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

Olivier Boyer, David Saadoun, Julien Abriol, Mélanie Dodille, Jean-Charles Piette, Patrice Cacoub, and David Klatzmann

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 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)
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 CD56 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.

**Functional 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 × 10⁵ CD4⁺CD25⁺ T cells with or without 12.5 × 10⁴ 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 (IL-2, 50 U/mL; Chiron France, Suresnes, France). At day 4, [³H]-thymidine (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.⁰¹⁰¹ Therefore, their proportion was determined as the frequency of CD4⁺T cells with the brightest CD25 expression level.¹⁰⁻¹⁷ Therefore, their proportion was determined as the frequency of CD4⁺/H11001 T cells within this compartment. We also found that the frequency of CD4⁺CD25⁺ T cells within the CD4⁺ compartment 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, ●), no MC nor aAb (8.8% ± 2.3%, n = 21, ◊), or 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; HCV⁺ inflammation, 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 cells¹⁸ in addition to Treg cells that are mainly CD62L⁻,¹⁹ we assessed the frequency of CD62L⁺ within this compartment. We also found that the frequency of CD25⁺CD62L⁺ T cells within the CD4⁺ compartment was significantly reduced in patients with symptomatic MC (2.1% ± 1.3%, n = 12, P < .05) as compared to asymptomatic MC or aAb (4.6% ± 3.1%, n = 14), no MC nor aAb (5.7% ± 2.5%, n = 16), or controls (6.5% ± 3.2%, 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.⁷⁸ 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 or aAb (4.6% ± 3.1%, n = 14), no MC nor aAb (5.7% ± 2.5%, n = 16), or controls (6.5% ± 3.2%, 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.⁷⁸ 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% ± 20% 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.¹¹¹ Another property of Treg cells is their anergy, which can be reversed by IL-2.¹⁹ 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 symptomatic 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 significantly 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.

Acknowledgments

We acknowledge Marita Andreu and Hélène Trébédèn-Nègre for their help; David Antonelli, Véronique Bon-Durand, Marie-Christine Burland, and Christiane Lourtat for technical assistance; and Benoît Salomon and José Cohen for helpful discussions.

References

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

Olivier Boyer, David Saadoun, Julien Abriol, Mélanie Dodille, Jean-Charles Piette, Patrice Cacoub and David Klatzmann