A murine model of severe immune thrombocytopenia is induced by antibody- and CD8+ T cell-mediated responses that are differentially sensitive to therapy

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Running title: Murine model of severe ITP

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Immune thrombocytopenia (ITP) is a bleeding disorder characterized by antibody-opsonized platelets being prematurely destroyed in the spleen although some patients with ITP may have a cell-mediated form of thrombocytopenia. Although several animal models of immune thrombocytopenia have been developed, few mimic primary chronic ITP nor have any demonstrated cell-mediated platelet destruction. To create this type of model, splenocytes from CD61 knockout (KO) mice immunized against CD61+ platelets were transferred into SCID (CD61+) mouse recipients and their platelet counts and phenotypes were observed. As few as 5 x 10^4 splenocytes induced a significant thrombocytopenia and bleeding mortality (80%) in recipients within 3 weeks post-transfer. Depletion of lymphocyte subsets before transfer demonstrated that the splenocyte’s ability to induce thrombocytopenia and bleeding was completely dependent on CD4+ T helper cells and that both CD19+ B cell (antibody)- and CD8+ T cell (cell)-mediated effector mechanisms were responsible. Treatment of the SCID mouse recipients with intravenous gammaglobulins (IVIg) raised platelet counts and completely prevented bleeding mortality induced by antibody-mediated effector mechanisms but did not affect cell-mediated disease. This novel model not only demonstrates both antibody- and cell-mediated immune thrombocytopenia and bleeding but suggests that these two effector mechanisms have a differential response to therapy.
Introduction

Immune thrombocytopenia (ITP) is one of the most common hematological autoimmune bleeding disorders characterized by premature platelet clearance by Fcγ receptor (R) mediated phagocytosis in the reticuloendothelial system (RES). ITP was distinguished by two forms termed acute and chronic but an international group of recognized experts have significantly revised the definitions and recommendations of the clinical diagnosis of ITP. Primary immune thrombocytopenia (ITP) is now defined as an autoimmune disorder characterized by isolated thrombocytopenia (peripheral blood platelet count <100x10^9/L) in the absence of other causes or disorders that may be associated with thrombocytopenia. The various phases of ITP are defined by the time since diagnosis; newly diagnosed ITP occurs within 3 months from diagnosis whereas persistent ITP is between 3 to 12 months from diagnosis and chronic ITP is now defined as thrombocytopenia lasting for more than 12 months. Severe ITP is now reserved for those patients where there is the presence of bleeding symptoms at presentation or the occurrence of new bleeding symptoms requiring therapeutic intervention. First line treatments for patients with chronic ITP include steroids and intravenous gammaglobulins (IVIg) and previous studies have shown that IVIg can also protect against passive-immune thrombocytopenia in mice.

At least 70 percent of patients with chronic ITP have identifiable serum autoantibodies that are primarily composed of IgG1 and IgG3 isotypes and generally target platelet glycoproteins (GP) IIb/IIIa and/or GPIbIX. On the other hand, some patients with ITP have no detectible anti-platelet autoantibodies yet are thrombocytopenic and it has been demonstrated that in these patients, cell-mediated immune mechanisms such as CD8+ T cell cytotoxicity may lead to the thrombocytopenia. At present, however, it is unknown whether cell-mediated platelet destruction
contributes to the severity of disease or the difficulty of treatment in some patients with ITP. Thus, an animal model that mimics chronic ITP pathophysiology would be desirable.

There have been several animal models of ITP including the secondary autoimmune model where immune thrombocytopenia is secondary to either other diseases e.g. lupus nephritis,17-20 or infections21-23 and the passive transfer model where continuous injection of platelet specific antibodies are required to maintain a steady state of thrombocytopenia.9-12 Although these models have been instrumental in understanding the pathophysiology of secondary ITP and the mechanisms of action of treatments such as IVIg, they are not ideal for chronic ITP pathophysiology where both T cell and B cell autoimmune attack are focused on platelet-specific antigens. Recently, however, a platelet specific immune model of neonatal alloimmune thrombocytopenia (NAIT) was developed using immune CD61 knockout (KO) mice 24 that were pregnant with wild type (WT) CD61+ fetuses. Fetal death due to bleeding occurred and in successful pregnancies, the neonatal pups were thrombocytopenic and exhibited a bleeding diathesis.24 In this report, we have created a novel murine model of severe chronic ITP that demonstrates both antibody-mediated and cell mediated platelet and megakaryocyte destruction and show that while antibody-mediated thrombocytopenia is responsive to IVIg treatment, cell-mediated disease is not. This model may be applicable for understanding the immune nature of severe ITP and the lack of response of cell-mediated thrombocytopenia and bleeding to IVIg therapy may suggest that other therapeutic strategies may be needed to treat this form of ITP.
Methods

