rituximab showed a depletion of peripheral blood B cells through 24 weeks during a randomized-control trial; CD19+ cell first decreased and then remained stable in the control group (oral methotrexate); however, all treatment groups had an initial decrease then rebound of T cells: CD3+, CD4+ and CD8+.¹⁹ All patients with relapsing-remitting multiple sclerosis who had lumbar puncture before and after rituximab infusion had decreased or undetectable cerebrospinal fluid (CSF) B cells, while the majority of patients had reduced CSF T cells.²⁰ The study objective was to determine the changes in activated and memory T cells from pretherapy to follow-up while on rituximab therapy and the correlations of these immune parameters with active disease, BVAS, among HCV-MC vasculitis patients.

2 | METHODS

2.1 | Study population

The study population is HCV-MC vasculitis patients treated with rituximab therapy. Patients were selected from an open-labelled, randomized controlled trial (NCT#00029107) conducted at the National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes of Health (NIH) Clinical Center, Bethesda, Maryland.¹⁰ The clinical trial eligibility criteria were patients with HCV infection and MC vasculitis who had failed interferon alpha and ribavirin therapy or who could not tolerate that therapy. Patients enrolled were randomized 1:1 to receive either treatment arm of rituximab or the control arm of best available therapy.¹⁰ Patients in the treatment arm received rituximab 375 mg/M² on

days 1, 8, 15 and 22 beginning at the time of randomization. This study used data from 20 patients who received rituximab therapy overall (11 patients from the treatment arm and nine patients from the control arm).¹⁰ All patients provided written informed consent, and the clinical trial was approved by the NIAID Institutional Review Board.

2.2 | Laboratory measurements

Patients had comprehensive clinical and laboratory evaluations at each monthly clinical trial visit: chemistries, urinalysis, complete blood counts, cryoglobulin levels (% of cryocrit), peripheral blood flow cytometry, HCV plasma RNA levels, and remission defined as a BVAS of 0 indicating no new or worsened activity or no persistently active disease manifestations within the previous month.¹⁰ Flow cytometry includes the concentration (cells/ μ L) of lymphocytes, CD4+, CD8+ T lymphocytes and CD19+ B lymphocytes from blood samples with a four-colour flow cytometry analysis multitest with Trucount tubes containing fluorescent beads as an internal standard, according to the manufacturer's instructions (BD Bioscience, Mountain View, CA, USA).¹⁶ A flow cytometric analysis of lymphocyte subpopulations was performed that used a panel of monoclonal antibodies, fluorescein isothiocyanate, phycoerythrin-cyanine 5 or allophycocyanine for the following surface protein: CD3+, CD4+, CD8+, CD16+, HLA-DR+ (DR+), CD25+, CD27+, CD38+ and CD56+.

2.3 | Statistical analysis

We performed exploratory analysis to determine differences between the treatment and control arm for immune parameters at clinical trial baseline (pretherapy) and at the 6-month primary endpoint using Student's *t*-test for continuous variables. The primary analysis was to determine individual differences in immune parameters from pretherapy to the 6 and 12 months follow-up while on rituximab therapy using a paired *t*-test. Immune parameters (cells/ μ L) were expressed in means $\pm$ standard deviation (SD). To determine the association of T-cell activation and remission, we determined the Pearson's correlation coefficient (*R*) of immune-activated T cells to both BVAS and cryoglobulin at pretherapy and during rituximab therapy. All analyses were conducted in SAS, version 9.4 (SAS Institute, Inc., Cary, NC) with a *P*-value $<.05$ (two-sided) indicating statistical significance.

3 | RESULTS

3.1 | Study population

The demographic and clinical characteristics of patients from the clinical trial and the results have been previously described.¹⁰ Briefly, the rituximab treatment arm achieved a statistically significant higher remission compared to the control arm (*P* $<.001$) at the 6-month primary endpoint.¹⁰ The following immune markers were statistically different at the 6-month primary endpoint between the treatment (*n*=11) and the control (*n*=11) arm: BVAS (*P* $=.03$), CD19+ (*P* $=.004$), CD3+ (*P* $=.01$), CD8+ (*P* $=.02$), CD14+ (*P* $=.007$) and cryoglobulin (*P* $=.02$), while no differences at pretherapy. The primary analysis for this study included all 20 patients who received rituximab therapy regardless of the initial clinical trial arm.

3.2 | Decline in activated T cells with B-cell depletion therapy and remission

The mean BVAS at pretherapy was 9.4 (SD: 5.6) and significantly decreased to 2.7 (SD: 4.3) at 6-month of rituximab ($P<.001$). The mean cryoglobulin level at pretherapy was 10.8% of cryocrit (SD: 9.6) and significantly decreased to 6.3% of cryocrit (SD: 7.6) at 6-month of rituximab ($P=.005$). Table 1 shows the immune parameters that were statistically significant comparing individual differences from pretherapy to 6-months of rituximab therapy. T-cell activation was shown to decrease with therapy and BVAS for CD4+ CD38+ DR+, CD4+ CD38+ DR+, CD8+ CD38+ DR+ and CD8+ DR+ (Table 1). Similar results for immune parameters were seen when increasing follow-up to 12 months with a significant decrease in T-cell activation with therapy and BVAS: CD4+ CD38+ DR+, CD8+ CD38, CD8+ CD38+ DR+ and CD8+ DR+ (Table 2).

