Pathogenesis of Fetal and Neonatal Immune Thrombocytopenia: Role of Anti-Beta3 Integrin Antibodies in Vascular Injury and Angiogenesis

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Laboratory Medicine and Pathobiology
University of Toronto

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2011

Abstract

Fetal and neonatal immune thrombocytopenia (FNIT) is a severe bleeding disorder which results from fetal platelet destruction by maternal antibodies against platelet antigens, including GPIIbIIIa (αIIbβ3 integrin) and GPIbα. β3 integrin is also expressed by angiogenic endothelial cells (ECs) and is required for angiogenesis. Therefore, we investigated whether anti-β3 antibodies in FNIT cross-react with blood vessels of the fetus/neonate and contribute to pathogenesis. Antibodies to GPIbα were used as controls. To mimic human FNIT, β3 integrin- or GPIbα-deficient female mice were immunized with wild-type platelets and bred with wild-type male mice. Pups in both groups had thrombocytopenia but intracranial hemorrhage was only observed in anti-β3-mediated FNIT. Anti-β3-mediated FNIT pups had increased apoptosis in the brain and impaired vascularization of the brain and retina. In addition, anti-β3 sera inhibited proliferation and vascular-like tube formation by ECs in vitro. Therefore, anti-β3 antibodies in FNIT likely impair angiogenesis in the developing fetus/neonate.
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra-Acetic Acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen binding</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable</td>
</tr>
<tr>
<td>FcRn</td>
<td>Neonatal Fc Receptor</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc-gamma receptor</td>
</tr>
<tr>
<td>Fg</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FNIT</td>
<td>Fetal and neonatal immune thrombocytopenia</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
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<td>GS IB4</td>
<td>Griffonia simplicifolia IB4</td>
</tr>
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<td>Hour</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HDN</td>
<td>Hemolytic disease of the newborn</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>Human platelet antigen</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<td>Immunoglobulin G</td>
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<tr>
<td>ISBT</td>
<td>International Society of Blood Transfusion</td>
</tr>
<tr>
<td>ITP</td>
<td>Idiopathic thrombocytopenic purpura</td>
</tr>
<tr>
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<td>Intrauterine growth restriction</td>
</tr>
<tr>
<td>IVIG</td>
<td>Intravenous immunoglobulin G</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>MAIPA</td>
<td>Monoclonal antibody-specific immobilization of platelet antigens</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>Micro-CT</td>
<td>Micro-computed tomography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
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</tr>
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<td>nm</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
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<td>Paraformaldehyde</td>
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<td>Postnatal day</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>PSI</td>
<td>Plexin/semaphorin/integrin</td>
</tr>
<tr>
<td>PTP</td>
<td>Post-transfusion purpura</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>Rh</td>
<td>Rhesus</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
<tr>
<td>UBM</td>
<td>Ultrasound biomicroscopy</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume concentration</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>Vascular endothelial growth factor receptor-2</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>w/w</td>
<td>Mass concentration</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>β3⁻/⁻</td>
<td>β3 integrin-deficient</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
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CHAPTER 1: Introduction

1.1. Platelets

Platelets are small, anucleate cells in the blood involved in vascular repair and hemostasis. Upon interaction with an injured surface, platelets adhere to the injured vessel wall and recruit additional platelets to the site, ultimately leading to the arrest of bleeding. Glycoprotein (GP) Ibα and GPIIbIIIa (αIIbβ3 integrin) are the major receptors on the platelet surface involved in platelet adhesion and aggregation. At the site of vascular injury, particularly at high shear stress, the binding of the platelet GPIb complex to von Willebrand factor (VWF) on the injured vessel wall initiates platelet tethering and subsequent adhesion. Platelet aggregation is then mediated by interaction between platelet αIIbβ3 integrin and fibrinogen (Fg) (Figure 1.1). However, thrombus formation can also occur in the absence of both VWF and Fg. During platelet activation and degranulation, P-selectin translocates to the platelet surface. Through interaction with sulfatides and the GPIb complex, P-selectin may contribute to the stabilization of platelet aggregates. We recently showed that Fg is required for maintenance of platelet intracellular and cell-surface P-selectin expression; β3 integrin engagement with the C-terminus of the Fg γ chain was required to maintain normal levels of platelet intracellular P-selectin, likely via de novo P-selectin synthesis by platelets. In addition, it is becoming increasingly apparent that platelets have a multitude of functions outside of their hemostatic roles, including inflammation, atherosclerosis, lymphatic development, angiogenesis, tumour growth and modulation of innate and adaptive immunity.
It is believed that expression of both GPIbα and GPIIb (the αIIb subunit of αIIbβ3 integrin) is exclusive to platelets and their precursor megakaryocytes, although expression of GPIbα has been demonstrated on endothelial cells under certain conditions\textsuperscript{10-13} but this remains controversial.\textsuperscript{14,15} GPIIIa (the β3 subunit of αIIbβ3 integrin) can be expressed on other cell types in addition to platelets and megakaryocytes, including angiogenic endothelial cells\textsuperscript{16,17} and endothelial progenitor cells (EPCs),\textsuperscript{18,19} microglia,\textsuperscript{20} astrocytes,\textsuperscript{21,22} as well as invasive cytotrophoblast\textsuperscript{23} and syncytiotrophoblast cells of the placenta.\textsuperscript{24,25}
Figure 1.1. Platelet adhesion and aggregation in hemostasis and thrombosis.

Platelets respond to sites of vascular damage by adhering to the injured vessel wall and recruiting additional platelets to the site, ultimately leading to the arrest of bleeding. GPIbα and GPIIbIIIa (αIIbβ3 integrin) are the major receptors on the platelet surface involved in platelet adhesion and aggregation. At sites of vascular injury, particularly at high shear stress, binding of the platelet GPIb complex to von Willebrand factor on the injured vessel wall initiates platelet tethering and subsequent adhesion. Platelet aggregation is then mediated by interaction between platelet αIIbβ3 integrin and fibrinogen (Fg). Inappropriate activation of these processes, as occurs following rupture of an atherosclerotic lesion, may lead to myocardial infarction or stroke. Figure adapted from Robbins SL, Kumar V and Cotran RS. (2010). Robbins & Cotran Pathologic Basis of Disease, 8th ed. Philadelphia: Saunders/Elsevier.
1.2. Immune-mediated thrombocytopenias

In addition to their roles in thrombosis and hemostasis, platelet surface GPIb\(\alpha\) and \(\alpha\)IIb\(\beta\)3 integrin are also frequent antigens targeted by both autoantibodies and alloantibodies, leading to platelet destruction and resultant thrombocytopenia. In adult idiopathic thrombocytopenic purpura (ITP) patients, greater than 70% of platelet autoantibodies are directed against \(\alpha\)IIb\(\beta\)3 integrin, while the remaining 20-40% have specificity for the GPIb\(\alpha\) complex, or both.\(^{26-28}\)

Antibodies to these receptors can also be generated following exposure to allogeneic platelets. This can occur after transfusion with donor platelets or during pregnancy following maternal exposure to fetal platelets and may result in alloimmune diseases such as post-transfusion purpura (PTP) and fetal and neonatal immune thrombocytopenia (FNIT), respectively.
Fetal and neonatal immune thrombocytopenia (FNIT) is a serious condition associated with significant fetal and neonatal morbidity and mortality. In healthy human fetuses, the platelet count is approximately $150 \times 10^9/L$ at 10 to 17 weeks of gestation\textsuperscript{29} and reaches $265 \pm 59 \times 10^9/L$ by 30 to 35 weeks of gestation.\textsuperscript{30} Therefore, thrombocytopenia in the fetus and neonate has been defined as platelet count less than $150 \times 10^9/L$. FNIT is the most common cause of severe thrombocytopenia in fetuses and neonates (platelet count $<20 \times 10^9/L$)\textsuperscript{31,32} and its frequency is estimated at 0.5-1.5 per 1000 liveborn neonates.\textsuperscript{32-37} However, this prevalence rate does not include the incidence of miscarriage in FNIT, since the rate in affected women is largely unknown.\textsuperscript{32,38-40} Analogous to ITP in adults, the major antigenic target in FNIT is $\alpha_{IIb}\beta_3$ integrin (75-95\% of cases).\textsuperscript{38,41,42}
1.2.1.1. **Pathophysiology of FNIT**

