CD20^+ B cell depletion therapy suppresses murine CD8^+ T cell-mediated immune thrombocytopenia (ITP).

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Running Title: CD20 therapy prevents T cell-mediated ITP.

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CD20 B cell depletion therapy prevents CD8+ T cell-mediated ITP in vivo.

Abstract

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder with a complex pathogenesis which includes both antibody- and T cell-mediated effector mechanisms. Rituximab (an anti-human CD20 monoclonal antibody) is one of the treatments for ITP and is known to deplete B cells but may also work by affecting the T cell compartments. Here we investigated the outcome of B cell depletion (Bdep) therapy on CD8+ T cell-mediated ITP using a murine model. CD61 knockout (KO) mice were immunized with CD61+ platelets and T cell-mediated ITP was initiated by transfer of their splenocytes into severe combined immunodeficient (SCID) mice. The CD61 KO mice were administrated an anti-mouse CD20 monoclonal antibody either before or after CD61+ platelet immunization. This resulted in efficient Bdep in vivo, accompanied by significant increases in splenic and lymph node CD4+ and CD8+ T cells and proportional increases of FOXP3+ in CD4+ and CD8+ T cells. Moreover, Bdep therapy resulted in significantly decreased splenic CD8+ T cell proliferation in vitro that could be rescued by interleukin-2 (IL-2). This correlated with normalization of in vivo platelet counts in the transferred SCID mice suggesting that anti-CD20 therapy significantly reduces the ability of CD8+ T cells to activate and mediate ITP.
Introduction

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by an isolated thrombocytopenia (<100 ×10⁹/L).¹ The pathogenesis of chronic ITP is incompletely understood and thought to be heterogeneous.¹ For example, approximately two thirds of patients with ITP have detectable anti-platelet antibodies that destroy platelets primarily by Fc-mediated phagocytosis within the spleen.²,³ On the other hand, patients with no detectable antibodies can harbour platelet- and megakaryocyte-specific T cells that can mediate ITP.⁴,⁵ These effector responses are closely correlated with dysfunctional CD4⁺ T regulatory cells (Tregs) suggesting T cells are critical for ITP development.⁶-⁸

B cell depletion (Bdep) therapy using rituximab has been shown to be a successful second-line treatment for patients with ITP.⁹ Although B cells function to primarily elicit humoral immunity, they also have other roles such as antigen presentation, co-stimulation and T cell activation/cytokine production.¹⁰,¹¹ Moreover, patients with ITP, whether anti-platelet antibody positive or not, can respond to rituximab and in those antibody-positive patients, their autoantibody titers do not necessarily change.¹² This suggests that anti-CD20 can significantly affect T cell compartments and this was originally confirmed by Stasi et al showing that rituximab normalizes the observed CD4⁺ T cell abnormalities in ITP.⁶,¹³ To better understand how anti-CD20 therapy affects CD8⁺ T cells, we investigated its effect on T cell-mediated thrombocytopenia in an established murine model of ITP.¹⁴ The data suggests that anti-CD20 Bdep significantly inhibits IL-2 dependent CD8⁺ T cell proliferation which blocks their ability to mediate ITP.
Methods

Mice

BALB/c mice (BALB/cAnNCrl, H-2\textsuperscript{d}, CD61\textsuperscript{+}, Charles River Laboratories, Montreal, QC, Canada) were used as platelet donors and BALB/c CD61 (GPIIIa) knockout (KO) mice were supplied by Dr. Heyu Ni and used as a source of immune splenocytes. CB.17 severe combined immune deficient (SCID) mice (CB17/Icr-Prkdc\textsuperscript{scid}/IcrIcoCrl, H-2\textsuperscript{d}, Charles River Laboratories) were used as splenocyte transfer recipients for induction of ITP. All mice were 8-12 weeks of age and all studies were approved by the St. Michael’s Hospital Animal Care Committee.