Mice

Female BALB/c (H-2<sup>d</sup>), C57BL/6 (H-2<sup>b</sup>) mice and CB.17 (H-2<sup>d</sup>) severe combined immunodeficient (SCID) mice, 8-12 weeks of age, were used as either platelet donors or spleen cell transfer recipients and were obtained from Charles River Laboratories (Montreal, PQ, Canada). BALB/c CD62 KO mice were bred in the laboratory of Dr. Heyu Ni and originally obtained from Dr. Richard O. Hynes (MIT, Boston, MA). All animal studies were approved by the St. Michael's Hospital Animal Care Committee (Protocol No. 942).

Platelet preparation and Immunization of CD61 KO mice

For platelet preparation the indicated donor mice were anesthetized and blood was drawn by cardiac puncture from into phosphate buffered saline (PBS) containing citrate/phosphate, dextrose, acetate (CPDA) buffer. The blood was pooled, diluted with PBS and centrifuged at 150 x g for 15 minutes and the platelet-rich plasma (PRP) was collected. The PRP was washed three times by centrifuging at 450 x g for 18 minutes and the washed platelets were resuspended in PBS, counted and their concentration adjusted to 1x10<sup>9</sup> cells/mL.

For immunization, BALB/c CD61 KO mice were transfused with 100 uL of 10<sup>8</sup> platelets from wild-type (WT) BALB/c mice weekly for three weeks. Sera from these immune KO mice have been previously shown to induce a profound thrombocytopenia when injected into naïve mice. To produce 3<sup>rd</sup> party immune control animals, BALB/c mice were transfused as above with platelets from MHC allogeneic C57BL/6 mice. Immunization was monitored by measuring IgG anti-platelet antibody production in the sera by flow cytometry.
**Splenocyte preparations and cell depletion.**

The anti-platelet immune CD61 KO mice were sacrificed and their spleens were removed and prepared into a splenocyte suspension by crushing and straining through a mesh filter in PBS. The suspension was washed 3 times by centrifugation at 400 x g for 15 minutes. The splenocytes were then adjusted in cell medium (PBS+ 2% FBS with 1mM EDTA) to a concentration of 1x10^8 cells/mL, titrations were made and transferred to SCID mice to determine the optimal dose for inducing thrombocytopenia (Supplemental Figure 1). Engraftment of 1x10^5 immunized splenocytes caused 67% death by day 15 post-transfer whereas engraftment of 1x10^4 splenocytes had little effect on the SCID mouse mortality (Supplemental Figure 1). An intermediate dose of 5x10^4 cells was chosen for all experiments so that the engrafted SCID mice would exhibit symptoms of thrombocytopenia and survive past at least two weeks after irradiation.

In the indicated cell depletion studies, splenocytes (1x10^8 cells/mL cell medium) were first depleted of either CD4+, CD8+, and/or CD19+ lymphocytes before transfer by the procedures and reagents provided by the EasySep Magnetic cell sorting kit (StemCell Technologies, Vancouver BC; CD4+kit Cat#18752, CD8+kit Cat#18753 and CD19+kit Cat#18754). Briefly, cells were coated with the kit specific CD antibody reagent and the tube was inserted passed into a magnet and the negatively selected non-bound cells were collected by decanting, counted and resuspended with RPMI-5% heat inactivated FBS for engraftment. Depletion efficiencies were determined by flow cytometry (Supplementary Figure 2) and were greater than 96% for the specific lymphocyte subpopulations removed.
**Splenocyte transfer and platelet counts.**

SCID mice were bled prior to the experiments and sera was screened for the presence of murine IgG by an ELISA as previously described; and any mouse with a serum IgG concentration greater than 15 ug/ml was deemed “leaky” and sacrificed.

