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Emergence of long-lived autoreactive plasma cells in the spleen of primary warm auto-immune hemolytic anemia patients treated with rituximab

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ABSTRACT

Primary warm autoimmune hemolytic anemia (wAIHA) is a rare autoimmune disease in which red blood cells are eliminated by IgG autoantibodies. We analyzed the antibody-secreting cells in the spleen and the peripheral blood of wAIHA patients in various contexts of treatment. Plasmablasts were observed in peripheral blood of newly diagnosed wAIHA patients and, accordingly, active germinal center reactions were present in the spleen of patients receiving short-term corticosteroid therapy. Long-term corticosteroid regimens markedly reduced this response while splenic plasma cells were able to persist, a fraction of them secreting anti-red blood cell IgG *in vitro*. In wAIHA patients treated by rituximab and who underwent splenectomy because of treatment failure, plasma cells were still present in the spleen, some of them being autoreactive. By using a set of diagnostic genes that allowed us to assess the plasma cell maturation stage, we observed that these cells displayed a long-lived program, differing from the one of plasma cells from healthy donors or from wAIHA patients with various immunosuppressant treatments, and more similar to the one of normal long-lived bone-marrow plasma cells. Interestingly, an increased level of B-cell activating factor (BAFF) was observed in the supernatant of spleen cell cultures from such rituximab-treated wAIHA patients. These results suggest, in line with our previous report on primary immune thrombocytopenia, that the B-cell depletion induced by rituximab promoted a suitable environment for the maturation and survival of auto-immune long-lived plasma cells in the spleen.

Abbreviations: wAIHA, warm autoimmune hemolytic anemia; BAFF, B-cell activating factor; ASC, antibody-secreting cells; LLPC, long-lived plasma cells; RTX, rituximab; ITP, immune thrombocytopenia; HD, healthy donors; RBCs, red blood cells; GC, germinal-center; PC, plasma cells; PCA, principal component analysis; PB, plasmablasts.

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1. Introduction

Long-term protective humoral immunity is, in part, provided by long-lived antibody-secreting cells (ASC) [1,2]. Newly-formed plasmablasts generated in a T-dependent response migrate towards the bone-marrow, where some of them reside for decades in an appropriate niche as long-lived plasma cells (LLPC) [1,2]. Such LLPC have been described in the spleen of normal mice, in the context of B-cell depletion induced by irradiation or by anti-CD20 therapy [2,3]. Long-lived autoreactive plasma cells have also been described in the spleen of auto-immune NZB/W mice [4]. These observations raised the question as to whether the spleen could similarly represent a site for plasma cell persistence in humans. We recently analyzed the transcriptional program of human splenic plasma-cells from immune thrombocytopenia (ITP) patients treated or not with anti-CD20 (Rituximab, RTX) and from healthy donors (HD) [5]. Surprisingly, splenic LLPC were found in patients treated with RTX, but were rare in untreated ITP patients or in healthy donors. Moreover, by using a signature of genes that distinguished LLPC from short-lived plasma cells, we observed that most plasma cells from healthy donors and from untreated ITP patients displayed an intermediate profile between a short- and a long-lived program upon analysis at the single-cell level. These results suggested that the B-cell depletion *per se* might has created an environment allowing such splenic plasma cells to differentiate into long-lived ones [6]. In order to understand whether this mechanism could have a general relevance, we studied here the transcriptional program of splenic plasma cells in another auto-immune syndrome, primary warm autoimmune hemolytic anemia (wAIHA), from patients treated or not with RTX.

Primary wAIHA is a rare auto-immune disease, with a prevalence of 17 cases per 10^5 and an incidence of 1–3 cases per 10^5/year in western countries, characterized by IgG auto-antibodies directed against red blood cells (RBCs) antigens, leading to their accelerated destruction [7,8]. Antibody-coated RBCs are removed mainly in the spleen by the Fcγ receptor pathway [9]. Diagnosis of primary wAIHA is based on the exclusion of underlying auto-immune diseases, primary immunodeficiency, infection, drug intake or lymphoproliferative disorder [10–12]. Most patients respond to corticosteroids but remain corticosteroid-dependent, and require long-lasting therapy [11,12]. The use of B-cell depletion with RTX in wAIHA leads to 60 % to 80% of overall response at one-year and beyond [13–16]. Nevertheless, 20–40% of patients may resist to RTX or relapse, and thus often require splenectomy. Although the role of auto-antibodies in wAIHA has been recognized for a long-time, most studies have focused so far on the role of autoreactive T-cells [17–21]. A description of the B-cell response in this disease is thus presently lacking.