B cell depletion in a murine model of ITP

A murine ITP model was used as previously described.\textsuperscript{14} CD61 KO mice were immunized against CD61\textsuperscript{+} BALB/c platelets and their non-depleted (ND) splenocytes were transferred into SCID mice to initiate ITP.\textsuperscript{14} Splenocytes from some CD61 KO mice were depleted of CD19\textsuperscript{+} B cells in vitro before transfer to initiate CD8\textsuperscript{+} T cell-mediated ITP (Bdep in vitro). Other KO mice were B cell depleted (Bdep) in vivo by 2 intravenous injections of an anti-mouse CD20 monoclonal IgG2a antibody (Biogen, Cambridge, MA, USA, 250 ug/mouse) either before or after platelet immunization to mimic Bdep therapy (Bdep pre or post, respectively). Details of the in vitro experimentation and the experimental design are shown in the supplemental methods.

Statistical analysis

Data are expressed as mean±SD, and were analyzed using GraphPad Prism 6.02 software for Windows (GraphPad Software, San Diego, CA).
Results and Discussion.

Anti-CD20 antibody efficiently depletes B cells and increases the T cell percentages in vivo

Murine monoclonal anti-CD20 antibodies derived from immunization of CD20 KO mice were first reported by Uchida et al and induce significant B cell depletion in vivo. In our hands, all the anti-CD20 antibody-treated mice were significantly depleted of B cells in the peripheral blood, spleen and mesenteric lymph nodes (Supplemental Figures S2A and S2B). In CD61KO mice Bdep before platelet immunization, a significant reduction in IgG anti-platelet antibody production was also observed (Supplemental Figures S2C and S2D). Bdep therapy in immune mice significantly increased the percentages of peripheral blood, splenic and lymph node CD3⁺CD4⁺, CD3⁺CD8⁺ T cells and FOXP3⁺ Treg subpopulations proportionally (Supplemental Figure S3 and S4), consistent with previous studies showing that Bdep therapy increases the proportion of non-B cell populations within immune compartments.

Bdep therapy suppresses CD8⁺ T cell proliferation in vitro

B cells have been previously shown to be involved in CD8⁺ T cell maintenance and memory cell formation and patients with ITP treated with rituximab have significant alterations of CD8⁺ T cells, including increased cytotoxicity of splenic CD8⁺ T cells in rituximab non-responders. Therefore we examined the proliferation potential of splenic CD8⁺ T cells from the non-depleted (ND) or Bdep immune CD61 KO mice. Purified splenic CD8⁺ T cells were stained with V450 (VPD 450, BD Biosciences, Mississauga, ON) and cultured with anti-CD3 antibody together with either anti-CD28 antibody and/or recombinant IL-2 for 72 hrs and the fluorescence intensity per cell was analyzed (Figure 1A). Compared with CD8⁺ T cells from either naïve BALB/c or ND CD61 immune KO mice, CD8⁺ T cells from the Bdep KO mice showed a significantly reduced proliferation upon anti-CD3 and anti-CD28 stimulation (Figure 1B). The deficient CD8⁺
T cell proliferation could be rescued by addition of recombinant IL-2 to the cultures (Figure 1C). Further examination of the splenic CD8+ T cells revealed increased intracellular Granzyme B expression after Bdep therapy (Supplemental Figure S5). This may be related to how CD20low4-1BBL+ B regulatory cells regulate Granzyme B expression in CD8+T cells. Although Granzyme B expression in CD8+ T cells was found to be increased in follicular lymphoma patients at diagnosis, which was associated with better prognosis, it appeared unchanged in patients with ITP. Taken together, our results suggest that in vivo Bdep therapy interferes with in vitro IL-2-dependent CD8+ T cell activation but more research is required to characterize how this phenomenon occurs.

**Bdep therapy induces normalization of platelet counts in a murine model of ITP**

We further examined the effect of Bdep therapy on the development of ITP. SCID mice were transferred with splenocytes from immune CD61 KO mice that were either non-depleted (ND) or depleted of CD19+ B cells in vitro to initiate antibody- and T cell-mediated ITP respectively. As previously described, both splenocyte populations induced significant thrombocytopenia when transferred into SCID mice (Figure 2, columns 1-3). In contrast, however, if the CD61 KO mice were Bdep in vivo either before or after platelet immunization, the ability of their splenocytes to induce ITP was completely prevented (Figure 2, columns 4-5).