On the day before splenocyte transfer, screened SCID mice were injected intraperitoneally (ip) with 50 uL of rabbit anti-asialo GM1 (Wako Pure Chemical Industries, Ltd, Waco, TX) to primarily remove NK cells and on the day of transfer, the SCID mice were subjected to 200 cGy total body gamma irradiation to inhibit recipient innate immune responses and enhance engraftment. Within 3h of irradiation, mice were bled from the Saphenous vein for pre-bleed platelet counts and then injected intraperitoneally with 100 uL of the indicated splenocyte preparations (at 5x10^4 cells/mL final). Platelet counts and phenotypes were then recorded weekly. For platelet counts, blood was diluted 1/100 in PBS:CPDA and the platelet count was measured by a Beckman Coulter Counter-LH750 hematology analyzer.

**IVIg treatment**

SCID mice were weighed and Intravenous immunoglobulin G (IVIg, 10% w/v, Gamunex; Talecris Biotherapeutics, Mississauga, Ontario, Canada) was administered ip at a dose of 2g/kg one day before splenocyte transfer and twice weekly thereafter to ensure that a relatively constant in vivo level was maintained. This dose was chosen based on its common use clinically in patients with ITP. Control human serum albumin (HSA, 25% w/v, Baxter Healthcare Corporation, Westlake Village, CA, USA) was diluted to a final concentration of 10% (w/v) with sterile PBS (pH 7.4) and administered ip at a dose of 2g/kg like IVIg. Although the albumin dose has a greater oncotic load than does IVIg, it did not adversely affect the mice.
Flow cytometry to detect serum platelet CD61-specific antibodies.

To determine the levels of platelet-specific antibodies in the engrafted SCID mice, platelets were prepared from BALB/c mice and 5x10⁶ platelets were incubated with 4µL of undiluted serum from the SCID mouse recipients for 30 minutes at room temperature. The platelet suspension was washed once with PBS and 100µL of a FITC conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA; 1/200 final) was added and incubated in the dark for 30 minutes at room temperature. The suspension was washed and then analyzed by flow cytometry.

Bone Marrow histology

Femurs were dissected from sacrificed mice and their ends were cut off and the long bones were placed in fixative (B+ Fixative) for 12 hours. Decalcification was achieved with 10% Nitric Acid followed by embedding in wax and staining with haematoxylin and eosin. To concentrate megakaryocytes in the bone marrows from selected mice, the long bones were prepared as above and bone marrow cells were flushed from the lumen in PBS. The cells were collected and concentrated onto glass microscope slides using a Shandon Cytospin 2 Centrifuge (Block Scientific, Nutley, NJ). Slides were fixed and stained with H&E stain.

Statistical analysis

Data are expressed as mean +/- SEM and were analyzed using Student's t test. P<0.05 was considered significant.
Results

Induction of Severe Immune Thrombocytopenia (ITP).

When irradiated CD61+ SCID control mice received either no transfer or splenocytes from BALB/c mice immunized against a 3rd party platelet antigen (H-2^b MHC class I from C57BL/6 platelets), an irradiation-induced thrombocytopenia occurred at day 7 but recovered in all mice by day 14 (Figure 1) and no mortality was observed. In contrast, CD61+ SCID mice engrafted with 5x10^4 splenocytes from CD61 KO mice immunized against CD61+ platelets exhibited a profound thrombocytopenia which did not recover (Figure 2a) and there was an 80% bleeding mortality within 21 days post-transfer (Figure 3a). Bleeding diathesis occurred in the intestines, lungs, abdomen, subcutaneous tissues and brain (Supplemental Figure 3) and veterinary necropsy confirmed that the morbid mice suffered from hyperplasia of splenic red pulp associated with anemia, thrombocytopenia and amegakaryocytopenia. Histological sectioning of the bone marrows between healthy and morbid mice indicated that the latter had virtually no identifiable megakaryocytes (Figure 4) and those megakaryocytes that were found after cytospin concentration appeared to have irregular, enlarged nuclei, membrane blebbing and cytoplasmic abnormalities consistent with apoptosis (Figure 4). Determination of serum antibody levels also showed that the transferred SCID mice had significant levels of IgG anti-platelet CD61-specific antibodies by the second week post-transfer (Figure 5).

Induction of Severe ITP is Dependent on T helper cells and Induced by Both Antibody- and Cell-Mediated Effector Cell Responses.