We described in this study the ASC present in the spleen and in the peripheral blood of newly diagnosed or chronic wAIHA patients. Moreover we characterize here the maturation stage of splenic plasma-cells from patients treated or not with RTX and of splenic and bone-marrow plasma cells from healthy donors (HD). This study reveals a population of auto-reactive LLPC in the spleen of RTX-treated patients that could explain the failure of the B-cell depletion therapy.

2. Methods

2.1. Patients

The study was approved by the Agence de la Biomédecine, the Comité de Protection des Personnes (CPP) Ile de France-II for healthy donors, the CPP Rennes for bone-marrow samples and by the CPP Ile de France-IX for ITP and wAIHA patients. This study was conducted in accordance with the Helsinki Declaration. All patients gave their informed consent and their blood samples were collected and stored at the French referral center for adult's cytopenia. All clinical data were available and analyzed at time of blood or spleen sample collection.

wAIHA, ITP patients and healthy donors (HD) were all adult individuals. Primary wAIHA and ITP diagnosis was made according to the American Society of Hematology guidelines. Patients with underlying immunodeficiency, hepatitis C virus infection, lymphoproliferative disorders, thyroid or liver disease, and definite systemic lupus erythematosus (≥4 American Rheumatism Association criteria) were excluded. The phases of the disease (i.e., newly diagnosed, persistent, or chronic) were defined by analogy to standard definitions used for ITP [22]. Patients with cold AIHA, as well as patients with a negative antiglobulin test were excluded. Complete response to splenectomy was defined as an increase of the hemoglobin level above 12 g/dl in the absence of any further therapy an/or recent transfusion.

Blood samples from healthy donors (n = 8) were obtained from EFS (Etablissement Français du Sang). Blood samples from newly diagnosed wAIHA patients (n = 11) and patients treated with steroids (n = 8) were obtained from the “Centre de référence des cytopénies auto-immunes de l'adulte”. Control spleen samples (n = 4) were obtained from organ donors. All died from stroke. Spleen samples from children were obtained from patients undergoing a splenectomy for sickle-cell disease, and used as control lymphoid tissues harboring germinal center reactions [23]. Clinical characteristics of the splenectomized ITP patient have been previously described (patient 1 in ref. 5). Clinical characteristics of the splenectomized wAIHA patients (7 wAIHA patients and 4 RTX-treated patients) are presented Table 1.

Bone marrow samples were obtained from patients undergoing cardiovascular thoracotomy.

2.2. Histological and confocal microscopy analysis of human spleens

Spleen deparaffinized tissue sections were stained using a three-step immunoperoxidase technique with the Leica Biosystems Bond-Max and Bond-III Autostainer, with the following antibodies: anti-CD20 (clone M0755, dilution 1:500), anti-IgD (clone A0093, dilution 1:800), anti-BCL2 (clone 124, dilution 1:800) from DAKO, anti-BCL6 (clone LN22, ready-to-use). Numbers of $CD20^+$ B-cell follicles and $BCL6^+$ positive follicles were counted and reported to the spleen surface measured (in cm^2).

For spleen confocal microscopy, pieces of splenic tissue were excised and embedded in OCT (Sakura), snap-frozen in liquid nitrogen, and stored at −80 °C. Cryosections (7–9 μm) were cut, fixed in cold (−20 °C) acetone for 10 min. In some conditions, sections were then fixed with 4% PFA and wash with 0.1% triton X-100 in PBS. Sections were rehydrated in wash buffer (Tris-buffered saline, pH 7.6), and incubated in blocking buffer (0.5% BSA, and 10% goat serum in PBS) for 30 min at room temperature. Sections were then incubated for 60 min at room-temperature, with the indicated primary antibodies in blocking buffer: mouse IgG1 anti-BCL6 (DAKO, clone PG-B6p, 1/20), polyclonal rabbit anti-IgD (DAKO, 1/300), mouse IgG2a anti-CD20 (DAKO, RTU, Clone L26). Sections were washed 3–4 times and incubated with the secondary antibody as indicated (30 min, room temperature): Cy5 goat anti-mouse IgG1 (Southern Biotech, 1/400), Alexa 488 goat anti-rabbit IgG (Invitrogen, 1/300), biotin anti-mouse IgG2a (Invitrogen, 1/200), Cy3-conjugated streptavidin (Jackson Immunoresearch, 1/500). Sections were washed and mounted in Fluoromont-G (Southern Biotech). Images were acquired by confocal microscopy with an SP5 (Leica). Fluorescence of single channels was measured and control reference