The Role of Anti-CD36 Antibodies in Immune Mediated Thrombocytopenia

Inauguraldissertation
zur Erlangung des Grades eines Doktors der Medizin (Zahnmedizin oder Humanbiologie)
des Fachbereichs Medizin
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Aus dem Fachbereich Medizin der Justus-Liebig-Universität Gießen

Institut für Klinische Immunologie und Transfusionsmedizin

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1. General Introduction

Platelets are small enucleated blood cells generated by megakaryocytes in the bone marrow and play key roles in the hemostatic process. In addition, recent studies have indicated the versatility of platelets, which play important roles in various conditions, such as inflammation, immune responses, cancer, angiogenesis, lymphatic vessel development, and atherosclerosis (Li et al., 2012; Murphy et al., 2013; Semple et al., 2011). The platelet count usually remains fairly stable throughout a normal human life, and the normal range of platelets in circulation is 150–400 $\times 10^9$/L. However, when platelet counts reach below 150,000/μL, thrombocytopenia bleedings may occur. Thrombocytopenia can be caused by several factors, including platelet underproduction, sequestration, consumption, or destruction through non-immune or immune-mediated mechanisms. Among these, immune-mediated thrombocytopenia results from antibody-mediated platelet destruction leading to several bleeding disorders, including autoimmune thrombocytopenia (ITP), drug-induced thrombocytopenia (DITP), heparin-induced thrombocytopenia (HIT), and alloimmune thrombocytopenias, such as platelet transfusion refractoriness (PTR), fetal and neonatal alloimmune thrombocytopenia (FNAIT), and post-transfusion purpura (PTP).

Alloimmune thrombocytopenia occurs due to *allo*antibodies against polymorphic structures being expressed on the platelet surface, such as ABO blood group antigens, as well as HLA class I and human platelet antigens (HPAs). Meanwhile, more than 30 different HPAs have been identified (https://www.versiti.org/medical-professionals/precision-medicine-expertise/platelet-antigen-database/hpa-gene-database). Among them, *allo*antibodies against HPA-1a formed by point mutation (Leu33Pro) on platelet glycoprotein (GP) IIIa (known as β3 integrin), are responsible for the majority of cases of alloimmune thrombocytopenia in Caucasians (Mueller-Eckhardt et al., 1989). However, how FNAIT is caused by anti-HPA-1a antibodies is not well understood in other populations.

Interestingly, recent accumulated data has indicated that immune-mediated thrombocytopenias caused by anti-CD36 antibodies (also known as anti-Nak^a) are frequently found in Asian and African populations (Wu et al., 2017; Xu et al., 2013; Curtis et al., 2002; Lee et al., 1999). However, little is currently known about the

clinical relevance of anti-CD36 antibodies in Asian and African populations. In this study, we aimed to determine the frequency of CD36 deficiency among Chinese, characterize the molecular basis of these CD36 deficiencies, develop suitable methods for the identification of CD36 defects and anti-CD36 antibodies, identify cases of immune-mediated thrombocytopenia caused by anti-CD36 antibodies, and develop an animal model to understand the patho-mechanism of anti-CD36 mediated thrombocytopenia and treatment approaches thereof.

1.1. CD36

Cluster of differentiation 36 (CD36) is an 88 kDa glycoprotein that is widely expressed on a variety of cells, such as monocytes, macrophages, platelets, endothelial cells, and epithelial cells (Greenwalt et al., 1992). It belongs to the class B scavenger receptor family, which binds many different ligands, including collagen, thrombospondin, oxidized phospholipids (Ox-PL), oxidized low-density lipoprotein (ox-LDL), and long-chain fatty acids (Nergiz-Unal et al., 2011; Yang et al., 2017) (**Figure 1**).

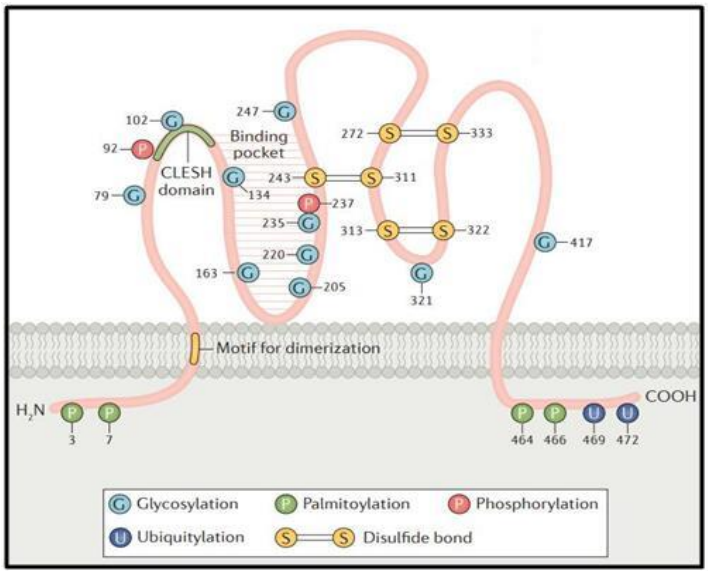

Figure 1: Multifunctional CD36 receptor

CD36 has two transmembrane domains and two short cytoplasmic tails that contain four palmitoylation sites. The extracellular loop contains ten N-linked glycosylation sites and two phosphorylation sites. A variety of ligands (such as thrombospondin, Ox-LDL, Ox-PL, long-chain fatty acid) and antibodies bind to CD36 via the hydrophobic binding pocket. This figure is adapted from *Yang X et al, Nat Rev Nephrol. 2017, 13(12):769-81*.

Recent studies have demonstrated that the binding of ox-LDL to platelet CD36 promotes platelet signaling, resulting in hyperactive platelets (Podrez et al., 2007). Furthermore, CD36 binds *Plasmodium falciparum*-infected erythrocytes, *Staphylococcus*, and *Mycobacterium* bacterial components (Febbraio et al., 2001). Accordingly, it is involved in diverse physiological and pathological processes, including thrombosis/hemostasis, atherogenesis, the innate immune defense, and diabetes (Silverstein et al., 2010; Cabrera et al., 2014; Ni et al., 2012; Zhu et al., 2012).

