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CD4⁺CD25⁺ regulatory T-cell deficiency in patients with hepatitis C–mixed cryoglobulinemia vasculitis

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Patients who are chronically infected with hepatitis C virus (HCV) often develop mixed cryoglobulinemia (MC), a B-cell proliferative disorder characterized by polyclonal activation and autoantibody production. We investigated if MC is associated with a deficit of CD4⁺CD25⁺ immunoregulatory T (Treg) cells, which have been shown to control autoimmunity. Because Treg cells express higher amounts of CD25 than activated CD4⁺ T cells, we analyzed blood CD4⁺CD25⁺ T cells in 69 untreated patients chronically infected with HCV. Treg cell frequency in patients without MC (8.8% ± 2.3%) or with asymptomatic MC (7.4% ± 2.1%) was comparable to that of healthy controls (7.9% ± 1.3%). In contrast, it was significantly reduced in symptomatic MC patients (2.6% ± 1.2%, \( P < .001 \)) even when compared to a panel of untreated HCV⁺ patients with different inflammatory disorders (6.2% ± 0.8%, \( P < .0001 \)). In symptomatic MC patients, the purified remaining CD4⁺CD25⁺ T cells retained suppressive activity in vitro. These results, together with experimental data showing that depletion of Treg cells induces autoimmunity, suggest a major role of Treg cell deficiency in HCV-MC vasculitis and this is the first report of a quantitative Treg cell deficiency in virus-associated autoimmunity. (Blood. 2004; 103:3428-3430)

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Introduction

Infection with hepatitis C virus (HCV) is associated with most cases of mixed cryoglobulinemia (MC), a B-cell proliferative disorder characterized by polyclonal activation and autoantibody (aAb) production. Cryoglobulins are immunoglobulins that precipitate at cold temperature and resolubilize when rewarmed. MC is composed of different immunoglobulins, including a monoclonal component in type II and only polyclonal immunoglobulins in type III. MC may be asymptomatic or lead to clinical manifestations ranging from an MC syndrome (purpura, arthralgia, asthenia) to a more serious vasculitides with neurologic and renal involvement. Although MC is found in 30% to 50% of patients with chronic HCV infection, only 10% to 15% of them will develop symptomatic MC. The observation of T cells in the vascular infiltrates and the presence of autoantibodies together with the observation that some HLA groups confer susceptibility to MC vasculitis in HCV-infected patients suggest that autoimmune processes are implied in this virus-linked pathology.

Compelling evidence now indicates that a population of CD4⁺CD25⁺ immunoregulatory T (Treg) cells plays a central role in the physiologic control of autoimmunity. Adoptive transfer of Treg-depleted T cells in mice leads to autoimmune manifestation. Administration of purified Treg cells allows control of autoimmune processes. Recently, the existence of Treg cells in healthy humans has been demonstrated and the search for their implication in autoimmune diseases is currently the object of intense investigation. The purpose of this study is to determine whether HCV-MC vasculitis is associated with a deficit in CD4⁺CD25⁺ T cells.

Patients and methods

Patients

The study was approved by institutional ethics committee and informed consent was obtained from all patients. Sixty-nine chronically HCV-infected patients (mean age, 52 years; range, 22-72 years) entered the study, of whom 22 had symptomatic MC and 26 had asymptomatic MC or aAb (rheumatoid factor, antinuclear antibodies; anti-HIV antibodies. MC⁺ patients had an MC level in their serum more than 0.05 g/L, at least at 2 determinations. Symptomatic MC was defined by serum MC associated with the triad of purpura-arthralgia-asthenia sometimes associated with renal or neurologic involvement. None of these 69 patients had received antiviral or immunosuppressive treatment for at least 6 months preceding their inclusion in the study. A population of untreated HCV⁺ patients suffering from a panel of inflammatory disorders (Sjögren syndrome, n = 3; systemic sclerosis, n = 2; systemic vasculitis, n = 1; sepsis, n = 2; systemic lupus erythematosus, n = 1; relapsing polychondritis, n = 1) was also included.

