Experimental Hematology

Experimental Hematology 2016;44:924–930

The spleen dictates platelet destruction, anti-platelet antibody production, and lymphocyte distribution patterns in a murine model of immune thrombocytopenia

Rukhsana Aslam[a,b], Rick Kapur[a,b,c], George B. Segel[d], Li Guo[a,b], Anne Zufferey[a,b], Heyu Ni[a,b,c,e,f], and John W. Semple[a,b,c,e,g]

[a]*Toronto Platelet Immunobiology Group, Toronto, ON, Canada;* [b]*Keenan Research Centre for Biomedical Science of St. Michael's Hospital, Toronto, ON, Canada;* [c]*Canadian Blood Services, Toronto, ON, Canada;* [d]*Division of Hematology and Department of Medicine, University of Rochester, Rochester, NY;* [e]*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada;* [f]*Department of Physiology, University of Toronto, Toronto, ON, Canada;* [g]*Departments of Medicine and Pharmacology, University of Toronto, Toronto, ON, Canada*

(Received 10 May 2016; revised 24 June 2016; accepted 2 July 2016)

For many years, splenectomy has been used to treat immune thrombocytopenia (ITP), and this procedure benefits approximately two-thirds of the treated patients. Although splenectomy may raise platelet counts, antibody-coated platelets and cytotoxic T lymphocytes appear to persist or can change over time. To better understand how the spleen may affect anti-platelet immune responses, we used a murine model of ITP demonstrating both antibody-mediated and T lymphocyte-mediated thrombocytopenia. Mice with severe combined immunodeficiency (SCID) were either splenectomized or not and transfused with splenocytes from CD61 (GPIIIa) knockout mice immunized against $CD61^+$ platelets. Platelet counts and anti-platelet antibody levels were performed weekly. After 4 weeks, the mice were sacrificed, and lymphoid organs were harvested and examined by flow cytometry to quantify $CD4^+CD25^+FoxP3^+$ Tregs and conventional cross-presenting $XCR1^+$ and tolerizing SIRPα+ dendritic cells. The results indicate that compared with control non-splenectomized mice, thrombocytopenia was improved and anti-platelet antibody production was significantly diminished in all splenectomized mice that received immune splenocytes. Splenectomized SCID mice also had a marked reduction in Tregs in the thymus together with an increased proportion of both thymic dendritic cell subsets that correlated with increased platelet counts. Of interest, although splenectomy diminished anti-platelet antibody production and raised platelet counts, marrow megakaryocyte densities were still significantly reduced in mice that received immune splenocytes. These results suggest that the spleen in murine ITP not only is the primary site responsible for platelet destruction, but it also controls, to a significant extent, the antibody response against platelets and the migration patterns of lymphocyte subsets.

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by an isolated thrombocytopenia defined as a blood platelet count less than 100×10^9/L [1,2]. ITP pathophysiology is not only complex and heterogeneous, but appears to be caused primarily by immunoglobulin G (IgG)-mediated platelet destruction in the spleen and/or megakaryocyte inhibition/destruction in the marrow [1–5]. Of interest, however, most studies have indicated that anti-platelet antibodies can be identified in approximately 65% of patients with ITP [6–11]. In 2003, Olsson et al. elegantly demonstrated that in addition to anti-platelet antibodies, cytotoxic T lymphoctyes (CTLs) can also be responsible for thrombocytopenia [12], and this has been subsequently confirmed by other studies [13–15].

First-line therapy for patients with ITP generally includes glucocorticoids and/or intravenous immunoglobulin (IVIg), and if those fail, treatments such as rituximab,

RA and RK contributed equally.

Offprint requests to: John W. Semple, St. Michael's Hospital, 30 Bond Street, Toronto, ON, Canada M5B 1W8; E-mail: semplej@smh.ca

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.exphem.2016.07.004.

0301-472X/Copyright © 2016 ISEH - International Society for Experimental Hematology. Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.exphem.2016.07.004