CD36 deficiency was first reported in a Japanese patient who developed platelet transfusion refractoriness caused by anti-CD36 *iso*antibodies (termed anti-Nak^a antibody) (Ikeda et al., 1989; Tomiyama et al., 1990). CD36 deficiency can be divided into two subgroups: type I, in which neither platelets nor monocytes express CD36, and type II, in which only platelets lack CD36 expression (Yamamoto et al., 1994). Moreover, it has been suggested that individuals with type I deficiency may be at risk of developing anti-Nak^a *iso*antibodies after receiving transfusions or during pregnancy (Yamamoto et al., 1994). Anti-CD36 antibodies can lead to serious immune-mediated thrombocytopenia, such as FNAIT, PTR, and PTP (Kankirawatana et al., 2001; Curtis et al., 2002; Fujino et al., 2001; Bierling et al., 1995). However, the exact mechanism of anti-Nak^a mediated disorders is not fully understood.

Although several cases of immune mediated thrombocytopenia caused by anti-CD36 antibodies have been reported in Japan (Ikeda et al., 1989; Fujino et al., 2001; Ogata et al., 2005; Okajima et al., 2006; Taketani et al., 2008), the clinical impact of these antibodies in Chinese populations has only recently been recognized (Xu et al., 2013; Xia et al., 2014; Wu et al., 2014 & 2017).

Besides immune thrombocytopenia, anti-CD36 antibodies can also cause platelet activation associated with non-hemolytic transfusion reactions (NHTRs) and hemolytic uremic syndrome (HUS) (Wakamoto et al., 2005; Rock et al., 2005). Interestingly, transfusion-related acute lung injury (TRALI) associated with anti-CD36 antibodies was recently reported (Nakajima et al., 2008).

1.2. The Frequency of CD36 Deficiency

Population studies have shown that the incidence of CD36 deficiency varies between different ethnic groups. CD36 deficiency is very rare in Caucasians (<0.3%) and is more common in African and Asian populations (4–8%) (Yamamoto et al., 1994; Curtis et al., 1996; Lee et al., 1999). The frequency of CD36 deficiency in different populations is shown in **Table 1**.

Table 1: Frequency of CD36 deficiency in various populations

Population	Number of individuals	Type I + Type II (%)	Type I (%)	References
Chinese				
Guangzhou	998	1.80% (18/998)	>0.5% (>5/998)	Xu X et al, 2013
Zhejiang	192	3.65% (7/192)	0.0% (0/192)	Xu X et al, 2014
Shanghai	1022	2.15% (22/1022)	0.2% (2/1022)	Li R et al, 2014
Guangxi	4425	4.27% (189/4425)	>1.06% (>47/4425)	
Han	3036	3.59% (109/3036)	>0.96% (>29/3036)	Zhong Z et al, 2014
Zhuang	1389	5.76% (80/1389)	>1.30% (>18/1389)	Zhong Z et al, 2014
Total	6637	3.56% (236/6637)	>0.81% (>54/6637)	
Japanese	135	8.15% (11/135)	1.5% (2/135)	Masuda Y et al, 2015
Indonesian	168	4.17% (7/168)	NT	Santoso S et al, 1993
Malaysian	200	2.00% (4/200)	NT	Xia W et al, 2016
Taiwanese	640	1.56% (10/640)	0.63% (4/640)	Lo SC et al, 2016
Thai	700	2.14% (15/700)	0.43% (3/700)	Rhuangtham R et al, 2020
Koreans	55	3.64% (2/55)	NT	Xia W et al, 2016
African	206	7.77% (16/206)	2.91% (6/206)	Lee K et al, 1999
Arabian	1328	2.64% (35/1328)	NT	Flesch BK et al, 2021

>: not all type I and type II individuals (CD36 negative on platelets) presented in the third column were tested for the absence of CD36 on monocytes to determine type I deficiency (CD36 negative on platelets and monocytes). Therefore, the percentage of type I deficiency was estimated as > as the numbers presented here. N.T. not tested.

A higher frequency of CD36-deficient individuals (type I and type II) was recently reported in Japan (8.15%) (Masuda et al., 2015). In China, the frequency of CD36 deficiency in platelets was about 3.56% (1.80–4.27%), similar to that in Korea (3.64%) (Xu et al., 2013; Xu et al., 2014; Li et al., 2014; Zhong et al., 2014). Lower frequencies were found in Malaysia (2.0%), Taiwan (1.56%), and Thailand (2.14%) (Xia et al., 2016; Lo et al., 2016; Phuangtham et al., 2020). However, a higher incidence of CD36 deficiency was found in the Chinese *Zhuang* ethnic group living in South China in Guangxi province (5.76%) (Zhong et al., 2014). The frequency of type I-deficient individuals in the *Han* and *Zhuang* ethic groups was about 0.5% and 1.3% lower than that in Japanese (1.50%), respectively (Xu et al., 2013; Zhong et al., 2014; Masuda et al., 2015). Recently, the prevalence of CD36 deficiency in platelets in an Arabian population living in Germany was reported to be 2.64% (Flesch et al., 2021).

1.3. Molecular Basis of CD36 Deficiency

The 46 kilobase gene encoding CD36 is located on chromosome 7q11.2 and is encoded by 15 exons (Fernandez-Ruiz et al., 1993; Armesilla et al., 1996) (**Figure 2**). However, only part of exon 3, exon 4 to 13, and part of exon 14 encode the CD36 protein (Armesilla et al., 1994). The remaining exons (exons 1, 2, and 15) form the 5′ and 3′ untranslated regions (UTRs), respectively.

The expression of the CD36 gene is complex and reflects the multifunctional role of CD36 in different tissues and conditions (Andersen et al., 2006). There are 5 alternative first exons (exons 1a, 1b, 1c, 1e, and 1f; **Figure 2**) that are controlled by 3 alternative promoters (Armesilla et al., 1994; Sato et al., 2002; Andersen et al., 2006). Exon 1a is most highly transcribed in adipose tissue, followed by heart, skeletal muscle, and liver tissue, while the transcript containing exon 1b is higher in adipose tissue compared to other tissues (Andersen et al., 2006). In addition, the functional diversity of the CD36 gene can also be generated by an alternative splicing mechanism (Omi et al., 2003).