To determine the nature of the adaptive immune responses that maybe responsible for the thrombocytopenia and bleeding, the splenocytes were first depleted of lymphocyte populations before transfer. In contrast with SCID mice transferred with non-depleted splenocytes (Figure 2a and 3a), depletion of CD4^+ T cells completely abolished their ability to induce thrombocytopenia, bleeding
mortality (Figures 2b and 3b respectively) or to produce serum anti-platelet antibodies upon transfer (Figure 5).

Further examination of the immune effector cell responses that maybe responsible for the effects showed that depletion of CD8+ T cells did not affect the transferred splenocyte’s ability to induce the development of anti-platelet antibodies (Figure 5), thrombocytopenia or bleeding mortality (Figures 2c and 3c respectively). On the other hand, as expected, depletion of CD19+ B cells significantly inhibited the transfer’s ability to generate anti-platelet CD61 antibody production in the SCID mouse recipients (Figure 5), however, it still induced a significant thrombocytopenia (Figure 2d) and bleeding mortality (Figure 3d) suggesting the presence of a cell-mediated effector mechanism. Characterization of this cell-mediated thrombocytopenia and bleeding by performing double depletions of both CD19+ B cell and CD8+ T cells confirmed the presence of CD8+ T effector cells (Figure 6); like controls, the double-depletion group completely recovered from the irradiation-induced thrombocytopenia and no anti-platelet antibody production (Figure 5) nor bleeding mortality was observed (Figure 6).

**IVIg Therapy has a Differential Effect on the Two Effector Responses.**

To determine the effect of a common first-line therapy clinically used in patients with ITP,1,2,6,8 2g/kg of IVIg was administered to the SCID mice the day before transfer, and twice weekly. When non-depleted splenocytes were transferred into either non-treated or BSA-treated SCID mice, significant thrombocytopenia and bleeding mortality occurred (Figures 7a and 7d respectively). In contrast, treatment of the transferred SCID mice with 2g/kg IVIg raised platelet counts (Figure 7a) and significantly reduced the bleeding mortality from 80% to 40% (Figure 7d). The IVIg efficacy could be completely separated if the SCID mice received splenocytes depleted of either CD8+ or CD19+ lymphocytes. IVIg treatment significantly prevented thrombocytopenia (Figure 7b) and completely
reversed the bleeding mortality (Figure 7e) in the SCID mice transferred with antibody-mediated effectors (CD8+ T cell-depleted splenocytes, Figure 7c) but not in those mice transferred with cell-mediated effectors (CD19+ B cell depleted splenocytes, Figures 7f).
Discussion

Severe chronic ITP is a diagnosis reserved for those patients with ITP that have bleeding symptoms either at presentation or with the occurrence of new bleeding symptoms requiring therapeutic intervention and over the past several years, information regarding the cellular immune pathophysiology of this disorder has been accumulating. For example, it is well established from studies in patients with chronic ITP that the disorder is associated with several T cell abnormalities including abnormally activated T helper cells with a Th1 cytokine bias, a deficiency of T regulatory cells (Tregs) that lead to autoantibody production and, in some patients, thrombocytopenia mediated by CD8+ T cells. To date, however, there are no known animal models that mimic these T cell abnormalities or demonstrate the severe form of chronic ITP with active bleeding. We report here the development of such a murine model that demonstrates both antibody (CD8 T cell-depleted)- and cell (CD19-depleted)-mediated effector responses that lead to thrombocytopenia and bleeding mortality. Of interest, the antibody-mediated bleeding mortality was significantly reduced by IVIg treatment but cell-mediated bleeding mortality was not. This model may offer several advantages in that it not only demonstrates two major immunopathologic mechanisms of platelet destruction, but it will be amenable to further elucidating the T cell-associated immunopathogenesis of the disorder and for testing novel therapeutics for severe chronic ITP.

Both the B cell and CD8+ T cell effector mechanisms of thrombocytopenia and bleeding were completely dependent on the presence of CD4+ T helper cells (Figure 2b) suggesting they are induced by an adaptive T helper cell immune response against platelet GPIIIa (CD61). This data appears to support previous observations in humans showing that T helper cell abnormalities are responsible for the initiation and perpetuation of the immune effector mechanisms responsible for the human disease. In addition, it suggests that the CD4+ T cells can be examined with relation to how a
relatively platelet-specific antigen (CD61) initiates their activation which has relevance to understanding and developing platelet-derived peptide-based therapies for reducing immune responses against platelets. These types of therapies have been proposed for patients with chronic ITP and those patients with human platelet antigen (HPA)-specific alloantibodies.\textsuperscript{44,45} Taken together, the complete dependence of thrombocytopenia and bleeding mortality on CD4+ T cells may, at least, mimic the pathophysiologic processes that lead to bleeding in those patients with severe ITP.