FNIT is believed to develop due to paternally-derived antigens present on fetal platelets which are lacking in the mother. The maternal immune system therefore regards the fetal platelets as foreign and mounts an immune response, consequently leading to the generation of alloantibodies against the platelet antigen. These antibodies are able to cross the placenta and opsonize fetal platelets, resulting in thrombocytopenia and a bleeding diathesis (Figure 1.2.1.1). FNIT is similar to the more common condition hemolytic disease of the newborn (HDN), in which maternal alloantibodies target antigens on fetal red blood cells, such as the Rhesus (Rh) antigen. FNIT carries significant risks including intrauterine growth restriction (IUGR), intrauterine fetal demise and fetal intracranial hemorrhage (ICH). ICH occurs in approximately 10-20% of affected neonates and is associated with death in 10% and neurological sequelae in 10-20% of these cases.\(^{38,43-46}\) In contrast to HDN, occurrence of FNIT in primiparous women (first pregnancy) is more common (up to 50% of cases),\(^{32,33,38}\) thus making diagnosis and treatment more difficult. Furthermore, there is a greater than 90% rate of recurrence of FNIT among subsequent platelet antigen-positive siblings,\(^{33}\) who are often more severely affected.\(^{47}\) The sole established predictor of severe disease in a subsequent affected sibling is antenatal ICH in the previous sibling, which is predictive of both ICH and severe fetal thrombocytopenia.\(^{47,48}\)
Figure 1.2.1.1. Fetal and neonatal immune thrombocytopenia (FNIT).

Fetal and neonatal immune thrombocytopenia (FNIT) is an alloimmune disorder associated with significant fetal and neonatal morbidity and mortality. Following exposure to paternally-derived fetal platelet antigens, the immune system of the antigen-negative mother may mount an immune response leading to the production of pathogenic alloantibodies. These antibodies are able to cross the placenta and opsonize fetal platelets leading to their destruction in the fetal mononuclear phagocytic system, e.g. the spleen. Figure adapted from Blanchette VS, Johnson J and Rand M. Baillieres Best Pract Res Clin Haematol. 2000; 13(3): 365-90.
1.2.1.1.1.  Alloantigens

Platelets share several antigen systems with other cell types, including the human leukocyte antigen (HLA) class I and the ABH blood group antigens on their surface; however, these antigen systems are rarely implicated in FNIT and their significance in the etiology of the disease remain controversial. FNIT is mainly caused by alloantibodies directed against epitopes in “platelet-specific” membrane glycoproteins. Currently, there are at least 16 platelet antigens listed by the Platelet Serology Working Party of the International Society of Blood Transfusion (ISBT). In 1990, this Working Party established a nomenclature designating platelet antigens as HPA for human platelet antigen, whereby each platelet antigen is numbered according to its date of discovery and alleles are labeled in alphabetical pairs based on serologic frequency (‘a’ for the high-frequency allele and ‘b’ for the low-frequency allele).

Despite the implication of a variety of antigens, only a small number are responsible for the majority of reported cases of FNIT. In White populations, incompatibility of HPA-1a (residue 33 Leu and Pro on β3 integrin) accounts for approximately 75% of FNIT cases. Although FNIT due to incompatibility of HPA-1 is extremely rare in African American and Asian populations, regardless of race, polymorphisms in the β3 integrin subunit are a major cause of FNIT. There are at least nine HPAs identified in β3 integrin, which occur throughout the extracellular portion of the molecule: the N-terminal PSI domain (HPA-1 and 10), I-like domain (HPA-4 and 7), cysteine-rich domain (HPA-6 and 14; Sec), and the C-terminal extracellular region (HPA-8) (Figure 1.2.1.1.1). Furthermore, isoantibodies have been detected in mothers deficient for the entire αIIbβ3 integrin molecule (a disorder termed Glanzmann thrombasthenia), which may be implicated in FNIT.
Two HPAs (HPA-3 and 9) have been located on the αIIb subunit of αIIbβ3 integrin, two HPAs (HPA-5 and 13) on GPIa (α2 integrin), one HPA (HPA-12) on GPIbβ, and one HPA (HPA-2) has been reported on GPIbα (Figure 1.2.1.1.1). Although reported cases of FNIT mediated by GPIbα are rare, we recently demonstrated in a murine model that antibodies to GPIbα causes spontaneous miscarriage, which could mask the severity and frequency of anti-GPIbα-mediated FNIT in humans.59
Figure 1.2.1.1.1. Human platelet antigens (HPAs) involved in FNIT.

There are at least nine human platelet antigens (HPAs) identified in β3 integrin, which occur throughout the extracellular portion of the molecule: the N-terminal PSI domain (HPA-1 and 10), I-like domain (HPA-4 and 7), cysteine-rich domain (HPA-6 and 14; Sec), and the C-terminal extracellular region (HPA-8). Incompatibility of HPA-1 on β3 integrin is responsible for approximately 75% of FNIT cases. Two HPAs (HPA-3 and 9) have been located on the αIIb subunit of αIIbβ3 integrin, two HPAs (HPA-5 and 13) on GPIa (α2 integrin), one HPA (HPA-12) on GPIbβ and one HPA (HPA-2) has been reported on GPIlbα. Figure adapted from Newman PJ, who adapted it from Humphries MJ and Mould PA. Science. 2001; 294: 316.
1.2.1.2. Maternal and fetal/neonatal immune responses

The exposure of the mother to paternally-derived fetal antigens is not well understood. It is unclear whether immunization occurs only during pregnancy, which would be akin to HDN. In HDN, immunization is thought to occur as a result of fetomaternal transplacental hemorrhage, which may occur due to trauma or during partuision. If immunization against platelet antigens also occurs only during pregnancy, the higher incidence of FNIT in a first pregnancy as compared with HDN may be attributable to the smaller size and thus greater mobility of platelets. However, results from a recent prospective study suggest that occurrence of FNIT in primiparous women may be less than previously believed. Nevertheless, it is thought that leakage of fetal platelets into the maternal circulation induces an immune response by the mother. Fetal platelet antigens have been shown to be expressed as early as 16 weeks of gestation and formation of immunoglobulin G (IgG) alloantibodies with destruction of fetal platelets may occur by 16–20 weeks of gestation. However, it has been demonstrated that β3 integrin is expressed on angiogenic endothelial cells, as well as invasive cytotrophoblast and syncytiotrophoblast cells of the placenta, suggesting that sensitization could occur independent of platelets. Furthermore, β3 integrin is expressed on human spermatozoa, which implies that preconceptional exposure may occur.

When platelet antigens are exposed to the maternal immune system, they are processed by maternal antigen presenting cells (e.g. macrophages) and are presented to antigen-specific T helper lymphocytes. These T cells can then stimulate antigen-primed B cells to differentiate into plasma cells and secrete specific IgG antibodies. However, not all women with an incompatible fetus will develop anti-platelet alloantibodies; several prospective studies have indicated that only approximately 6-11% of women homozygous for the HPA-1b allele (and therefore
susceptible to immunization by an HPA-1a-positive fetus) will develop anti-HPA1a antibodies during pregnancy with an incompatible fetus.\textsuperscript{35,38,40,41,65} This may be explained by the strong association between HPA-1a alloimmunization and the HLA class II DRB3*0101 allele,\textsuperscript{40,41,66,67} which suggests that specific maternal antigen presentation pathways may be involved in the immune response to fetal peptides. Indeed, it has been demonstrated that the HLA DRB3*0101 locus has a binding affinity for the Leu at residue 33 on HPA-1a, but not for the Pro33 substitution present on HPA-1b,\textsuperscript{68} indicating that maternal genetic background is involved in alloimmunization to HPA-1a.