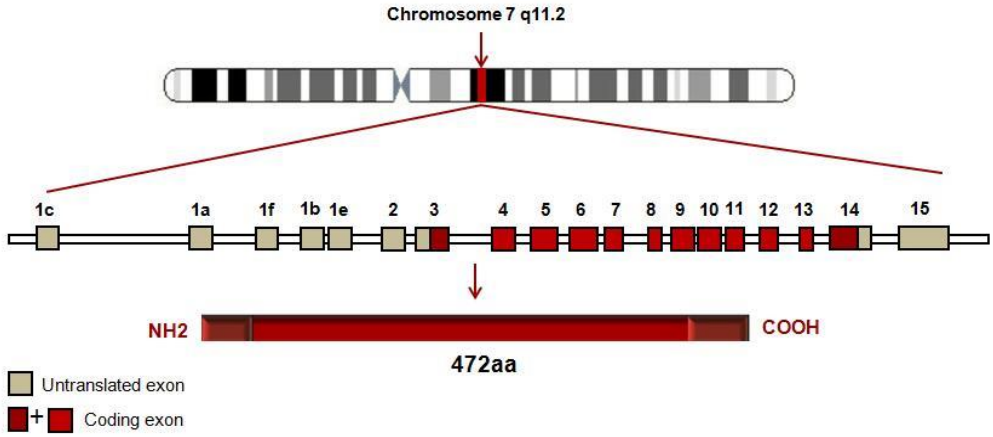

Figure 2: Schematic representation of human CD36 gene

To date, more than 30 mutations due to nucleotide substitutions/deletions, short insertions, duplications, or nucleotide rearrangements in the coding region of CD36 gene have been described as responsible for type I and type II CD36 deficiency (Li et al., 2014). Homozygous or compound heterozygous mutations in the CD36 gene associated with the total absence of CD36 surface expression on platelets and monocytes have been discovered. **Table 2** shows 35 mutations located in exons and introns of the CD36 gene associated with type I and type II deficiencies found in various populations. Three common mutations, C268T, 949*ins*A, and 329–330 *del*AC, have been identified in Japanese individuals, and of these, the C268T nucleotide substitution is the most frequent mutation (>50%) (Kashiwagi et al., 2001). In contrast, 329–330 *del*AC and 1228–1239 *del*ATTGTGCCTATT represent the most common mutations in Chinese population (Xu et al., 2013; Li et al., 2014). In Thailand, 1163A > T seems to be the most frequent mutation associated with CD36 deficiency (Phuangtham et al., 2020). Due to the heterogeneity of these mutations, a practicable genotyping approach for identifying individuals with CD36 deficiency has been difficult to develop.

Table 2: Genetic variation of CD36 responsible for CD36 deficiency in various populations

Exon	mRNA	Amino	Nucleotide change	Amino acid change	References
3	-89 to +120	1-40	G14A	Arg5Gln	Li R et al,2014
4	121-281	41-94	C268T	Pro90Ser	Kashiwagi H et al, 1993
			121-126*del*gCAAGTT	unknown	Xu X et al, 2013
			C220T	Gln74Term	Xu X et al, 2013
			198-205*del*GATCTTTG	frameshift at AA66	Xia W et al, 2016
5	282-429	94-143	329-330*del* AC	frameshift at AA 110	Kashiwagi H et al,1994
			C371T	Pro124Leu	Li R et al, 2014
			C380T	Ser127Leu	Omi K et al, 2003
			T410C	Val137Ala	Imai M et al, 2002
			429+4*ins*g	exon 6 skipping	Xu X et al, 2013
6	430-609	144-203	T538C	Trp180Arg	Li L et al, 2016
			560*ins*T	frameshift at AA 187	Tanaka T et al, 2001
7	610-701	204-234	619-624*del*ACTGCA/*ins*AAAAC	frameshift at AA 207	Kashiwagi H et al, 2001
			691-696*del*AAAGGT	inframe del AA 231-232	Atiman TJ et al, 2000
8	702-748	234-250	T737A	Ile246Asn	Xia W et al, 2016
9	749-818	250-273	T760C	Phe254Leu	Hanawa H et al, 2002
			771*del*T	frameshift at AA 285	Liu J et al, 2020
10	819-1006	273-336	949*ins*A	frameshift at AA 317	Kashiwagi H et al, 1996
			T975G	Tyr325Term	Aitman TJ et al, 2000
11	1007-1125	336-375	T1079G	Leu360Term	Lepretre F et al, 2004
			C1123T	Pro375Ser	Liu J et al, 2020
12	1126-1199	376-400	*Del* tttagAT	skipping exon 12	Tanaka T et al, 2001
			T1142G	Leu381Term	Liu J et al, 2020
			G1150C+1155*del*A	Ala384Pro+frameshift at AA 385	Atiman T et al, 2000
			C1156T	Arg386Trp	Okajima et al, 2006
			A1163T	Gln388Leu	Li R et al, 2014
13	1200-1254	400-418	*Del*tattacagAG	skipping exon 13	Tanaka T et al, 2001
			1200-5*inv*49bp	exon13 skipping	Xu X et al, 2013
			*dupl.*1204-1246	frameshift at AA 416	Tanaka T et al, 2001
			1218-1224 *del*GAGGAAC	frameshift at AA 406	Kashiwagi H et al, 2001
			1228-1239 *del*ATTGTGCCTATT	deletion of Ile-Val-Pro-Ile410-413	Tanaka T et al, 2001 Kashiwagi H et al,2001
			A1226G	Tyr409Cys	Li R et al, 2014
14	1255-1419	419-472	1236*del*T	frameshift at AA 413	Liu J et al, 2020
			A1237C	Ile413Leu	Hanawa H et al, 2002
			C1409T	Thr470Ile	Liu J et al, 2020

*GenBank NM001001548; The first mRNA nucleotide encoding CD36 protein is +1.

In comparison to CD36 type I deficiency, the molecular or genomic background of CD36 type II is not clearly understood. Kashiwagi H and colleagues hypothesized that silencing of a "platelet-specific allele" may responsible for the controlling of CD36 expression in platelets (Kashiwagi et al., 1995). However, no specific DNA sequence corresponding to such a platelet-specific silent allele has been identified so far.