Of perhaps greater interest, this model may help to understand the immunopathophysiology of chronic ITP, particularly with respect to why some patients with ITP bleed while others with similar platelet counts, do not. For example, immune thrombocytopenic mice display significant bleeding when they are subjected to various different types of inflammatory conditions e.g. contact dermatitis, stroke or endotoxin-induced pulmonary inflammation.\textsuperscript{51} In addition, when some patients with ITP become infected, they can have precipitous drops in their platelet counts and increased bleeding symptoms. It is possible that the pro-inflammatory nature of the adaptive anti-platelet immune response in the SCID mice enhances the propensity to bleed. For example, when the transferred immune splenocytes first encounter CD61\textsuperscript{+} platelets in the SCID mice, they may promote pro-inflammatory cytokine cascades which, together with the ensuing immune thrombocytopenia, enhances the bleeding diatheses. We are currently studying this.

Perhaps the most astonishing observation was that the majority of the SCID mice transferred with immune KO splenocytes have a significant bleeding mortality which is not observed in the vast majority of patients with chronic ITP. In addition, the level of thrombocytopenia in the transferred mice does not necessarily account for the increased bleeding since it is known that mice rendered thrombocytopenic by, for example, passive transfer of anti-platelet antibodies do not bleed excessively.\textsuperscript{9-12,46} The excessive bleeding in our model suggests that additional immune factors are
involved. For example, the IgG anti-CD61 antibody response in immune CD61 KO mice was extremely strong (titres >1:12,000) and contains antibodies with multiple CD61 epitope specificities\textsuperscript{24,25} that not only induce FcR-mediated phagocytosis but also significantly inhibit platelet function.\textsuperscript{47} Thus, SCID mouse recipients that contain these anti-platelet antibodies are virtually devoid of platelet function and this may be a major reason why they bleed excessively. Alternatively, the higher bleeding mortality observed in relation to the antibody-mediated platelet destruction may be also partially explained by the observations that CD61 can be expressed on endothelial cells during pro-inflammatory conditions.\textsuperscript{48} It is possible that a pro-inflammatory immune response developing in a SCID mouse recipient may activate endothelial cells allowing the anti-CD61 antibodies to bind and perhaps disrupt or interfere with hemostatic mechanisms.\textsuperscript{48} Nonetheless, this model may at least mimic the bleeding observed in patients with severe ITP and may be useful in understanding why some patients with ITP go on to develop bleeding.

Compared with the severe antibody-mediated bleeding, the cell-mediated platelet destruction was associated with a lower bleeding mortality (Figure 3). The reasons for this are unclear but may be due to several reasons related to the nature of immune destruction mediated by autoantibodies compared with cell-mediated mechanism. Activated CD8\(^+\) T cells (CTL) kill target cells directly by two different pathways; the major pathway is mediated by perforin and a series of serine proteases (granzymes) and the second minor pathway is via the Fas-Fas Ligand (L) interactions ligands bind to the target cells Fas receptors to induce apoptosis.\textsuperscript{49} H&E staining of the few megakaryocytes found by concentrating the bone marrows from morbid mice revealed abnormalities indicative of apoptosis (Figure 4) which is, at least, consistent with cytotoxic T cell mediated damage. In contrast, antibody-mediated immune destruction in ITP is primarily mediated by FcR-mediated phagocytosis within the spleen. Perhaps FcR-mediated phagocytosis is more efficient in platelet clearance and megakaryocyte
destruction leading to bleeding compared with CTL mediated mechanisms. For example, platelet autoantibodies in patients with ITP have been shown to bind to and either destroy or inhibit megakaryocytes which decreases platelet production. Histological sections of the bone marrow of morbid mice revealed almost a complete lack of megakaryocytes in comparison to healthy SCID mice (Figure 4). Perhaps cell-mediated platelet destruction together with an inability to generate new platelets may also predispose the SCID mouse to bleeding but to a lesser extent than the antibody-mediated form of ITP.