It has been demonstrated that the neonatal Fc receptor (FcRn) plays an important role in transplacental IgG transport.\textsuperscript{69} In addition, FcRn has been shown to regulate IgG homeostasis by protecting IgG from degradation.\textsuperscript{70,71} Therefore, FcRn was suspected to be involved in FNIT, but its specific role in the pathogenesis of the disease had not been previously studied. In a murine model of FNIT, we recently demonstrated that FcRn is indispensable for the transfer of all IgG isotypes to the fetus and thus, is essential for the induction of FNIT. Furthermore, we clarified that fetal, and not maternal, FcRn is required for transport of IgG to the fetus.\textsuperscript{72}

The mechanisms of platelet clearance in FNIT are thought to be similar to that in ITP: antibodies bind to platelets via their Fab portion and bridge the platelet to a monocyte/macrophage through interaction between the antibody Fc portion and monocyte/macrophage Fc receptors, resulting in clearance of opsonized platelets by the mononuclear phagocytic system (reticuloendothelial system; e.g. the spleen).\textsuperscript{73,74} However, some antibodies, particularly those targeting GPIb\textsubscript{a}, may mediate platelet clearance via an Fc-independent pathway.\textsuperscript{75} Interestingly, we recently demonstrated in a murine model of FNIT that anti-GPIb\textsubscript{a} antibodies may deliver signals leading to platelet activation and subsequent aggregation, which may lead to platelet destruction via an
alternative mechanism (e.g., consumption). In addition, since megakaryocytes express similar membrane glycoproteins as platelets, impaired platelet production may also contribute to thrombocytopenia in FNIT, as is becoming increasingly apparent in ITP. However, neonates may differ in their response to thrombocytopenia compared with adults, and further studies are required to determine effects on megakaryocytes in FNIT.
1.2.1.2. Diagnosis and Management of FNIT

In the absence of routine screening, the diagnosis of FNIT often occurs following the delivery of a symptomatic child. This may occur following detection of thrombocytopenia in standard blood tests, or when a neonate presents with bleeding symptoms such as petechiae or ICH. Laboratory testing is then necessary to confirm the alloimmune origin of the thrombocytopenia. Detection of alloantibodies can be achieved by incubating maternal serum with paternal platelets and analyzing the bound anti-platelet antibodies via flow cytometry. The antibodies can then be identified by an antigen-capture technique, such as the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay, using paternal platelets. Experienced laboratories can also perform platelet antigen genotyping to determine the exact antigen incompatibility, which is important for determining risk for subsequent pregnancies. It is in these subsequent at-risk pregnancies that antenatal therapy can be initiated in hopes of preventing severe complications, including ICH and associated sequelae.

Although several treatments, such as intravenous immunoglobulin G (IVIG), corticosteroids, and fetal and neonatal platelet transfusions, have been used to manage FNIT, antenatal management of FNIT has not been standardized. IVIG, a pooled blood product from thousands of healthy donors, has been used to treat a number of auto- and alloimmune diseases, including ITP. However, the mechanism(s) of action of IVIG in the treatment of these diseases is still uncertain. Among the proposed mechanisms of action are anti-idiotypic antibody activity, blockade of the mononuclear phagocytic system, enhanced pathological antibody clearance through occupancy of FcRn, inhibition of phagocytosis via upregulation of macrophage inhibitory Fcγ receptor FcγRIIB and modulation of dendritic cell function. IVIG has also been employed as a therapy for FNIT with varying results. Nevertheless,
IVIG is widely used in the antenatal management of FNIT, but optimal treatment regimens remain to be determined.\textsuperscript{103}

We recently tested the use of a monoclonal antibody to FcRn to block transplacental transport of pathogenic maternal antibodies in a murine model of FNIT\textsuperscript{72} and the results look promising.\textsuperscript{104} Anti-FcRn was able to downregulate levels of anti-\(\beta_3\) integrin antibodies in the maternal circulation and elevated neonatal platelet counts. All pups delivered from mothers treated with anti-FcRn appeared healthy and no abnormalities were found. In addition, no obvious adverse effects were observed in pregnant mice or their pups after treatment with a dose of anti-FcRn greatly exceeding that required for the amelioration of FNIT.\textsuperscript{72} These data suggest that FcRn is a potential therapeutic target for treatment of FNIT.
1.2.1.3. Animal model of FNIT

Since FNIT is potentially life-threatening, randomized controlled trials employing an untreated control group are not possible, thus making it difficult to determine optimal treatment. In addition, limited research can be performed with FNIT patients to study the pathogenesis of the disease and mechanisms of action of various treatments. Therefore, a murine model of FNIT has been established in our laboratory. Our lab previously demonstrated that mice deficient for β3 integrin (β3−/−) generate antibodies against β3 integrin following transfusion of wild-type (WT) platelets. When immunized β3−/− female mice were bred with WT male mice, thrombocytopenia and bleeding disorders, including ICH, were observed in the heterozygous pups, similar to FNIT in human patients. Spontaneous fetal demise was observed if maternal antibody titre was sufficiently high. Maternal administration of IVIG was able to ameliorate FNIT, indicated by decreased anti-β3 integrin antibody in the maternal and neonatal circulation as well as elevated platelet counts, reduced bleeding manifestations and decreased mortality in pups. In addition, another FNIT model has been established in our laboratory using GPIbα-deficient (GPIbα−/−) mice. We found that anti-GPIbα antibodies caused miscarriage (complete lack of parturition) in most affected mothers and markedly enhanced fibrin deposition in their placentas, leading to impaired placental function. This is different from the traditional concept of FNIT, which is primarily characterized by bleeding symptoms in neonates. Maternal administration of IVIG or a monoclonal antibody against FcRn could prevent this devastating consequence.
1.3. Vascular development

During mammalian development, blood vessels arise from haematopoietic precursor angioblasts, which assemble into a primitive vascular bed, a process known as vasculogenesis. The vascular plexus expands by the growth and sprouting of these pre-existing vessels (termed angiogenesis) leading to the formation of a highly organized vascular network.\textsuperscript{106,107} In addition, tissues may also become vascularized by a process known as intussusceptive angiogenesis, in which transluminal tissue pillars develop within small blood vessels and subsequently fuse, thus creating new vessels.\textsuperscript{108,109} The distinction between vasculogenesis and angiogenesis is not absolute and overlaps, as both processes involve endothelial cell proliferation, migration and tube formation.\textsuperscript{110} Furthermore, while angiogenesis is thought to be the major mechanism of postnatal blood vessel growth, the discovery of circulating EPCs in adults suggests that vasculogenesis may not only be important in early embryogenesis, but may also play a role in both physiological and pathological neovascularization in the adult.\textsuperscript{111,112}
1.3.1. **Integrins**

Integrins are the principle adhesion receptors used by endothelial cells to interact with the extracellular environment and play critical roles in the development of blood vessels. The integrins are a family of cell surface receptors that mediate adhesion to the extracellular matrix, but can also interact with cell surface or soluble ligands. They are heterodimeric, each comprised of an α- and a β-subunit. Integrins are expressed in the simplest metazoans and in higher mammals, 18 α- and 8 β-subunits combine to form at least 24 different heterodimers. Each integrin heterodimer has its own ligand specificity, with most integrins recognizing several extracellular matrix proteins, including vitronectin, fibronectin and collagen. Integrins can signal bidirectionally across the cell membrane: the change in conformation of integrins conferring ability to bind ligands is regulated from within the cell (“inside-out” signaling), while the binding of ligands elicits signals that are transduced into the cell (“outside-in” signaling).113,114

Among the integrins, 9 have been implicated in angiogenesis, including the collagen receptors: α1β1, α2β1; the laminin receptors: α3β1, α6β1, α6β4; the fibronectin receptors: α4β1 and α5β1; and the vitronectin receptors: αvβ3 and αvβ5. Expression of each of these receptors has been described on endothelial cells and varies depending on the location and activation state of the cell.115-117
1.3.1.1. \( \alpha \beta 3 \) integrin

It has been demonstrated that \( \alpha \beta 3 \) integrin is expressed on proliferating endothelial cells during the processes of angiogenesis and vascular remodelling\(^{16} \) (Figure 1.3.1.1). Disruption of \( \alpha \beta 3 \) integrin ligation by cyclic arginine-glycine-aspartic acid (RGD; the canonical integrin-binding motif)-containing peptide antagonists or the blocking antibody LM609 induced apoptosis of angiogenic vascular cells\(^{17} \) and prevented formation of blood vessels.\(^{118-120} \) Interestingly, ablation of \( \beta 3 \) integrin in mice did not result in vasculature malformations\(^{121} \) and these mice exhibited enhanced pathological angiogenesis and tumour growth.\(^{122,123} \) This enhanced pathological angiogenesis may be due to an upregulation of vascular endothelial growth factor (VEGF) receptor-2 (VEGFR-2) in the absence of \( \beta 3 \) integrin.\(^{124} \) It has been demonstrated that engagement of \( \alpha \beta 3 \) integrin with its ligands (e.g. vitronectin) is involved in the full activation of VEGFR-2 mediated by VEGF.\(^{125} \) In addition, crosstalk between the two receptors has been shown to determine the response of endothelial cells to both VEGF and integrin ligation and is required for pathological angiogenesis.\(^{126-128} \) However, several other hypotheses have been proposed to explain the seemingly contradictory results observed in the \( \alpha \beta 3 \) integrin blocking versus genetic ablation studies.\(^{129} \) The role of \( \beta 3 \) integrin in angiogenesis, particularly developmental angiogenesis, requires further investigation.
Figure 1.3.1.1. Structure of αvβ3 integrin.