1.4. Laboratory Diagnostic Tests for CD36 Antigens and Antibodies

During the last two decades, different approaches have been developed to detect platelet-specific alloantibodies, including in-house assays such as monoclonal antibody-specific immobilization of platelet antigens (MAIPA), the platelet suspension immunofluorescence test (PSIFT), and commercial assays such as the solid-phase assay (PakPlus) and a Luminex bead-based platelet antibody detection assay (Pak Lx) (Porcelijn et al., 2014). However, all of these methods can have some drawbacks. False-negative results often occur in MAIPA due to the competitive inhibition between the capture mouse monoclonal antibodies and human anti-CD36 antibodies. PSIFT is a simple and fast flow cytometry-based method. However, it requires fresh CD36-deficient platelets as a negative control to exclude the presence of other platelet-reactive antibodies in serum samples (e.g., anti-HPA or anti-HLA antibodies). Commercial kits such as PakPlus and PakLx are expensive and require special instruments, and false-negative results caused by the destruction of antigen during the purification process cannot be ruled out.

Additionally, transfected cell lines stably expressing CD36 antigen have been introduced for the detection of CD36 antibodies by flow cytometry (Hayashi et al., 2009). Compared to the MAIPA approach, however, a flow cytometry-based method cannot completely exclude the false-positive reactions that may occur between antibodies and other platelet proteins besides CD36 antigen. Recently, a monoclonal antibody independent antigen capture assay (ACA) has been successfully developed for the detection of alloantigen against human neutrophil antigen 3 (HNA-3). Through this approach, recombinant HNA-3 antigens harboring the V5-peptide (GKPIPNPLLGLDST) were expressed on the surface of mammalian HEK293 cells (Bayat et al., 2012). After incubation of these cells with human sera, bound anti-HNA-3 antibodies could be detected in the cell lysates after immobilization on a microtiter plate pre-coated with anti-V5 antibodies. This approach overcomes the problems of using

fresh neutrophils and prevents the false-negative reactions caused by competitive inhibition by monoclonal antibodies as mentioned above. Such a method is also attractive to develop in the near future for reliable detection of anti-CD36 antibodies. However, testing of CD36 expression both on platelets and on monocytes by flow cytometry is necessary to dissect the real target of anti-CD36 antibodies.

1.5. Immune-mediated Thrombocytopenia caused by CD36 Antibodies

Immune-mediated thrombocytopenia is a common cause of bleeding disorders and is responsible for <15% of cases; however, the pathomechanisms of immune-mediated thrombocytopenia are still not fully understood. Recently, several lines of evidence have indicated that both innate and adaptive immune responses are involved in the mechanism of platelet clearance. However, humoral immune responses mediated by anti-platelet antibodies seem to be a major cause (Lazarus et al., 2013; Li et al., 2013; Ni et al., 2006). Until recently, *allo*antibodies against more than 30 HPAs have been identified, and anti-HPA-1a is the most important factor for alloimmune thrombocytopenia in populations of European ancestry. However, alloimmunization against HPA-1 is hardly present in Asian populations. Recently, the first case of FNAIT due to anti-HPA-1a antibodies was found in a blood-related Chinese family (Armawai et al., 2015). In contrast, immune-mediated thrombocytopenia caused by anti-CD36 antibodies is more commonly reported in Chinese populations (Xu et al., 2013; Wu et al., 2017). In **Table 3**, cases of immune thrombocytopenia mediated by CD36 antibodies reported in Asia, including 6 cases of FNAIT and 12 cases of PTR, are summarized. The majority of patients (16/18) were immunized either by transfusions or by transplantation (12 cases) or as a result of pregnancy (4 cases). In China, most cases of immune mediated-thrombocytopenia caused by CD36 antibodies occur in Guangxi province, which has a higher frequency of CD36-deficient residents than other provinces. The reason for the accumulation of CD36-deficient individuals in this region is currently unknown.

Table 3: CD36-type I deficiency and immune-mediated thrombocytopenia in Asian populations

Subjects	Age/ Sex	Population	Diagnosis	No. of transfusions	No. of pregnancies	Cases	References
P1	22/M	Chinese	T- lymphoblastic tumour	Multiple	N/A	PTR	Xu X et al, 2013
P2	30/F	Chinese	Pregnancy	0	4	FNAIT	Xu X et al, 2013
P3	11/M	Chinese	Acute leukemia	Multiple	N/A	PTR	Xia W et al, 2014
P 4	35/F	Chinese	Myelodysplastic syndromes	Multiple	3	PTR	Xia W et al, 2014
P 5	35/F	Chinese	Pregnancy	1	7	FNAIT	Xia et al, 2014
P6	46/M	Chinese	Alcohol cirrhosis	Multiple	N/A	PTR	†
P7	5/M	Chinese	Acute leukemia	1	N/A	PTR	†
P8	7/M	Chinese	Comminuted fractures	Multiple	N/A	PTR	Yin X et al, 2011
P9	26/F	Chinese	Acute leukemia	2	1	PTR	Wu G et al, 2014
P10	21/F	Chinese	Pregnancy with bleeding	4	2	PTR	Wu G et al, 2014
P 11	63/F	Chinese	Acute leukemia	Multiple	2	PTR	Wu G et al, 2014
P 12	?/F	Thai	Pregnancy	0	1	FNAIT	Kankirawatana S, et al, 2001
P13	36/F	Japanese	Acute myeloid leukemia	Multiple	0	PTR	Ikeda H, et al, 1989
P14	19/M	Japanese	Seminoma	Multiple	N/A	PTR	Fujino H, et al, 2001
P15	47/M	Japanese	HBV-related liver cirrhosis	Liver transplant	N/A	PTR	Ogata T, et al, 2005
P 16	?/F	Japanese	Pregnant	?	3	FNAIT	Taketani T et al, 2008
P17	36/F	Japanese	Pregnant	0	2	FNAIT	Okajima S et al, 2006
P18	26/F	Taiwanese	Pregnant	0	2	FNAIT	Lin M et al, 2018

† Unpublished case which found in Guangzhou Blood Centre; ?: Unknown; HBV: Hepatitis B virus; Multiple: the number of transfusions is more than four times.