thrombopoietin (TPO) receptor agonists, and/or splenectomy are available [16,17]. Splenectomy to date is still considered the gold standard in the second-line treatment of therapy-resistant ITP as approximately 60% of patients remain in remission after 5 years from their surgeries [18]. It was originally thought that the mechanism of action of splenectomy was simply removal of the site of platelet destruction. However, antibody-coated platelets and CTLs can persist or wax and wane after splenectomy, and this may be related to failures or relapses [19–21]. Several reports have indicated that splenectomy, in other disorders, causes significant changes in host immunity [22–30]; however, relatively few studies have addressed how splenectomy directly affects anti-platelet immune responses in patients with ITP. For example, Martinez-Gamboa et al. analyzed the distribution and phenotypic characteristics of B-cell subsets in non-splenectomized and splenectomized patients with ITP and noted decreased frequencies of memory B cells in the splenectomized individuals with a decline of $CD27^+IgD^+$ and $CD27^+IgD^-$ and $CD27^-/IgD^-$ cells [31]. Glycoprotein IIb/IIIa (GPIIb/IIIa, also known as integrin $\alpha_{IIb}\beta_3$) is an integrin complex found on platelets, and in ITP, antibodies are often formed against platelet GPIIb/IIIa. Kuwana et al. reported that the frequencies of circulating GPIIb/IIIa-reactive T and B cells were significantly decreased after splenectomy in patients with a complete response but were unchanged in non-responders [32]. These findings suggested that GPIIb/IIIa-reactive T- and B-cell interactions that induce anti-platelet antibody production in patients with ITP occur primarily in the spleen [32]. Furthermore, autologous mixed lymphocyte reaction-induced suppressor cell function was studied in nine patients before and after splenectomy. Although the platelet counts increased significantly as a result of the splenectomy, suppressor cell function exhibited no significant improvement [33]. In mice, Mizutani et al. studied the effect of splenectomy on anti-platelet antibody production and observed that platelet-associated antibody levels underwent a transient decrease, but there was no change in serum anti-platelet antibodies [34], in contrast to our current observations in the murine model. They suggested that suppression of anti-platelet antibody production is essential for the successful treatment of ITP [34]. Taken together, although it appears that splenectomy in ITP may affect immune responses, little is known of the exact details of this modulation. To address this, we used a well-characterized murine model of ITP that expresses both antibody- and T cell-mediated thrombocytopenia in an attempt to determine how splenectomy affects anti-platelet immune responses and lymphocyte distribution patterns in ITP. This murine model of ITP is initiated by first immunizing CD61 (GPIIIa) knockout mice with $CD61^+$ platelets. After 4 weeks, the immune splenocytes (or control non-immune splenocytes from naïve mice) were transferred into severe combined immunodeficient (SCID) mice, which were splenectomized (or splenocytes were transferred into control non-splenectomized SCID mice). Prior to splenocyte transfer, the SCID recipients were irradiated. Weekly platelet counts and anti-platelet antibody levels were assessed in the SCID recipient mice, and after 4 weeks, the mice were sacrificed and lymphoid organs were harvested and examined by flow cytometry to quantify thymic $CD4^+CD25^+FoxP3^+$ Tregs and conventional cross-presenting $XCR1^+$ and tolerizing $SIRP\alpha^+$ dendritic cells (DCs). In addition, bone marrow megakaryocyte densities were determined.

Methods

Mice

Female BALB/c mice (AnNCrl, H-2^d, $CD61^+$, 8–12 weeks of age, Charles River Laboratories, Montreal, QC, Canada) were used as platelet donors. BALB/c CD61 KO mice ($CD61^-$/H-2^d) were bred in the laboratory of Dr. Heyu Ni and used as anti-platelet immune splenocytes. Nonsplenectomized and splenectomized female CB.17 SCID mice (H-2^d, CB17/Icr$^-$*Prkdc*^{scid}/IcrIcoCrl, 8–12 weeks of age, Charles River) were used as spleen cell transfer recipients for induction of ITP. All mice were housed in the Li Ka Shing Knowledge Institute's Research Vivarium, and all animal studies were approved by St. Michael's Hospital Animal Care Committee.

Platelet preparation and immunization of CD61 KO mice

Leuko-reduced platelets from donor mice were prepared as previously described [15]. Briefly, blood was collected from the indicated donor mice, diluted with 1× phosphate-buffered saline (PBS) containing 10% citrate–phosphate–dextrose with adenine (PBS/CPDA buffer) and centrifuged at 120*g*. Platelet-rich plasma was then collected and washed at 450*g*. The washed platelets were resuspended in PBS, adjusted to 1×10^9 cells/mL, and 100 μL was transfused into BALB/c CD61 KO (CD61-/H-2^d) mice weekly for 4 weeks.

Preparation of splenocytes

Immune CD61 KO mice (CD61-/H-2^d) were sacrificed, and their spleens were removed, homogenized in RPMI-1640 medium, and washed twice by centrifugation at 400*g* for 15 min. Splenocyte suspensions were further treated with ammonium chloride–potassium red blood cell (RBC) lysing solution and washed with 1× PBS to remove lysed RBCs.

ITP induction

SCID mice were prescreened for the presence of serum IgG using an enzyme-linked immunosorbent assay (ELISA), and any mouse with a serum IgG concentration greater than 20 μg/mL was deemed "leaky" and excluded from study. ITP was induced as previously described [15]. Briefly, on day –1, CB.17 SCID mice were bled via the saphenous vein, and pretreatment platelet counts were performed using a Beckman Coulter Counter LH750 hematology analyzer. Natural killer cells were depleted in the mice by intraperitoneal infusion of 50 μL of a rabbit anti-asialo GM1 antibody (Wako Pure Chemical Industries). On day 0, the mice were sublethally gamma irradiated (200 cGy) and then received 100 μL (4×10^4 cells total) of nondepleted splenocytes (intraperitoneally) from the immune CD61 KO mice. Control transfers were