The extracellular portion of the αv subunit of αvβ3 integrin consists of a β-propeller region, a thigh region and the calf domains (calf-1 and calf-2). The extracellular portion of the β3 subunit consists of the βA domain, hybrid domain, PSI domain, EGF domains, and the β tail domain. HPA-1 is located in the PSI domain. Integrin αvβ3 binds to a variety of plasma and extracellular matrix proteins containing the conserved arginine-glycine-aspartic acid (RGD) amino acid sequence, e.g. vitronectin, and modulates cell adhesion. Integrin αvβ3 is expressed on endothelial cells and has been demonstrated to play an important role in angiogenesis. Figure adapted from Arnaout MA, Goodman SL and Xiong JP. Curr Opin Cell Biol. 2002; 14:641–651.
1.3.1.1. Antagonists for clinical use

Since expression of $\alpha_v\beta_3$ integrin is upregulated during angiogenesis and is undetectable in quiescent blood vessels, antagonists of $\alpha_v\beta_3$ integrin were developed as anti-angiogenic agents. The first of these agents to enter clinical trials was etaracizumab (Abegrin; formally known as Vitaxin), a humanized form of the monoclonal antibody LM609. Etaracizumab was shown to promote disease stabilization in late stage cancer patients without causing significant toxicity in a phase I trial\textsuperscript{130} and is in phase II trials. Cilengitide (EMD 121974), an RGD-mimetic cyclic peptide inhibitor of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, is currently in clinical trials as therapy for several types of cancer, including phase III trials for glioblastoma. Although effective in pre-clinical models,\textsuperscript{131,132} the efficacy of $\alpha_v\beta_3/\alpha_v\beta_5$ integrin inhibitors in suppressing tumour growth in humans has been limited (except for glioblastoma, where effects may be anti-tumour rather than anti-angiogenic).\textsuperscript{133,134} In fact, low concentrations of RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ integrin inhibitors have recently been shown to actually stimulate tumour growth and angiogenesis.\textsuperscript{135} Therefore, although $\beta_3$ integrin is clearly involved in vascular development, its specific role in these processes remains unclear.
1.4. Rationale

Integrin αIIbβ3 is a key glycoprotein expressed on the platelet surface and is the major target for auto- and alloantibodies, resulting in ITP and FNIT. However, bleeding is more severe in FNIT, with a frequency of ICH 10-100 times greater than in ITP.\(^{136}\) Since platelet destruction is thought to be the major contributor to bleeding in FNIT, little information is available regarding the effect of anti-platelet antibodies on vascular injury and angiogenesis. It is unknown whether the more severe bleeding diathesis observed in FNIT is due to a cross-reaction of maternal antibodies with fetal endothelial cells, which causes vascular injury and synergizes with the low platelet count. Most anti-platelet antibodies in FNIT target the β3 integrin subunit, which is expressed as αvβ3 integrin on angiogenic endothelial cells.\(^{16,17}\) Therefore, it is possible that anti-β3 integrin antibodies may cross-react with the angiogenic endothelial cells of the developing fetus/neonate (particularly in the brain, a major site of angiogenesis during fetal development)\(^{106}\) and cause a more severe bleeding diathesis. Expression of HPA-1 on human endothelial cells has been reported\(^{137-141}\) and ICH in FNIT patients is most commonly associated with incompatibility of HPA-1.\(^{38,142,143}\) Injury to the blood vessels or anti-angiogenic effects may explain the propensity for intraparenchymal hemorrhage in FNIT, even though intraventricular hemorrhage is more commonly observed in prematurity and other conditions.\(^{33,144}\) In addition, emerging evidence suggests that low platelet counts alone do not cause bleeding\(^{145-147}\) and that impairment of angiogenesis may be the major reason for fetal brain hemorrhage.\(^{148,149}\) However, platelets have been shown to contribute to pathological angiogenesis\(^{150-154}\) and it is therefore possible that thrombocytopenia itself may impair angiogenesis during fetal development.
1.5. **Hypothesis**

The cross-reaction of anti-β3 integrin antibodies with angiogenic endothelial cells leads to vascular injury and/or impairment of angiogenesis, which contributes to pathogenesis of FNIT.

1.6. **Specific aims**

1) To examine vascular injury and anti-angiogenic effects in a murine model of β3 integrin-mediated FNIT (a GPIbα-mediated FNIT model will be used as a control for the effects of thrombocytopenia).

2) To examine the effects of anti-β3 integrin antibodies on angiogenic endothelial cells in vitro.
CHAPTER 2: Materials and Methods

2.1. Reagents

Isoton II Diluent was purchased from Beckman Coulter Canada (Mississauga, ON, Canada). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig, thiazolyl blue tetrazolium bromide and dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich Canada (Oakville, ON, Canada). Rabbit anti-human (cross-reacts with mouse) von Willebrand factor, rabbit anti-mouse collagen IV antibodies and rabbit anti-mouse active (cleaved) Caspase 3 antibody were purchased from Millipore (Billerica, MA, USA). Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated Isolectin GS-IB4 were acquired from Invitrogen Canada (Burlington, ON, Canada). Growth factor reduced Matrigel was obtained from BD Biosciences (Mississauga, ON, Canada).
2.2. Mice

β3−/− mice105,121 were kindly provided by Dr. Richard O. Hynes (Massachusetts Institute of Technology, Boston, MA, USA). GPIbα−/− mice155 were kindly provided by Drs. Jerry Ware and Zaverio M. Ruggeri (The Scripps Research Institute, La Jolla, CA, USA). Both β3−/− and GPIbα−/− mice have been backcrossed onto a BALB/c background 10 times, respectively. To further minimize genetic background differences, β3−/− mice were bred with GPIbα−/− mice to generate β3+/−/GPIbα+/− mice. These mice were then crossed to re-generate the β3−/− and GPIbα−/− mice used in this study. Genotypes of experimental animals were confirmed by polymerase chain reaction analysis. Wild-type (WT) BALB/c mice (6-10 weeks old) were purchased from Charles River Laboratories (Montreal, QC, Canada). All mice were housed in the Research Vivarium at St. Michael’s Hospital and the experimental procedures were approved by the Animal Care Committee.
2.3. **Blood collection and platelet preparation**

WT mice were anaesthetized with 2.5% tribromoethanol (0.015mL/g) and bled via the retro-orbital plexus using a heparin-coated glass capillary tube. Blood was collected into a tube containing 3% acid citrate dextrose (38mM citric acid, 75 mM trisodium citrate, 100 mM dextrose; 1/9, v/v). Platelet-rich plasma (PRP) was obtained by centrifugation at 300 × g for 7 min at room temperature (RT). Supernatant was collected into a fresh tube and centrifuged at 300 × g for 5 min RT. Platelets were isolated from the PRP using a Sepharose 2B column in PIPES buffer (5mM PIPES, 1.37mM NaCl, 4mM KCl, 0.1% (w/v) glucose, pH 7.0).
2.4. Immunization of β3−/− and GPIbα−/− mice and detection of anti-β3 integrin or anti-GPIbα antibodies via flow cytometry

β3−/− and GPIbα−/− mice (6-10 weeks old) were immunized with two or four weekly transfusions of WT platelets (2×10⁷ - 1×10⁸) via the tail vein. Whole blood was collected from the immunized mice via the saphenous vein, both prior to and following platelet immunizations and allowed to clot at RT. Sera were obtained by centrifuging clotted blood at 9600 × g for 5 min and were diluted in phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄, pH 7.4). WT platelets (10⁶) were incubated with sera (final dilutions of 1:50, 1:100, 1:200) for one hour at RT. Samples were washed with PBS by centrifugation at 600 × g for 10 min and then incubated with FITC-conjugated anti-mouse IgG for 30 min at RT. Samples were analyzed on a FACSCaliber flow cytometer (Becton Dickinson, San Jose, CA, USA). The value for anti–platelet IgG was calculated as a fold increase ratio. Fold = mean fluorescence intensity (MFI) of immunized serum/MFI of preimmune serum.
2.5.  Induction of FNIT