1.5.1. Fetal and Neonatal Alloimmune Thrombocytopenia (FNAIT)

FNAIT, as a life-threatening bleeding disorder, is the most common cause of severe thrombocytopenia in live born neonates (Bussel et al., 2008). In Caucasians, FNAIT occurs in approximately one per 1,000 births, and the majority (~80%) of cases is

caused by incompatibility in the human platelet antigen (HPA-1) between the mother and fetus (Mueller-Eckhardt et al., 1989; Kjeldsen-Kragh et al., 2007). An antigen-negative mother becomes immunized by an antigen-positive fetus and develops maternal *allo*antibodies against the human platelet antigen expressed on fetal platelets (HPA). These maternal *allo*antibodies can cross the placenta via the neonatal Fc receptor (FcRn) during pregnancy, enter fetal circulation, bind fetal platelets, and cause fetal thrombocytopenia (**Figure 3**).

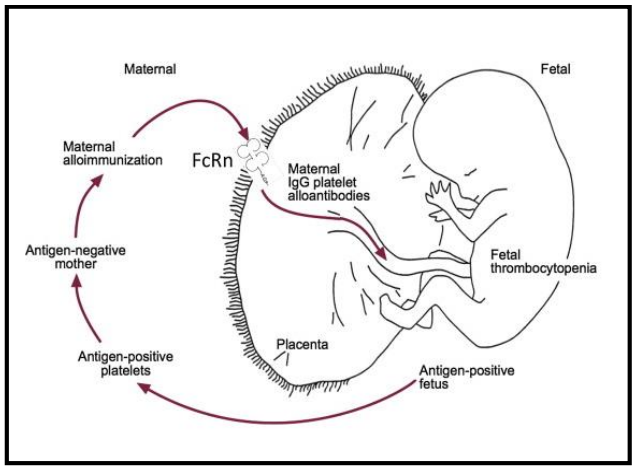

Figure 3: Pathogenesis of fetal and neonatal alloimmune thrombocytopenia

Following exposure to fetal platelets, a mother becomes immunized and develops maternal antibodies against human platelet antigens which expressed on fetal platelets. These maternal antibodies can pass through the placenta via the FcRn and cause platelet destruction, leading to fetal thrombocytopenia. This figure is adapted from *Blanchette VS, Johnson J and Rand M, Baillieres Best Pract Res Clin Haematol. 2000, 13(3):365-90.*

The manifestations of FNAIT vary from asymptomatic thrombocytopenia discovered incidentally and minor bleeding, i.e., petechial or purpura, to intracranial hemorrhage (ICH) (Peterson et al., 2013). However, the most severe clinical complications of FNAIT, such as ICH in full-term infants, can lead to death or persistent neurological sequelae in approximately 10% of clinically symptomatic cases (Herman et al., 1986; Bussel et al., 2009; Tiller et al., 2013). It is currently unclear which factors determine whether ICH will occur or not. Several strategies have been developed to reduce the incidence of ICH, such as weekly high-dose intravenous immunoglobulin (IVIG) and intrauterine platelet transfusion (IUPT). The recurrence rate of ICH in subsequent pregnancies was reported to be 79% (Radder et al., 2003). Therefore, early

identification of ICH is mandatory for antenatal management to reduce the risk of morbidity and mortality in subsequent pregnancies.

Until recently, little was known about the relevance of platelet antibodies in the development of FNAIT in China. In contrast to Caucasians, several studies have demonstrated that the gene frequency of HPA-1b is low among Asian populations (Tanaka et al., 1996; Nie et al., 2010). Accordingly, immunization against HPA-1a is very rare in China (Wu et al., 2017). However, FNAIT caused by anti-CD36 antibodies seem more common than other drivers of anti-HPAs antibodies in China (Wu et al., 2014). Similar to what was previously shown; *allo*antibody-mediated FNAIT cases the degree of thrombocytopenia caused by anti-CD36 *iso*antibodies vary widely from moderate to severe. ICH has also been reported to co-occur. However, hydrops and recurrent miscarriages seem the most devastating risks of FNAIT caused by anti-CD36 when compared with anti-HPA-1a antibodies (Okajima et al., 2006; Xu et al., 2018; Wu et al., 2020). The reason for this phenomenon is currently unknown, and consequently, a treatment strategy for FNAIT based on the clinical experience with anti-HPA-1a cannot be easily adapted for anti-CD36 antibodies.

1.5.2. Platelet Transfusion Refractoriness (PTR)

PTR is defined as the repeated failure to achieve satisfactory responses to platelet transfusion, and the response is assessed by measuring the post-transfusion platelet count increment. Many causes, immune and nonimmune, can lead to platelet refractoriness. Antibodies against human leucocyte antigen (anti-HLA) are the most common cause (>80%) of PTR. However, platelet-specific *allo*antibodies or platelet-specific *iso*antibodies (such as anti-Nak^a) alone or in combination with anti-HLA antibodies, can cause PTR. In Asia, several cases of PTR caused by anti-Nak^a have been reported (**Table 3**). Transfusion of HLA- and/or HPA-matched donor platelets is the best management for PTR (Hod et al., 2008). For those patients with PTR caused by anti-CD36 antibodies, transfusion with CD36-deficient platelets is the method of choice for preventing bleeding (Fujino et al., 2001).

1.5.3. Post-transfusion Purpura (PTP)

PTP is a rare but serious complication of blood transfusion, characterized by the sudden onset of severe thrombocytopenia (platelet counts often less than 10×10^9/L), usually within 7–10 days of a blood transfusion. Its incidence is approximately 1 in 50,000–100,000 blood transfusions, and it usually affects multiparous women. This disorder is mediated by *allo*antibodies against specific platelet antigens, commonly HPA-1a (Padhi et al., 2013). Other HPAs and CD36 have also been implicated in this life-threatening thrombocytopenia (Vu and Leavitt, 2018; Bierling et al., 1995). However, the mechanism of destruction of the patient's own platelets remains uncertain. Administration of immunoglobulin (IVIG) with and without corticosteroids has been used as a first-line therapy (Padhi et al., 2013).