IVIg treatment of the SCID mice transferred with non-depleted splenocytes did raise the platelet counts but it appeared not to be a complete response back to normal platelet numbers (Figure 7). In addition, IVIg treatment significantly reduced the bleeding mortality from 80% to 40% (Figure 7). These observations, particularly with regards to the IVIg therapeutic platelet response do not appear to correlate to the significant platelet responses observed in most patients with ITP treated with IVIg. However, when the antibody-mediated disease was isolated from the cell-mediated effects by CD8+ T cell depletions, IVIg treatment significantly elevated the platelet counts to normal levels and completely protected the mice from bleeding mortality. IVIg responses were not observed in those mice exhibiting only cell-mediated disease. It is possible that the combination of both forms of immune effector mechanisms in the non-depleted transfers is so robust that IVIg cannot counteract all the effects and only when the two mechanisms are isolated e.g. by cell depletions, can the full IVIg response be seen. Alternatively, it has been shown in passive mouse models of immune thrombocytopenia that antibodies with different platelet-specificities may affect the efficiency of IVIG to alleviate thrombocytopenia. For example, IVIG was more effective in ameliorating thrombocytopenia caused by anti-platelet GPIIbIIIa antibody than anti-GPIb\(\alpha\) antibody. It is thought that anti-GPIb\(\alpha\) may cause
thrombocytopenia thru a different mechanism (Fc-independent) from anti- GPIIbIIIa (Fc-dependent pathway).\textsuperscript{9,25} Perhaps related to the latter points, human IgG was used in the mouse model which may have introduced extraneous xenogenic effects such as cross-reactivity with murine red blood cells (RBC). However, other studies have successfully used human IVIg in different animal models of ITP with no apparent xenogeneic effects\textsuperscript{10-12,24,25} and in our hands, human IVIg at least, does not bind with murine RBC (JWS, unpublished data). Thus, taken together, our data suggest that IVIg therapy is efficacious for antibody-mediated thrombocytopenia but not the CD8+ T cell-mediated form of the disease.

In summary, we have described a murine model of ITP that demonstrates two major platelet/megakaryocyte destructive pathways e.g. antibody- and cell-mediated thrombocytopenia and show that while antibody-mediated thrombocytopenia is responsive to IVIg treatment, cell-mediated disease is not. This model will not only be important in understanding the immunopathologic processes in ITP but will also be useful in testing new therapeutic modalities to treat the different modes of platelet destruction.
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Authorship and Conflict of Interest Statements:

LC, designed research, performed/supervised all experiments, collected data, analyzed and interpreted data, performed statistical analysis, wrote manuscript first draft; RA, performed experiments, collected and analyzed data; ERS, performed experiments, collected and analyzed data; MK, performed experiments, collected and analyzed data; NC, performed bone marrow experiments, collected data, analyzed and interpreted data; MLW, performed IVIg experiments, collected data, analyzed and interpreted data; PC, performed IVIg experiments, collected data, analyzed and interpreted data; KS, performed experiments, collected data, analyzed and interpreted data; HN, designed research, manuscript editing, contributed animals; AHL, designed research, interpreted data, manuscript editing; MBG, experimental design, analyzed bone marrow histology and interpreted data, manuscript editing; JF, designed research, interpreted data, manuscript editing; JWS, financial resources, designed research, analyzed and interpreted data, manuscript editing. The authors have no conflict of interests or competing financial interests.
References


Figure Legends

Figure 1

Control SCID mice recover from radiation induced thrombocytopenia. Platelet counts in control irradiated SCID mice transferred with either nothing (☐, N=9) or 5x10⁴ splenocytes from BALB/c mice immunized against 3rd-party antigenic platelets from C57BL/6 mice (○, N=15). Thrombocytopenia at day 7 occurred in all mice irradiated (asialo GM-1 treatment had no effect on platelet counts). The data are expressed as platelet counts (x10⁹/L) (+/-SEM) over time (days). The solid horizontal line represents the normal mean platelet count (+/- SEM hatched lines) from 149 healthy SCID mice [mean platelet count = 960+310 (SD)]. The star (★) refers to statistical significance (p=0.0003) between the observed platelet count for all control mice and the normal mean platelet count.