For $\beta^3\text{--}^*$ mice, only mice that were sufficiently immunized (MFI ratio $\geq 10$) were utilized. For GPI$\alpha\text{--}^*$ mice, since there is a high rate of miscarriage, 59 mice with detectable anti-platelet antibodies (MFI ratio $\geq 2$) were included. One week after the second immunization of WT platelets, female $\beta^3\text{--}^*$ and GPI$\alpha\text{--}^*$ mice were bred with WT BALB/c male mice. Naïve $\beta^3\text{--}^*$ and GPI$\alpha\text{--}^*$ female mice (no platelet transfusions) were bred with WT male mice as controls.
2.6. Platelet enumeration

Whole blood (10μL) was isolated from postnatal day (PND) 2 mouse pups via the carotid artery and immediately diluted 1:25 in 240μL of 1% (v/v) EDTA/PBS, pH 7.4. PRP was isolated via centrifugation at 220 × g for 2 min and 50μL PRP was diluted 1:200 in 9.95mL Isoton II Diluent. Platelet counts were determined via a Z2 Series Coulter Counter (Beckman Coulter, Brea, CA, USA). Thrombocytopenia was defined as a platelet count ≤100×10⁹/L, and in the FNIT groups, only thrombocytopenic pups were included.
2.7. Preparation of mouse tissues for histological analysis

Following blood collection, PND2 mouse pups were fixed in formalin and paraffin-embedded. A microtome was used to cut 6-8μm sections which were transferred to glass slides. Tissue sections were stained with hematoxylin and eosin (H&E). Paraffin-embedding, sectioning of paraffin-embedded tissue and H&E staining was performed by the Pathology department at St. Michael’s Hospital. Alternatively, tissues were snap-frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA). 12μm sections were cut using a cryostat, transferred to aminoalkylsilane-treated slides and stored at -80°C.
2.8. Immunohistochemistry

Frozen tissue sections were thawed at RT and fixed with 4% (w/v) paraformaldehyde (PFA) in PBS for 5 min. After washing with PBS, samples were permeabilized with 1.5% (v/v) fetal bovine serum (FBS)/PBS containing 0.3% (v/v) Triton X-100 for 30 min. Sections were washed with PBS and antigen unmasking was performed with Citric Acid Based Antigen Unmasking Solution (Vector Laboratories, Burlington, ON, Canada). After washing with PBS, samples were incubated in 1.5% (v/v) FBS/PBS for 30 min, followed by primary antibody in FBS/PBS for 2 h at RT. Samples were washed in PBS and incubated with secondary antibody in FBS/PBS for 30 min at RT. Images were acquired using a Nikon E800 fluorescence microscope (Nikon Canada, Mississauga, ON, Canada). The fluorescence-positive areas were analyzed by Image J software (U.S. National Institutes of Health, Bethesda, MD, USA).
2.9. Detection of apoptosis in mouse tissues via terminal deoxynucleotidyl transferase dUTP nick end labelling

Frozen tissue sections were prepared as for histology. Frozen sections were fixed in PFA for 5 min at RT and then washed with PBS. Permeabilization was performed with 0.3% (w/v) Triton X-100 for 2 min on ice and then samples were washed with PBS. Positive controls were treated with 100U DNase I for 30 min at 37°C. Apoptosis was assayed via a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (In Situ Cell Death Detection Kit; Roche Diagnostics Canada, Laval, QC, Canada) according to the manufacturer’s protocol. Images were acquired using a Nikon E800 fluorescence microscope. The fluorescence-positive areas were analyzed by Image J software.
2.10. Examination of vessel development in the postnatal mouse retina

The globe was removed from PND2 pups and fixed in 4% (w/v) PFA in PBS for 30 mins, then rinsed in PBS. The retina was extracted and stored in methanol at -20°C. Retinas were washed in PBS and blocked in 5% (w/v) bovine serum albumin (BSA)/PBS for 30 min at RT. Retinas were incubated with 1:300 anti-collagen IV in BSA/PBS overnight at 4°C. Samples were washed in PBS and incubated with 1:400 Alexa Fluor 488-conjugated anti-rabbit IgG and 1:300 Alexa Fluor 594 conjugated Isolectin GS-IB4 in BSA/PBS for 2 h at RT. Retinas were whole-mounted and imaged with a Images were acquired using a Nikon E800 fluorescence microscope. For analysis of vessel lengths, the distance from the optic nerve head to the most distal point on a single vessel selected in each of eight sectors: 45°, 90°, 135°, 180°, 225°, 270° and 315° was measured using a radial grid with ImageJ software. The mean distance was calculated for each retina. The dissection of the retina from the globe, whole-mounting and image analysis was performed by Xu (Christine) Zhao in Dr. Shelley Boyd’s laboratory at St. Michael’s Hospital.
2.11. **Endothelial cell culture**

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in endothelial basal medium plus growth supplements (EGM-2; Lonza, Basel, Switzerland) in a humidified incubator at 37°C with 5% CO₂.
2.11.1. **Endothelial cell proliferation assay**

HUVECs were seeded into wells of a 96-well plate in 100μL of EGM-2 media. After 24h, cells were incubated with fresh media containing 1:25 naïve β3⁻ sera, anti-β3 sera or anti-GPIbα sera. After 12h, cells were incubated with 20μL of 5mg/mL thiazolyl blue tetrazolium bromide (MTT) for 3.5h. The supernatant was aspirated and cells were incubated with DMSO for 15mins. Absorbance was read at 570nm, with a reference wavelength of 630nm, on an optical plate reader (BioTek Instruments, Winooski, VT, USA). The extent of cell proliferation was determined by subtracting the value obtained at the reference wavelength from the absorbance at 570nm.
2.11.2. **Endothelial cell vascular-like tube formation assay**

Wells of a 24-well plate were coated with 50μL Matrigel. HUVECs were mixed with mouse sera in EGM-2 media and then added to coated wells at 50,000 cells/mL. Cells were incubated overnight and then imaged with an inverted microscope under phase-contrast. Tube formation was quantitated by counting the number of enclosed ring structures.
2.12. Statistical analysis

Data are presented as mean ± SEM and were analyzed using a one-way analysis of variance (ANOVA) test with post hoc comparisons using Tukey’s test. A $P$-value of <0.05 was considered significant.
CHAPTER 3: Results

3.1. Anti-β3 integrin or anti-GPIbα antibodies were generated after immunizing β3−/− or GPIbα−/− female mice with wild-type mouse platelets

β3−/− and GPIbα−/− female mice were immunized twice with weekly transfusions of WT platelets. Following platelet immunization, IgG antibodies to platelet antigens were detected by allowing sera to bind WT platelets. Bound antibodies were detected with a FITC-conjugated anti-mouse IgG via flow cytometry. The mice were immunoresponsive to platelet antigens and anti-platelet antibody was detected following 2 weekly transfusions of WT platelets (Figure 3.1).
Figure 3.1. β3−/− and GPIbα−/− mice were immunoresponsive to wild-type platelets.

The flow cytometry histograms display cell count (events) on the y-axis and FITC fluorescence on a logarithmic scale on the x-axis. WT mouse platelets were incubated with a 1:100 dilution of sera from immunized β3−/− mice (red line), GPIbα−/− mice (blue line) or their preimmune sera (black), followed by detection with FITC-conjugated anti-mouse IgG. Anti-platelet antibody was detected in both strains of mice following two weekly transfusions of WT platelets.
3.2. Reduced platelet counts of pups delivered by immunized $\beta 3^{-/-}$ and GPIb$\alpha^{-/-}$ mice

Following immunization with WT platelets, $\beta 3^{-/-}$ and GPIb$\alpha^{-/-}$ mice were bred with WT males to generate the murine models of FNIT. Naïve $\beta 3^{-/-}$ and GPIb$\alpha^{-/-}$ female mice (no platelet transfusions) were bred with WT males as controls. Platelet counts were detected in pups at PND2. Platelet counts were lower in pups delivered by immunized $\beta 3^{-/-}$ and GPIb$\alpha^{-/-}$ mice compared to their respective naive controls ($P<0.001$; Figure 3.2).
Figure 3.2. Reduced platelet counts of pups delivered by immunized β3⁻/⁻ and GPIbα⁻/⁻ mice.