Cell separation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density gradient and stained with combinations of the following monoclonal antibodies: PerCP-labeled anti-CD4 (13B2.2), fluorescein (FITC)–labeled anti-CD25 (B1.49.9), FITC–labeled mouse IgG2a (U7.27), or phycoerythrin (PE)–labeled mouse IgG1 isotypic control (679.1Mc7) from Coulter Immunotech (Marseille, France); PE-labeled anti-CD25 (M-A251) or FITC-labeled anti-CD62L (SK11) from BD Biosciences (San Diego, CA). A population of 69 untreated HCV⁺ patients suffering from a panel of inflammatory disorders (Sjögren syndrome, n = 3; systemic sclerosis, n = 2; systemic vasculitis, n = 1; sepsis, n = 2; systemic lupus erythematosus, n = 1; relapsing polychondritis, n = 1) was also included.

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CD4⁺CD25⁺ T cells were isolated from PBMCs by a first step of negative sorting using a cocktail of hapten-conjugated CD8, CD11b, CD16, CD19, CD36, and CD36 antibodies and microbeads coupled to an anti-hapten monoclonal antibody (CD4⁺ T-cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany). This was followed by a step of positive selection of CD25⁺ cells by microbead separation (CD25 microbeads; Miltenyi Biotec), a procedure yielding to 90% or more purity as assessed by flow cytometric counting of CD4⁺CD25⁺ cells.

**Function assays**

Costar 96-well plates (Corning, NY) were incubated with 2.5 μg/mL anti-CD3 monoclonal antibody (OKT3, Orthoclone; Iansen-Cilag, Paris, France) for 1 hour at 37°C, then for 30 minutes at 4°C, and washed. Then, 12.5 x 10⁵ CD4⁺CD25⁺ T cells with or without 12.5 x 10⁵ autologous responder T cells (negative fraction of CD25 sorting) were cultured in RPMI medium supplemented with 10% human AB serum in these anti-CD3–coated plates in the presence of soluble anti-CD28 (1 μg/mL, clone CD28.2; BD Biosciences Pharmingen, Le Pont de Claix, France) with or without recombinant human interleukin 2 (50 UI/mL; Chiron Corporation, Chirone France, Suresnes, France). At day 4, [³H]-thymidine uptake (2 μCi/well [0.074 MBq/well]) was added for 16 hours before proliferation was assayed. Percent inhibition of proliferation was determined as follows: 1−(median [³H]-thymidine uptake of 1:1 CD4⁺CD25⁺-CD4⁺CD25⁻ coculture/median [³H]-thymidine uptake of CD4⁺CD25⁺ cells).

**Results**

Sixty-nine chronically infected HCV patients were screened for the presence of blood Treg cells by flow cytometry. Within the CD4⁺ subset, Treg cells are contained in the population that displays the highest CD25 expression level. Therefore, their proportion was determined as the frequency of CD4⁺T cells with the brightest CD25 expression level. Therefore, their proportion was determined as the frequency of CD4⁺ cells among CD4⁺ blood cells (Figure 1). The frequency of Treg cells (mean ± SD) was significantly reduced in patients with symptomatic MC (2.6% ± 1.2%, n = 22, P < .001, Mann-Whitney test) as compared to those with asymptomatic MC or aAb (7.4% ± 2.1%, n = 26, n = 14, 9.9% ± 2.0%, n = 14; no MC nor aAb 9.7% ± 1.7%, n = 16; and controls, 9.3% ± 3.3%, n = 5. Because it cannot be formally excluded that the CD4⁺CD25⁺ subset also contains conventional activated CD62L⁺ T cells in patients with symptomatic MC or aAb (5.7% ± 3.3%, n = 14), no MC nor aAb (8.8% ± 2.3%, n = 21, and healthy controls (7.9% ± 1.3%, n = 5). It was also significantly reduced as compared to a panel of untreated HCV⁺ patients with different inflammatory disorders (6.2% ± 0.8%, n = 10. P < .0001). We confirmed this Treg cell deficiency in symptomatic HCV-MC patients on a subset of patients using another CD25 antibody labeled with PE: symptomatic MC, 3.5% ± 1.1%, n = 12, P < .005; symptomatic MC, 7.0% ± 0.9%, n = 10; asymptomatic MC or aAb, 9.9% ± 2.0%, n = 14; no MC nor aAb 9.7% ± 1.7%, n = 16; and controls, 9.3% ± 3.3%, n = 5. Because it cannot be formally excluded that the CD4⁺CD25⁺ subset also contains conventional activated CD62L⁺ T cells in patients with symptomatic MC or aAb (5.7% ± 3.3%, n = 14), no MC nor aAb (8.8% ± 2.3%, n = 21, and healthy controls (7.9% ± 1.3%, n = 5). Together, these results reveal a quantitative deficiency of Treg cells in symptomatic HCV-MC patients.