1.6. Current Therapies for FNAIT

During the last two decades, significant improvements have been made in the diagnosis and management of FNAIT, but existing therapies and prophylactic treatments have limitations. In contrast to the Rhesus D-associated hemolytic disease of newborns, FNAIT may occur in a first pregnancy and affect the fetus as early as the 20^{th} week of gestation, so that antenatal management becomes challenging and necessary (Vadasz Brian et al., 2015; Bussel et al., 2008; Bussel et al., 2010). Fetuses are at high risk for an adverse outcome if their mothers gave birth to a FNAIT child. Thus, recognition of FNAIT and appropriate therapy are both important for the affected neonate and for the management of subsequent pregnancies (Sainio et al., 2000).

Currently, administration of IVIG to a pregnant woman, with or without additional steroids, and intrauterine platelet transfusion have been used to prevent FNAIT. However, these three options have limitations (Bussel et al., 2009). Although intrauterine platelet transfusion is effective in amelioration of neonatal thrombocytopenia, it cannot be recommended as a first-line treatment for FNAIT, mainly due to the short half-life of transfused platelets and the high risk of fetal loss (Birchall et al., 2003; Kamphuis et al., 2001). Immunoglobulins are effective in protecting a fetus from bleeding in the majority of FNAIT cases, and usually, 1 g/kg of IVIG is administered weekly starting from 16 to 20 on (Sachs, 2013). However, IVIG is an expensive multi-donor human blood product with dose-related side effects, including

severe headaches, fatigue, and myalgia (Salomon et al., 2013; Brojer et al., 2016). High doses of steroids as a sole treatment for FNAIT are controversial, but in combination with IVIG, they have resulted in improved clinical outcomes, especially when corticosteroids are administered during the last trimester (Serrarens-Janssen, et al., 2008; Bertrand et al., 2014). For full-term neonates with suspected FNAIT mediated by anti-HPA-1a, random platelet transfusion is required if they present with severe bleeding or a platelet count of less than 30×10^9/L, or administration of IVIG (1 g/kg/d) for 1–3 days (Salomon et al., 2013; Bertrand et al., 2014). Thrombocytopenia usually resolves in 2 weeks, although rare cases may persist for up to 6 weeks. Ultrasound examination is recommended for every newborn with significant thrombocytopenia to exclude ICH. In comparison to anti-HPA-1a antibodies, little is known about the management of FNAIT caused by anti-CD36 *iso*antibodies.

1.7. New Therapeutic Approaches for FNAIT

Nowadays, non-invasive treatment with IVIG represents the first line of antenatal therapy. However, the precise mechanism of IVIG action is still unclear (Winkelhorst et al., 2017). Several mechanisms have been proposed, including decreasing maternal antibody production due to the inducing of immune tolerance, increasing pathogenic antibody clearance, and antibody transport by saturated FcRn (Hansen et al., 2002; Liu et al., 2018; Wabnitz et al., 2020). In a model of FNAIT caused by anti-β3 antibodies, Ni et al. (2006) in Toronto evaluated the efficacy of IVIG therapy. They found that administration of IVIG during pregnancy could down-regulate anti-β3 antibodies in both maternal and foetal circulation via FcRn-dependent and -independent pathways (Ni et al., 2006; Chen et al., 2010). However, such strategies may inhibit the transfer of immune-protective maternal IgG, which may cause an increased risk of infections during pregnancy as well during the first weeks after birth (Mathiesen et al., 2013). Therefore, more specific and effective antenatal therapies are desirable.

To begin with, it was demonstrated in mouse models that anti-FcRn antibodies could decrease miscarriages and improve angiogenesis by inhibition of fetal FcRn (Zdravic et al., 2016; Yougbaré et al., 2017; Chen et al., 2019). FcRn is a pH-dependent FcγR that only binds IgG at acidic pHs (Roopenian and Akilesh, 2007). Following pinocytosis of endothelial cells into endosomes, IgG binds to FcRn (due to the acidic milieu) and

thereby escapes rapid degradation. The FcRn-IgG complex can be translocated back to the cell surface, and IgG can be released after dissociation from FcRn (due to the neutral milieu). This recycling process is responsible for the relatively long half-life of IgG. Consequently, inhibition by anti-FcRn leads to rapid IgG degradation and clearance from circulation (Roopenian and Akilesh, 2007). This effect reduces platelet antibodies, which has been shown in ITP patients treated with high doses of IVIG (Ling et al., 2019). Anti-FcRn antibodies may have two modes of action in FNAIT, namely, the reduction of maternal antibody concentration and the inhibition of FcRn-mediated transfer via the placenta into the fetus (Bussel et al., 2021). Currently, a clinical trial with the inhibitory anti-FcRn blocking mAb nipocalimab (M281) has been initiated for the therapy of hemolytic disease of the fetus and newborn (HDFN). Thus, it seems warranted to consider this therapy for FNAIT if this approach turns out to be effective for HDFN therapy (Bussel et al., 2021).

Secondarily, an attractive alternative strategy would be to administer a non-destructive IgG that shares the allospecificity of maternal antibodies and which is able to inhibit binding of a pathogenic maternal *allo*antibody to fetal platelets. Such a therapeutic agent would not trigger effector functions, as it is unable to interact with fetal FcγRs, but would still retain the ability to be transported across the placenta. Ghevaert et al. (2013) introduced such a strategy by the development of human recombinant anti-HPA-1a (termed B2G1Δnab). These modified mAbs against HPA-1a were able to pass the placenta and ease FNAIT in the babies of immunized mothers (Ghevaert et al., 2013 & 2008).

1.8. Deglycosylated Antibodies may be Effective in the Prevention of FNAIT

In recent years, the therapeutic potential of deglycosylated IgG (deg-IgG) antibodies for autoimmunity treatment has been widely recognized (Crispin, 2013). Removal of the N-glycan (linked to asparagines 297), located on the Fc part, leads to a significant reduction of IgG binding with FcγRs expression on macrophages and its ability to activate complement C1q. Interestingly, these deg-IgG antibodies can still be transported from maternal circulation to a fetus via FcRn (Nimmerjahn and Ravetch, 2008). Accordingly, our previous *in vivo* study in mice demonstrated that deg-mAbs specific for HPA-1a (mAb SZ21) could pass through the placenta and prevent the clearance of fetal platelets mediated by maternal anti-HPA-1a antibodies. This

observation indicated that the use of epitope-specific antibodies for the antenatal therapy of severe FNAIT was feasible (Bakchoul et al., 2013).