Blood was collected from pups delivered by naive β3⁻/⁻ mice (n=65), naive GPIbα⁻/⁻ mice (n=15), immunized β3⁻/⁻ mice (n=15), and immunized GPIbα⁻/⁻ mice (n=8). Platelets were counted via a Coulter Counter. Platelet counts were lower in pups delivered from immunized β3⁻/⁻ and GPIbα⁻/⁻ mice compared to their respective naive controls (P<0.001).
3.3. Intracranial hemorrhage in anti-β3 integrin-mediated FNIT pups

Heads from anti-β3 integrin-mediated FNIT, anti-GPIbα-mediated FNIT and naive control pups were H&E stained. ICH was observed in anti-β3 integrin-mediated FNIT pups (3/15, 20%), but not in anti-GPIbα-mediated FNIT pups (0/8) or naive controls (0/65), as shown in Figure 3.3.
Figure 3.3. ICH was observed in pups delivered by immunized β3−/− mice.

Paraffin-embedded heads of pups were coronally-sectioned and H&E stained. ICH was observed in anti-β3 integrin-mediated FNIT pups (3/15; arrow indicated), but not in naive controls (0/65) or anti-GPIbα mediated FNIT pups (0/8).
3.4. Increased apoptosis in the brain of anti-\( \beta \)3 integrin-mediated FNIT pups

The brain of anti-\( \beta \)3 integrin-mediated FNIT, anti-GPIb\( \alpha \)-mediated FNIT and naive control pups were analyzed for apoptosis via a TUNEL assay. Increased apoptosis was detected in the brain of anti-\( \beta \)3 integrin-mediated FNIT pups compared to naive controls (\( P<0.01 \)) or anti-GPIb\( \alpha \)-mediated FNIT pups (\( P<0.05 \)), as shown in Figure 3.4. These results were confirmed by immunostaining the brain for active Caspase 3, another marker of apoptosis (Figure 3.4.1).
Figure 3.4. Increased apoptosis was observed in the brain of anti-β3 integrin-mediated FNIT pups via TUNEL.

Heads of pups were snap-frozen and sectioned. Apoptosis was detected via a TUNEL assay. Positive control was treated with DNaseI. No terminal deoxynucleotidyl transferase enzyme was added to the negative control. Positive fluorescence area was quantitated using ImageJ. Increased apoptosis was detected in the brain of anti-β3 integrin-mediated FNIT pups compared to naive controls \((P<0.01)\) and anti-GPlβα mediated FNIT pups \((P<0.05)\). Results are representative of at least 3 independent experiments \((n=3-6\) mice per group).
Figure 3.4.1. Increased apoptosis was observed in the brain anti-β3 integrin-mediated FNIT pups via immunostaining for active Caspase 3.

Heads of pups were snap-frozen and sectioned. Apoptosis was detected via immunostaining for active Caspase 3. Positive fluorescence area was quantitated using ImageJ. Increased apoptosis was detected in the brain of anti-β3 integrin-mediated FNIT pups compared to naive controls and anti-GPIbα mediated FNIT pups ($P<0.05$). Results are representative of at least 3 independent experiments (n=3-5 mice per group).
3.5. Reduced vessel density in the brain anti-β3 integrin-mediated FNIT pups

Heads of anti-β3 integrin-mediated FNIT, anti-GPIbα-mediated FNIT and naive control pups were stained with an anti-von Willebrand factor antibody to detect blood vessels. Reduced vessel density in the brain of anti-β3 integrin-mediated FNIT pups was observed compared to anti-GPIbα-mediated FNIT or naive control pups ($P<0.05$), as shown in Figure 3.5.
Heads of pups were snap-frozen and sectioned. An antibody to von Willebrand factor was used to stain blood vessels. Positive fluorescence area was quantitated using ImageJ. Anti-β3 integrin-mediated FNIT pups had reduced blood vessel density compared to anti-GPIbα mediated FNIT pups or naive controls (P<0.05). Results are representative of at least 3 independent experiments (n=3-6 mice per group).
3.6. Decreased retinal vascularization in anti-β3 integrin-mediated FNIT pups

Globes were removed from anti-β3 integrin-mediated FNIT, anti-GPIbα-mediated FNIT and naive control pups and the retina was extracted. Retinal whole-mounts were incubated with an anti-mouse collagen IV antibody or isolectin GS-IB4 to visualize the blood vessels. Vessel development in the retina of anti-β3 integrin-mediated FNIT pups was significantly inhibited compared to naive controls or anti-GPIbα-mediated FNIT pups ($P<0.001$), as shown in Figure 3.6.
Figure 3.6. Decreased vascularization in the retina of anti-β3 integrin-mediated FNIT pups. The globe was removed from pups and the retina was mounted. The retina was immunostained for collagen IV and imaged via a fluorescent microscope. The mean vessel lengths were quantitated. Vessel development in the retina of pups from anti-β3 integrin-mediated FNIT pups was significantly impaired compared to naive controls or anti-GPIIbα mediated FNIT pups ($P<0.001$). Results are representative of at least 3 independent experiments (n=3-6 mice per group).
3.7. **Effects of anti-β3 integrin sera on in vitro angiogenesis**

To further elucidate the effects of anti-β3 integrin antibodies on angiogenesis, studies were performed with endothelial cells in culture.

3.7.1. **Anti-β3 integrin sera decreased endothelial cell proliferation**

The effects of anti-β3 integrin antibodies on endothelial cell proliferation were investigated using an MTT assay. HUVECs were incubated with sera from immunized β3⁻/⁻ mice, immunized GPIbα⁻/⁻ mice or naïve controls. Following incubation for 12h, the MTT assay was performed (Figure 3.7.1). Anti-β3 integrin sera significantly inhibited HUVEC proliferation compared to naive sera ($P<0.05$).
Figure 3.7.1. Anti-β3 integrin sera inhibits endothelial cell proliferation.

HUVECs were seeded into wells of a 96-well plate. After 24h, cells were incubated with fresh media containing 1:25 naive control sera, anti-β3 integrin sera or anti-GPIbα sera. After 12h, cells were incubated with a MTT solution for 3.5h. Supernatant was aspirated and cells were incubated with DMSO for 15mins. Absorbance was read at 570nm (with a reference wavelength of 630nm) on an optical plate reader. Anti-β3 integrin sera significantly inhibited HUVEC proliferation compared to naive control sera ($P<0.05$). Results are pooled from 4 independent experiments performed with triplicate samples for each group.
3.7.2. Sera from immunized β3−/− mice inhibited vascular-like tube formation by endothelial cells

HUVECs were seeded into Matrigel coated wells and incubated overnight in EGM-2 media with dilutions of sera from immunized β3−/− mice, immunized GPIbα−/− mice and naïve controls. Tube formation was quantified by counting the number of enclosed ring structures. While HUVECs in the presence of sera from naive mice or immunized GPIbα−/− mice readily formed tubes under these conditions, this process was inhibited by the addition of anti-β3 integrin sera in a dose-dependent manner (Figure 3.7.2).
**Figure 3.7.2. Anti-β3 integrin sera inhibits vascular-like tube formation by endothelial cells.** HUVECs were seeded into Matrigel-coated wells in EGM-2 media and incubated with sera from naive or immunized mice as indicated. Cells were incubated overnight and imaged via a phase-contrast microscope. HUVECs treated with naive sera or anti-GPIbα sera formed tubes, while anti-β3 integrin sera inhibited this process in a dose-dependent manner. Tube formation was quantitated by counting the number of enclosed ring structures. * indicates $P<0.05$ when compared to either naive control sera or anti-GPIbα sera. Results are representative of at least 3 independent experiments for each group.
CHAPTER 4: Discussion