3.3 | Correlation of activated T cells to disease state

At pretherapy, there was no significant or strong correlation between BVAS and cryoglobulin. However, at the 6-month therapy measurement, BVAS and cryoglobulin showed a significant and strong correlation of 0.72 ($P=.0005$). There was no significant or strong correlation between BVAS and immune markers. The following immune markers showed significance ($P<.05$) and moderate correlation with cryoglobulin at pretherapy: CD8+ DR+ ($R=.64$), CD4+ CD38+ DR+ ($R=.63$), CD4+ DR+ ($R=.69$), CD8+ CD38+ DR+ ($R=.61$) and CD8+ ($R=-.71$). At the 6-month therapy measurement, the following immune parameters showed a significant ($P<.05$) and moderate correlation with cryoglobulin: CD8+ DR+ ($R=.61$), CD3+ CD38+ DR+ ($R=.57$), CD3+ DR+ ($R=.50$), CD4+ CD38+ DR+ ($R=.53$), CD4+ DR+ ($R=.52$) and CD8+ CD38+ DR+ ($R=.67$).

4 | DISCUSSION

We demonstrated an association between T-cell activation and HCV-MC vasculitis, which significantly declined with depletion of B cells while on rituximab therapy. There was a decrease in T-cell activation from pretherapy to the 6 and 12 months of follow-up, while on rituximab therapy as BVAS decreased. These immune markers of T-cell activation showed significant and moderate correlation with cryoglobulin levels. These results provide novel insights into the pathogenesis of HCV-MC vasculitis. The monoclonal expansion of B cells that leads to immune complex-mediated small vessel vasculitis also leads to T-cell activation. Rituximab therapy results in rapid depletion of circulating and tissue B cells and our study showed a decrease in T-cell activation, as B-cell activation likely mediates T-cell activation and other immune cells among patients with HCV-MC vasculitis. This could be caused by a direct effect triggering T-cell stimulation by the presentation of antigen in a specific manner or indirectly by secretion of cytokines. T-cell activation could contribute to disease pathogenesis and/or serve as markers of remission. Future work can demonstrate whether activated T cells are HCV-specific or not. However, questions remain how this performs against BVAS, cryoglobulin levels or B-cell counts. Similar to our findings, in other disease states, there was a decrease in both B and T cells after rituximab treatment among patients with rheumatoid arthritis and multiple sclerosis.^{19,20}

DAA therapy has revolutionized HCV treatment paradigm and is likely to reduce the prevalence of HCV-MC vasculitis by eliminating CHC. As such regimens become more prevalent, we anticipate a decline in HCV-MC vasculitis, although it is unclear whether normalization of B cells and/or T cells occurs as a consequence. A recent study of immunologic recovery with DAA therapy suggested a dichotomous response of T and B cells with SVR.²¹ Barrett et al. demonstrated normalization of T-cell activation and exhaustion, while persistence of B-cell abnormalities in the peripheral blood of patients who achieved SVR with sofosbuvir and ribavirin.²¹ Ongoing studies will address the clinical impact of these persistent B-cell abnormalities.

Our study had limitations. The sample size was small, although treatment did show a response in all. Second, the follow-up was short, while long-term follow-up would have allowed us to study T-cell activation with B-cell recovery and disease relapse. Third, we were limited in this secondary analysis to study the specificity of T cells and explore HCV-specific responses due to limited stored samples and testing, which would have answered an important question of T-cell– B-cell interaction in chronic HCV-MC vasculitis patients.

B-cell depletion therapy of HCV-MC vasculitis patients offered us a unique perspective into T-cell– B-cell interaction in chronic inflammatory processes in a disease model, where primarily B cells are considered to play a pathogenic role. Future studies should focus on evaluating B- and T-cell phenotype and specificity in HCV-MC vasculitis patients treated with DAAs, to understand whether T-cell– B-cell interactions occur in an antigen-specific manner or as part of the non-specific inflammatory process and whether this abnormal cellular activation persists despite eradication of HCV and remission of vasculitis.

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Abbreviations

BVAS	Birmingham vasculitis activity score
CHC	chronic hepatitis C
CSF	cerebrospinal fluid
DAA	direct-acting antiviral
EHM	extra-hepatic manifestations
HCV	hepatitis C virus
MC	mixed cryoglobulinemia
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
SD	standard deviation

SVR sustained virologic response

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TABLE 1

Significant mean individual changes between pretherapy and 6 months on rituximab therapy

Immune markers	Mean difference	SD	P value
CD3+	10.1	7.9	<.0001
CD3+ CD38+ DR+	-4.6	5.1	.003
CD3+ DR+	-3.4	5.0	.01
CD4+	7.3	6.2	<.0001
CD4+ CD38+ DR+	-2.3	2.1	.001
CD8+	2.8	3.6	.003
CD8+ CD27+ CD45RO+	-2.4	4.3	.05
CD8+ CD38+ DR+	-8.4	9.5	.004
CD8+ DR+	-6.1	9.3	.01
CD19+	-10.9	7.6	<.0001

SD, standard deviation; DR+, HLA-DR+.

TABLE 2

Significant mean individual changes between pretherapy and 12 months on rituximab therapy

Immune markers	Mean difference	SD	P value
CD3+	8.7	7	<.0001
CD3+ CD38+ DR+	-6.5	8.4	.01
CD3+ DR+	-4.3	7.3	.02
CD4+	7.6	7.1	<.0001
CD4+ CD27	3.1	4.9	.03
CD4+ CD38+ DR+	-2.6	2.7	.002
CD8+ CD27	-3	5.1	.04
CD8+ CD38	-7.1	11	.01
CD8+ CD38+ DR+	-11.9	13	.003
CD8+ DR+	-8.2	11	.01
CD19+	-8.2	8.8	.001

SD, standard deviation; DR+, HLA-DR+.