More recently, we tested the use of deg-anti-CD36 antibodies for the antenatal treatment of FNAIT caused by maternal anti-CD36 antibodies in a mouse model of FNAIT (**Figure 4**). The high frequency of fetal death could be significantly reduced (40% to 2.17%) by the antenatal administration (5 mg/kg body weight) of degylcosylated mouse mAb 32-106 (deg-32-106). However, similar antenatal treatments with IVIG administered at a dose of 1 g/kg body weight on days 10, 15, and 20 after breeding did not result in increased platelet counts and did not reduce fetal death rates (40.00%). Only IVIG administration to the immunized *Cd36*-/- mothers three days earlier (e.g., days 7, 12, and 17) reduced fetal death (40.00% to 12.70%) (Xu et al., 2021). Our results indicated that deg-32-106 could prevent the disturbance of placenta angiogenesis caused by maternal antibodies (Xu et al., 2021). Interestingly, deg-32-106 was shown to react to human CD36. Thus, development of humanized deg-32-106 as a drug to prevent severe FNAIT caused by anti-CD36 was shown to be feasible.

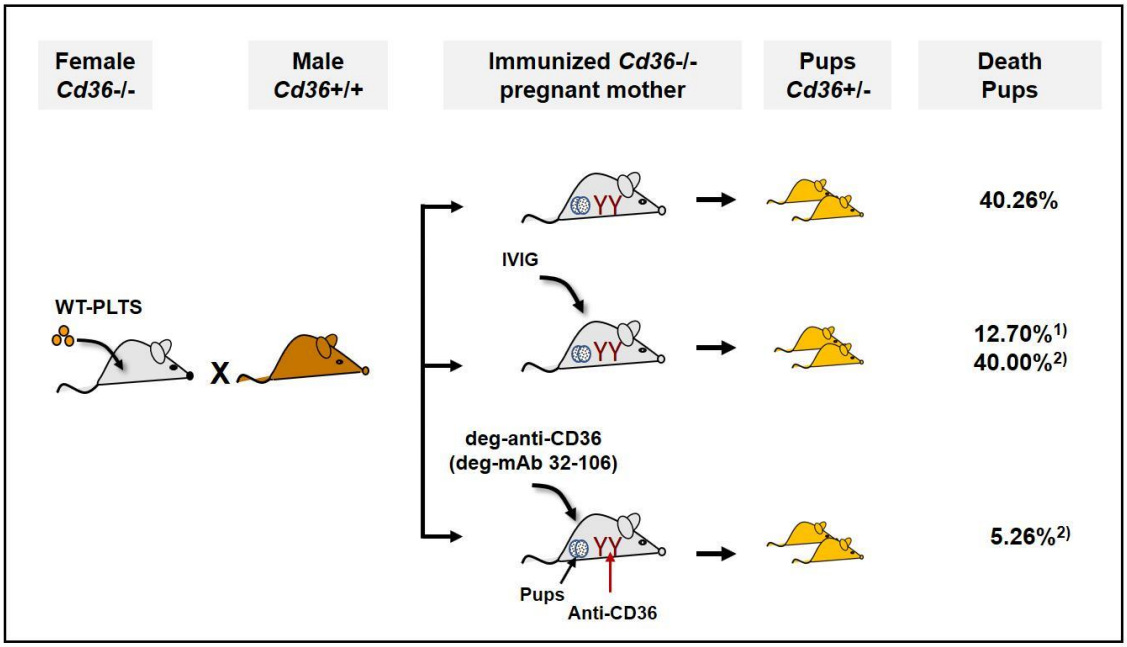

Figure 4：The efficacy of IVIG and deg-anti-CD36 for the antenatal treatment of anti-CD36-mediated FNAIT in a murine model

Immunized female *Cd36*-/- mice were immunized with wild-type platelets and crossed with wild-type male mice. Pregnant mothers were then untreated or treated with IVIG or deg-anti-CD36 three times 1) on days 7, 12 and 17, or 2) on days 10, 15 and 20. The number of dead pups was evaluated. This figure is adapted from *Xu et al, Blood. 2021,138(18):1757-67.*

2. Overview of the Manuscripts

Manuscript #1:

Author:

Xiuzhang Xu, Xin Ye, Wenjie Xia, Jing Liu, Haoqiang Ding, Jing Deng, Yangkai Chen, Yuan Shao, Jiali Wang, Yongshui Fu, Sentot Santoso

Title:

Studies on CD36 deficiency in South China: Two cases demonstrating the clinical impact of anti-CD36 antibodies

Journal:

Thrombosis and haemostasis

Thromb Haemost. 2013 Dec;110(6):1199-1206. doi: 10.1160/TH13-05-0435. Epub 2013 Aug 22. PMID: 23966019.

Impact Factor: 5.760

Summary of the Manuscript:

In publication "Studies on CD36 deficiency in South China: Two cases demonstrating the clinical impact of anti-CD36 antibodies", 998 healthy blood donors were typed for CD36 deficiency using flow cytometry. Nucleotide sequencing was performed to identify the molecular basis underlying the CD36 deficiency. Anti-Nak^a antibodies in CD36 deficient individuals were analysed by ELISA and flow cytometry. We found that more than 0.5% of CD36 type I deficient individuals are at risk to be immunized through blood transfusion or pregnancy in China. Testing of anti-Nak^a antibodies should be considered in FNAIT and PTR suspected cases. A registry of CD36 deficient donors should be established to allow treatment of immune-mediated bleeding disorders caused by anti-Nak^a antibodies.