In this study, we investigated whether the cross-reaction of anti-β3 integrin antibodies with angiogenic endothelial cells leads to vascular injury and/or impairment of angiogenesis and contributes to the pathogenesis of FNIT. To mimic FNIT observed in human patients, β3 integrin- or GPIbα-deficient female mice were transfused with WT platelets and bred with WT male mice to generate heterozygous fetuses. We found that pups in both groups had reduced platelet counts, but ICH was only observed in neonates targeted by anti-β3 integrin antibodies. Anti-β3 integrin-mediated FNIT pups had increased apoptosis in the brain. Blood vessel development was examined by immunostaining both the brain and retinal vasculature and anti-β3- (but not anti-GPIbα-) mediated FNIT reduced vascularization of both organs. In addition, anti-β3 sera inhibited both proliferation and capillary-like tube formation by endothelial cells in vitro. Therefore, anti-β3 integrin antibodies in FNIT likely target blood vessels of the developing fetus/neonate and impair angiogenesis, thus contributing to the pathogenesis of this disease.
4.1. Vascular injury and anti-angiogenic effects in a murine model of β3 integrin mediated FNIT

FNIT is an alloimmune disorder associated with significant fetal and neonatal morbidity and mortality. The most feared complication of the disease is ICH, which occurs in approximately 10-20% of affected neonates. ICH may lead to death in 10% and neurological impairments in 10-20% of these cases. Analogous to ITP in adults, the major antigen targeted in FNIT is platelet surface αIIbβ3 integrin; however, the frequency of ICH in FNIT is 10-100 times greater than in ITP. Since platelet destruction is thought to be the major contributor to bleeding in FNIT, it is unclear whether the more severe bleeding observed in FNIT is due to a cross-reaction of maternal antibodies with fetal angiogenic endothelial cells, which causes vascular injury and synergizes with the low platelet count. On platelets, the β3 integrin subunit is frequently expressed with the αIIb subunit (αIIbβ3, ~80,000 copies per platelet, the most abundant platelet surface protein), but it can also pair with the αv subunit (αvβ3, a few hundred copies per platelet). However, αvβ3 integrin is also expressed on vascular endothelial cells during angiogenesis and antagonists of this integrin lead to apoptosis of angiogenic vascular cells.

In White populations, incompatibility of HPA-1a on β3 integrin is responsible for approximately 75% of FNIT cases. Studies suggest that ICH in FNIT patients is most frequently associated with incompatibility of HPA-1 and expression of HPA-1 on human endothelial cells has been reported. Therefore, it is possible that anti-platelet HPA-1 antibodies cross-react with angiogenic endothelial cells of the developing fetus/neonate and contribute to pathogenesis. Although our model using mice deficient for the entire β3 integrin subunit may be more similar to isoimmunization than alloimmunization, it closely recapitulated the clinical indices of
There are at least nine HPAs reported throughout the extracellular portion of \( \beta_3 \) integrin\(^{33,56,57} \) and regardless of race, polymorphisms in \( \beta_3 \) integrin are a major cause of FNIT.\(^{51,53-55} \) In addition, isoantibodies have been detected in mothers with Glanzmann thrombasthenia (deficiency of \( \alpha_{IIb}\beta_3 \) integrin), which may be implicated in FNIT.\(^{58} \) Therefore, our murine model of \( \beta_3 \) integrin-mediated FNIT is a useful model to study the pathogenesis of the disease and mechanisms of action of various treatments.\(^{72,104,105} \)

We demonstrated in our murine model of FNIT, that pups targeted by anti-\( \beta_3 \) integrin antibodies have increased apoptosis in the brain (Figures 3.4 and 3.4.1) concomitant with reduced blood vessel density (Figure 3.5). Therefore, anti-platelet \( \beta_3 \) integrin antibodies in our FNIT model may bind fetal angiogenic endothelial cells and lead to vascular injury and/or anti-angiogenic effects. However, platelets contain pro-angiogenic molecules, including VEGF,\(^{157,158} \) platelet-derived growth factor and basic fibroblast growth factor,\(^{159} \) and are involved in pathological angiogenesis.\(^{150-154} \) Therefore, thrombocytopenia itself may impair angiogenesis during fetal development. To control for the possible effects of thrombocytopenia on angiogenesis in our model of FNIT, we employed a GPIb\( \alpha \)-mediated FNIT model. GPIb\( \alpha \) is a platelet surface antigen targeted in FNIT that is not expressed on the endothelium, although some have reported expression under specific conditions\(^{10-13} \) but this remains controversial.\(^{14,15} \) In the GPIb\( \alpha \)-mediated FNIT model, we did not observe a difference in apoptotic cells or vessel density in the brain when compared to naive controls (Figures 3.4, 3.4.1 and 3.5), despite similar levels of thrombocytopenia (Figure 3.2). This suggests that the enhanced apoptosis and the reduced vessel density in the brain of FNIT pups targeted by anti-\( \beta_3 \) integrin antibodies (Figures 3.4, 3.4.1 and 3.5) may be due to direct effects of the antibodies on endothelial cells and not as a result of the thrombocytopenia.
Interestingly, ICH was only observed in β3 integrin-mediated FNIT pups (3/15, 20%), whereas no ICH was observed in the GPIbα-mediated FNIT model (0/8) (Figure 3.3). Since the brain requires extensive angiogenesis during development, injury to endothelial cells may impair angiogenesis and lead to ICH. Indeed it has been demonstrated that impairment of angiogenesis during development can result in fetal hemorrhage, particularly in the brain. Kisucka J et al. showed that platelets stimulate angiogenic blood vessel growth and are critical for preventing hemorrhage from the angiogenic vessels. However, subsequent papers from the same group clarified that thrombocytopenia alone was not sufficient to induce hemorrhage, but the presence of inflammation, specifically tissue-infiltration of leukocytes, was required to cause hemorrhage in thrombocytopenic mice. This is consistent with earlier studies from other groups indicating that low platelet counts alone do not cause bleeding. In one study, Shivdasani RA et al. generated mice deficient in the hematopoietic subunit of the transcription factor NF-E2, which completely lacked circulating platelets. Surprisingly, these mice survived in utero and no hemorrhage was observed in neonates delivered by Cesarean section. This is consistent with our results in the GPIbα-mediated FNIT model where despite thrombocytopenia (Figure 3.2), no ICH was detected in pups. In FNIT patients, up to 80% of ICH occur in utero, suggesting that ICH may not be due to the trauma of birth in a thrombocytopenic neonate. Furthermore, ICH occurs in some FNIT patients despite near normal platelet counts. Since ICH was only observed in β3 integrin-mediated FNIT pups, it is possible that anti-β3 integrin antibodies bind to angiogenic endothelial cells and impair angiogenesis leading to ICH.

In the anti-β3 integrin mediated FNIT model, pups had a significant reduction in vascularization of the retina when compared to naive controls or GPIbα-mediated FNIT pups (Figure 3.6). The murine retina is an excellent system to investigate angiogenesis because the vasculature develops...
over the first two postnatal weeks and is relatively small and thin, allowing it to be visualized in whole-mounted retinas. In addition, the vascular development and patterning of the murine retina parallels that of humans.\textsuperscript{164} Our results are consistent with a previous study in which impaired vascular development was observed in the retina of pups injected postnatally with a peptide inhibitor of both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins.\textsuperscript{120} Therefore, it is likely that the anti-$\beta_3$ integrin antibodies in our FNIT model cross-react with endothelial cells and inhibit angiogenesis in the developing fetus/neonate. Interestingly, in a study comparing sibling pairs with FNIT, where the first child was untreated and the younger sibling received antenatal treatment with IVIG and/or corticosteroids, untreated siblings had significantly more vision problems that were not related to ICH.\textsuperscript{165} This suggests that anti-angiogenic effects in FNIT may irreversibly damage the retina and affect patients’ vision. Since in humans the development of retinal vessels begins before midgestation and is nearly complete by birth,\textsuperscript{166} the fetus is exposed to the pathogenic antibodies for a greater proportion of development and could have more detrimental effects.