Mechanistically, Bdep therapy in vivo may actively suppress or exhaust the proliferative potential of pathogenic CD8+ T cells upon activation and thereby limit their ability to induce ITP. Support for this possibility comes from studies showing that T cells, in the absence of B cells, have proliferation and memory development defects. In addition, although B cell depletion in vivo may modulate the balance between pro- and anti-inflammatory T cell-derived cytokines, we did not observe any significant differences in IFN-γ production by CD8+ T cells.
(Supplemental Figure S5) or intracellular expression of IFN-γ/IL-4 in CD4+ T cells (N=5-8, data not shown) after Bdep therapy. Furthermore, in accordance with previous studies, the activation marker CD44 was not increased on the CD8+ T cells after Bdep therapy (Supplemental Figure S5).18,19,25 Of interest, interruption of B and T cell interactions by, for example, CD40L antibody, shows a similar therapeutic effect as rituximab in ITP suggesting a direct interaction between B cells and T cells is essential for ITP induction.12

In summary, our study suggests that the effectiveness of anti-CD20 therapy is due to induction of a significant CD8+ T cell activation/proliferation defect via IL-2 blockade that correlates with their inability to induce thrombocytopenia. This may provide an additional explanation for the therapeutic effects of rituximab in T cell-mediated ITP.
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Authorship

Contribution: L.G. designed research, performed experiments, collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript first draft; R.K. analyzed and interpreted data, performed statistical analysis and edited the manuscript; R.A., E.R.S, Y.Z., A.Z. and M.K. performed experiments, collected and interpreted data; A.H.L. provided CD44 antibodies and interpreted data; H.N. supplied mice; J.W.S. provided financial resources, designed research, analyzed and interpreted data, and edited the manuscript.

Conflict-of-interest disclosure

The authors declare no competing financial interests.
References


Figure Legends

Figure 1.

**B cell depletion inhibits CD8⁺ T cell proliferation in vitro.** CD8⁺ T cells were purified from the spleens of BALB/c naïve mice (Bc naïve), platelet immunized CD61 KO mice (KO ND) and platelet immunized CD61 KO mice that received Bdep therapy during immunization (KO Bdep) and stained with the proliferation dye V450 and cultured in vitro with anti-CD3 ± anti-CD28/IL-2 for 72h. (A) Representative flow cytometric dot plot analysis of CD8⁺ T cell proliferation when either not stimulated (top panels) or stimulated with anti-CD3/CD28 antibodies for 72 hours. A cell division cycle is characterised by sequential halving of the V450 fluorescence. Cumulative data of (B) CD8⁺ splenic T cells stimulated with anti-CD3/anti-CD28 and (C) anti-CD3/IL-2. Data in B and C are expressed as the mean ± SD of percent CD8⁺ T cells proliferating (n=5-8 mice per group). Data were analyzed using one-way ANOVA with a Tukey’s post-hoc test (***P<0.001, ****P<0.0001, ns, non-significant).

Figure 2.

**In vivo B cell depletion results in a normalization of platelet counts in a murine model of T cell-mediated ITP.** Platelet counts in transferred recipient SCID mice after 21 days post engraftment of 3×10⁴ splenocytes. SCID mice were either transferred with PBS or with non-depleted splenocytes from platelet-immunized CD61 KO mice (ND), splenocytes from ND KO mice but depleted of CD19⁺ B cells in vitro (ND Bdep in vitro) or splenocytes from platelet-immunized CD61 KO mice depleted in vivo with anti-CD20 antibody before platelet immunization (B cell dep in vivo, pre) or after platelet immunization (B cell dep in vivo, post). Each data point represents one SCID mouse. Data were analyzed using one-way ANOVA with a Tukey’s posttest (*P<0.05, ***P<0.001).
Figure 1

A

B

C

CD8+ T cell Proliferation (%)

anti-CD3/anti-CD28

anti-CD3/IL-2
Figure 2

![Diagram showing platelet counts in various conditions: PBS, ND, ND Bdep in vitro, B cell dep in vivo, pre, and B cell dep in vivo, post. The x-axis represents different conditions, and the y-axis represents platelet counts (x 10^9/L). The diagram includes error bars and statistical significance markers (* and ***) to indicate differences between groups.](image-url)
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