Figure 2

Evidence of both antibody and cell-mediated thrombocytopenia. Platelet counts in irradiated SCID mice transferred with a) 5x10⁴ non-depleted splenocytes from CD61 KO mice immunized against WT BALB/c platelets (N=15) or 5x10⁴ splenocytes depleted of either b) CD4⁺ T cells (N=15), c) CD8⁺ T cells (N=18) or d) CD19⁺ B cells (N=14). Data is expressed as in Figure 1. For Panels a and c, platelet counts could not be determined past day 14 post transfer because of the poor health of the surviving mice. The numbers above the data points are the p values between the observed platelet counts for all mice and the normal mean platelet count (NS; not significant).

Figure 3

Bleeding mortality associated with cell-mediated thrombocytopenia is weaker than that of antibody-mediated thrombocytopenia. Kaplan-Meyer survival plots of irradiated SCID mice transferred with a) 5x10⁴ non-depleted immune splenocytes from CD61 KO mice immunized against
BALB/c platelets (N=15) or 5x10^4 immune splenocytes depleted of either b) CD4^+ T cells (N=15),
c) CD8^+ T cells (N=18) or d) CD19^+ B cells (N=14). The data are expressed as Percent Survival and
calculated from the mouse groups in Figure 2.

Figure 4

**Megakaryocytes are depleted and abnormal in ITP SCID mice:**  Histological section of A) healthy bone marrow and B) bone marrow from a morbid mouse engrafted with 5x10^4 non-depleted splenocytes (40x magnification; numerical aperture, plan apo; objective lens, 0.75). The yellow arrows in A point to megakaryocytes and each scale bar in A and B represents 50um. Because of the paucity of megakaryocytes in SCID mouse ITP bone marrows, the marrows were prepared by a cytospin method to concentrate them on a slide and stained with H&E. C) An example of a megakaryocyte from the bone marrow of a healthy SCID mouse. D) H&E staining of a rare megakaryocyte found in the bone marrow of a SCID mouse with ITP (x60 magnification; numerical aperture, plan apo; objective lens, 1.4). Each scale bar in C and D represents 10um. All pictures were taken with Nikon Eclipse E800 upright microscope.

Figure 5

**Anti-platelet CD61 antibodies in the sera of transferred SCID mice.**  Anti-CD61 specific antibody production in the sera of SCID transferred with either nothing (N=10), non depleted splenocytes (N=23), CD8^+ T cell depleted splenocytes (N=16), CD19^+ B cell depleted splenocytes (N=7) or CD19 and CD8 double-depleted splenocytes (N=5). Platelets from either BALB/c mice or CD61 KO mice were incubated with sera from the indicated groups of SCID mice and analyzed by flow cytometry. The data is expressed as fold increase and was calculated by: mean channel fluorescence (MCF) of test serum / MCF of naïve serum. All the positive sera were not reactive against platelets from CD61 KO mice.
**Figure 6**

Cell-mediated thrombocytopenia and bleeding is specific to CD8⁺ T cell lymphocytes. A.) Platelet counts in irradiated SCID mice transferred with either 5x10⁴ non-depleted immune spleen cells (N=9; ) or 5x10⁴ immunized spleen cells depleted of both CD8⁺ T cells and CD19⁺ B cells (N=8; ○). b) Kaplan-Meyer survival plot of each group shown in panel a.

**Figure 7**

IVIg treatment (□) is effective against antibody-mediated ITP but not cell-mediated ITP.

Platelet counts in irradiated SCID mice transferred with a) 5x10⁴ non-depleted immune splenocytes from CD61 KO mice immunized against BALB/c platelets (N=15; ○) or 5x10⁴ immune splenocytes depleted of either b) CD8⁺ T cells (N=18; ○) or c) CD19⁺ B cells (N=14; ○). Platelet counts in the SCID mice depletion groups treated with 2 g/kg IVIg (□) are also shown. Data is expressed as in Figure 1. The numbers above the data points are the p values calculated for the IVIg treatment groups as in Figure 1 (NS; not significant). Kaplan-Meyer survival plots for each group in panels a-c are shown in panels d-f. In panel d, the survival plot for control SCID mice that received human serum albumin (2g/kg; *) is also shown.
Figure 3

a. 

b. 

c. 

d. 

Percent Survival

Days Post Transfer
Figure 5

Serum Anti-CD61 Antibody (Fold increase)

- ND
- CD8 depleted
- CD19 depleted
- CD19/CD8 depleted
Figure 6

A. Platelet count (x10^9/L) over days post transfer.

B. Percent survival over days post transfer.
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