Although FNIT is thought to be antibody-mediated, we cannot exclude the possibility that there is a direct cytotoxic effect of T cells, as has recently been demonstrated in ITP.\textsuperscript{167-170} In a mouse model, Le T et al. showed that maternal T cells could not only traffic to the fetus, but could limit engraftment of allogeneic stem cell transplants.\textsuperscript{171} Therefore, maternal T cells reactive to fetal platelet antigens could cross the placenta and contribute to the pathogenesis of FNIT. However, after birth, the duration of thrombocytopenia depends on the rate of removal of the anti-platelet antibody from the neonatal circulation\textsuperscript{172} and thrombocytopenia will usually spontaneously resolve within 7 to 10 days.\textsuperscript{173} Since maternal cells have been shown to persist in humans well into adult life,\textsuperscript{174} it is unlikely that cytotoxic effects of maternal T cells are playing a significant role in mediating thrombocytopenia or other pathology in FNIT.
4.2. Anti-β3 integrin antibodies impair in vitro angiogenesis

In order to directly assess the effects of anti-platelet β3 integrin antibodies from our FNIT model on angiogenic endothelial cells, we performed in vitro angiogenesis assays. We observed that anti-β3 integrin sera significantly inhibited endothelial cell proliferation compared to naive control sera (Figure 3.7.1). In addition, using a vascular-like tube formation assay, we determined that anti-β3 integrin sera inhibited tube formation by endothelial cells in a dose-dependent manner, while sera from naive control mice or immunized GPIbα-/- mice had no effect on tube formation (Figure 3.7.2). Interestingly, lower doses of anti-β3 integrin sera seemed to slightly enhance tube formation compared to naive control sera, although the results were insignificant. It has been demonstrated that low concentrations of RGD-mimetic αvβ3/αvβ5 integrin inhibitors may stimulate angiogenesis by altering αvβ3 integrin and VEGFR-2 recycling within the cell. Whether similar mechanisms are responsible for the enhanced tube formation observed in response to low doses of anti-β3 integrin sera in our model requires further investigation.

van Gils JM et al. demonstrated that maternal antibodies to HPA-1a reduced endothelial cell spreading and monolayer integrity. In contrast, a previous study by Radder CM et al. showed that maternal anti-HPA-1a antibodies had no effect on endothelial cell activation or monolayer integrity. The discrepant results regarding the effects of anti-HPA-1a antibodies on endothelial cell monolayer integrity could be explained by the greater sensitivity of the technique used by van Gils JM et al. to measure this parameter. However, it has been demonstrated that there is a heterogeneity in binding of anti-HPA-1a antibodies to the antigen. For some antibodies, binding required only an intact amino-acid terminus while others bound more efficiently when other structural requirements within the entire glycoprotein were intact. This heterogeneity in
antibody binding to HPA-1a may affect the function of β3 integrin. This may account for the different results obtained in assays studying the effects of anti-HPA-1a antibodies on endothelial cell function. Furthermore, heterogeneity in antibody binding could explain the variability in the natural history of FNIT, such as variation in the extent of thrombocytopenia and occurrence of ICH.
4.3. Future Studies

4.3.1. Effect of maternal IVIG treatment on vascular injury and anti-angiogenic effects in β3 integrin-mediated FNIT

IVIG is widely used as antenatal therapy for FNIT, but optimal treatment regimens remain to be determined. In addition, the mechanism(s) of action of IVIG in the treatment of immune-mediated diseases is still uncertain. In our murine model of anti-β3 integrin-mediated FNIT, maternal administration of IVIG was able to reduce anti-β3 integrin antibody in the maternal and neonatal circulation, alleviate thrombocytopenia and reduce bleeding and mortality in pups. We recently determined that the mechanism of action of IVIG in our model involved both FcRn-dependent and -independent pathways. Interestingly, in FNIT patients, IVIG seems to offer protection against ICH, even with the persistence of severe thrombocytopenia. Therefore, if vascular injury and anti-angiogenic effects (and not thrombocytopenia) lead to ICH in FNIT, IVIG may be able to prevent these effects. IVIG can be administered to immunized β3-/- mice during pregnancy, and vascular injury and anti-angiogenic effects can be compared to untreated pregnancies. Preliminary data from our lab suggest that maternal administration of IVIG is able to restore angiogenesis in our model of anti-β3 integrin-mediated FNIT, but this requires further study.
4.3.2. Anti-angiogenic effects in pups directly injected with anti-β3 integrin sera

To determine whether the impaired retinal vascularization observed in our anti-β3 integrin-mediated FNIT model was directly due to the effects of the antibodies (rather than a general inhibition of fetal development), pups can be directly injected with anti-sera after birth. Pups heterozygous for β3 integrin can be randomly assigned to one of three groups and marked by tattooing the foot pad, as described.180 Pups can be injected intraperitoneally with either naive control sera, anti-β3 integrin sera or anti-GPIbα sera and retinal vascularisation can be evaluated. Preliminary results suggest that direct administration of anti-β3 integrin sera inhibits retinal angiogenesis and leads to ICH and neonatal mortality (data not shown).
4.3.3. Effects of anti-β3 integrin antibodies on placental vasculature in β3 integrin-mediated FNIT

In our murine model of β3 integrin-mediated FNIT, we observed some spontaneous fetal demise occurring around embryonic day 15.5 and when the uterus was extracted, fetuses appeared much smaller than naive controls of the same gestational age (data not shown), suggesting that intrauterine growth restriction (IUGR) may occur. Placental development includes extensive angiogenesis in fetal placental tissues and abnormal placental angiogenesis has been linked with the development of compromised pregnancies, such as IUGR. In addition, invasive trophoblasts adopt a vascular phenotype during placentation, expressing many adhesion receptors characteristic of endothelium, including αvβ3 integrin. Therefore, the role of anti-β3 integrin in placental angiogenesis/development and its potential involvement in IUGR in our β3 integrin-mediated FNIT model deserves further investigation.

Placental vascular development can be examined via evaluation of hemodynamics with ultrasound biomicroscopy (UBM) and analysis of vascular branching and vessel configuration with micro-CT (collaborations with Dr. Lee Adamson and Dr. John Sled). UBM can be employed to measure fetal and placental sizes and doppler velocity waveforms can be recorded in the umbilical, uterine and placental circulations to evaluate volume blood flow, as described. Micro-CT can be used to visualize the 3-dimensional vasculature of the placenta via a vascular corrosion casting method. Imaging data can be obtained using a micro-CT scanner and vessel diameters, lengths, number of branches, surface areas, and volumes can be quantified. Preliminary experiments suggest that these techniques are feasible with mice from our β3 integrin-mediated FNIT model. Effects of anti-β3 integrin antibodies on trophoblast
function can be examined in culture with trophoblast cell lines and co-cultures of trophoblasts and endothelial cells.
4.3.4. β3 integrin signalling and cross-activation of VEGFR-2 in β3 integrin-mediated FNIT

It has been demonstrated that engagement of αvβ3 integrin with its ligands (e.g. vitronectin) is involved in the full activation of VEGFR-2 mediated by VEGF. In addition, crosstalk between the two receptors has been shown to determine the response of endothelial cells to both VEGF and integrin ligation and is required for successful angiogenesis. Therefore, the anti-angiogenic effects observed in our murine model of β3 integrin-mediated FNIT may be due to defective cross-activation of VEGFR-2 resulting from inhibition of β3 integrin. Expression of activated αvβ3 integrin and VEGFR-2 can be evaluated in neonatal tissue by immunostaining and interaction of these receptors can be evaluated by co-localization or co-immunoprecipitation from lysed tissues followed by Western blotting. Phosphorylation of these receptors can be evaluated by Western blotting in order to determine whether activation is impaired by anti-β3 integrin antibodies in our FNIT model. These studies will help elucidate the mechanisms involved in the anti-angiogenic effects observed in our murine model of β3 integrin-mediated FNIT.
4.4. Conclusions

We investigated whether the cross-reaction of anti-β3 integrin antibodies with angiogenic endothelial cells leads to vascular injury and/or impairment of angiogenesis in FNIT. Using murine models of β3 integrin- or GPIbα-mediated FNIT, we observed that while both types of antibodies caused thrombocytopenia in pups, ICH was only observed in anti-β3 integrin-mediated FNIT. Anti-β3 integrin-mediated FNIT pups had increased apoptosis and reduced vessel density in the brain, when compared to naive control or GPIbα-mediated FNIT pups. Whether ICH results from injury to developing blood vessels in the brain by anti-β3 integrin antibodies remains to be determined. We observed that anti-β3 integrin-mediated FNIT pups had reduced vascularization of the retina compared to naive control or thrombocytopenic GPIbα-mediated FNIT pups. This suggests that anti-β3 integrin antibodies impair angiogenesis in FNIT pups independent of thrombocytopenia. Furthermore, we determined that anti-β3 integrin sera impaired endothelial cell proliferation and capillary-like tube formation in vitro. Therefore, anti-β3 integrin antibodies in FNIT likely cross-react with blood vessels of the developing fetus/neonate and impair angiogenesis, thus contributing to the pathogenesis of the disease.
CHAPTER 5: References