Treg cells are suppressors cells that inhibit the proliferation of conventional T cells in vitro. We thus evaluated if this quantitative deficiency was associated with a functional defect. For this, we determined the capacity of immunomagnetically sorted CD4⁺CD25⁺ T cells to suppress the proliferation of autologous responder T cells on activation with anti-CD3 plus anti-CD28. At a ratio of 1:1, CD4⁺CD25⁺ suppressed the proliferation of responder cells by an average factor of 67% in symptomatic MC patients (Figure 2A). Using this assay, the mean suppressive activity of CD4⁺CD25⁺ cells from healthy controls was 78% ± 5% (n = 3, difference not statistically significant; data not shown). The addition of exogenous IL-2 abrogated this suppression (Figure 2A), in accordance with other reports. Another property of Treg cells is their anergy, which can be reversed by IL-2. As expected, the sorted CD4⁺CD25⁺ population was hyporesponsive to anti-CD3.
plus anti-CD28 activation (Figure 2B) as compared to their CD25− counterpart (Figure 2A), whereas addition of IL-2 reversed this anergy (Figure 2B). Together, these results indicate that the remaining CD4+CD25+ T-cell population in symtomatic MC patients contains functional Treg cells but whether or not these cells exert some control on vasculitis-associated effector T cells is unknown.

**Discussion**

Recent studies have aimed to correlate different autoimmune diseases with Treg cell defects.12,14-16 Importantly, a Treg cell deficit due to a mutation in the Foxp3 gene has unambiguously been shown to cause aggressive autoimmunity and early death.20 The present study is the first report of a virus-linked autoimmunity associated to a quantitative Treg cell deficiency. Because Treg cell frequency in HCV-MC patients was signifcantly reduced as compared to patients with different inflammatory disorders, it is unlikely that it is only the feature of any systemic inflammatory response. It cannot be formally excluded that Treg cells have been recruited to sites of inflammation and consequently depleted from peripheral blood. Nevertheless, it was recently reported that accumulation of Treg cells in inflamed joints of patients with rheumatoid arthritis was not associated to a detectable reduction in the blood Treg cell count.13 A causal role for such Treg cell deficiency in MC vasculitis remains to be assessed in a prospective longitudinal study. It can be hypothesized that a Treg cell deficit may augment the helper function provided by conventional CD4+ T cells to autoantibody-secreting B cells. Along this line, we observed increased oligoclonal CD4+ T-cell expansions after IL-2 culture in symptomatic as compared to asymptomatic MC patients (not shown), suggesting that more antigen-primed helper T cells are present in the former patients. In addition, cognate interaction of virus-specific CD4+ helper T cells with virus-infected B cells may yield to hypergammaglobulinemia and autoantibody secretion in mice.21 It is therefore possible that a similar process operates in hepatitis C because HCV sequences are found in B cells,22 and that chronic HCV infection is associated with hypergammaglobulinemia, autoantibodies, and MC-linked disorders.

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**References**


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