Manuscript #2:

Author:

Wenjie Xia, Xin Ye, **Xiuzhang Xu**, Haoqiang Ding, Jing Liu, Jing Deng, Yangkai Chen, Yuan Shao, Jiali Wang, Hui Li, Yongshui Fu, Sentot Santoso

Title:

Two cases of platelet transfusion refractoriness and one case of possible FNAIT caused by antibodies against CD36 in China

Journal:

Transfusion medicine

Transfus Med. 2014 Aug;24(4):254-256. doi: 10.1111/tme.12137. PMID: 25124074.

Impact Factor: 1.308

Summary of the Manuscript:

In paper "Two cases of platelet transfusion refractoriness and one case of possible FNAIT caused by antibodies against CD36 in China", two cases of PTR and one possible FNAIT case caused by anti-CD36 antibodies were reported, and indicated further the clinical significance of anti-Nak^a antibodies in our population.

Manuscript #3:

Author:

Xiuzhang Xu, Lin Li, Wenjie Xia, Haoqiang Ding, Dawei Chen, Jing Liu, Jing Deng, Yangkai Chen, Zhiming He, Jiali Wang, Yuan Shao, Sentot Santoso, Xin Ye, Qun Fang

Title:

Successful management of a hydropic fetus with severe anemia and thrombocytopenia caused by anti-CD36 antibody

Journal:

International journal of hematology

Int J Hematol. 2018 Feb;107(2):251-256. doi: 10.1007/s12185-017-2310-5. Epub 2017 Aug 16. PMID:28815406.

Impact Factor: 2.251

Summary of the Manuscript:

In publication "Successful management of a hydropic fetus with severe anemia and thrombocytopenia caused by anti-CD36 antibody", one case of foetal and neonatal alloimmune thrombocytopenia (FNAIT) caused by anti-CD36 *iso*antibodies was reported. Serial intrauterine transfusions with red blood cells (RBC) and platelets from a CD36*null* donor were performed to improve the severe anemia and thrombocytopenia. This report suggests that intrauterine transfusions with compatible RBC and CD36*null* platelets are useful in preventing the deleterious clinical effects of anti-CD36-mediated severe FNAIT.

Manuscript #4:

Author:

Yongbin Wu, Dawei Chen, **Xiuzhang Xu**, Mingqin Mai, Xin Ye, Chengyao Li, Sentot Santoso, Wenjie Xia,Yongshui Fu

Title:

Hydrops fetalis associated with anti-CD36 antibodies in fetal and neonatal alloimmune thrombocytopenia: Possible underlying mechanism

Journal:

Transfusion medicine

Transfus Med. 2020 Oct;30(5):361-368. doi: 10.1111/tme.12705. Epub 2020 Jul 27.

PMID: 32720360.

Impact Factor: 2.019

Summary of the Manuscript:

In paper "Hydrops fetalis associated with anti-CD36 antibodies in fetal and neonatal alloimmune thrombocytopenia: Possible underlying mechanism", maternal anti-CD36 antibodies isolated from a case of FNAIT with signs of hydrops fetalis (HF) were incubated with haematopoietic stem cells (HSCs), and then the formation of burst-forming unit-erythroid and colony-forming unit-erythroid (CFU-E/BFU-E) cells was analyzed. In this study, we found that anti-CD36 antibodies could lead to a significant reduction in CFU-E/BFU-E cell formation, and this result was associated with an increased number of apoptotic CD34+ erythroid/myeloid precursor cells.

Manuscript #5:

Author:

Xiuzhang Xu, Dawei Chen, Xin Ye, Wenjie Xia, Yuan Shao, Jing Deng, Yangkai Chen, Haoqiang Ding, Jing Liu, Yaori Xu, Sentot Santoso, Yongshui Fu

Title:

Improvement of anti-CD36 antibody detection via monoclonal antibody immobilization of platelet antigens assay by using selected monoclonal antibodies

Journal:

Annals of Laboratory Medicine

Ann Lab Med. 2023 Jan;43(1):86-91. doi: 10.3343/alm.2023.43.1.86. Epub 2022 Sep 1. PMID: 36045061.

Impact Factor: 4.9

Summary of the Manuscript:

In manuscript "Improvement of anti-CD36 antibody detection via monoclonal antibody immobilization of platelet antigens assay by using selected monoclonal antibodies", we developed a panel of monoclonal antibodies against CD36, which do not easily compete with the human anti-CD36 antibodies. We found two hybridomas, termed GZ-70 and GZ-608, which could recognize all anti-CD36 antibodies tested so far. This significant improvement allows the identification of anti-CD36 antibodies by antigen capture assay.

Manuscript #6:

Author:

Marie Lin, **Xiuzhang Xu**, Hui-Lin Lee, Der-Cheng Liang, Sentot Santoso

Title:

Fetal/neonatal alloimmune thrombocytopenia due to anti-CD36 antibodies: antibody evaluations by CD36-transfected cell lines

Journal:

Transfusion

Transfusion. 2018 Jan;58(1):189-195. doi: 10.1111/trf.14369. Epub 2017 Oct 13. PMID: 29030871.

Impact Factor: 3.111

Summary of the Manuscript:

In paper "Fetal/neonatal alloimmune thrombocytopenia due to anti-CD36 antibodies: antibody evaluations by CD36-transfected cell lines", we reported the characterization of a patient with fetal/neonatal alloimmune thrombocytopenia in a Taiwanese family caused by anti-CD36 isoantibodies using a novel antigen-capture method. Analysis of maternal serum with CD36-transfected HEK293 cells by flow cytometry, immunoprecipitation, and antigen-capture assay could demonstrate the presence of anti-CD36 *iso*antibodies in maternal serum.

Manuscript #7:

Author:

Xiuzhang Xu, Sentot Santoso

Title:

Role of CD36 in immune-mediated thrombocytopenia in Asian populations

Journal:

ISBT Science Series

ISBT Science Series. 2018; 13, 317-322. First published: 14 February 2018.

https://doi.org/10.1111/voxs.12414.

Impact Factor: None

Summary of the Manuscript:

It has been suggested that type I cluster of differentiation 36 (CD36)-deficient individuals are at risk of developing anti-CD36 isoantibodies (also known as anti-Nak^{a}) after receiving platelet transfusions or during pregnancy. These antibodies are responsible for several immune thrombocytopenic disorders including fetal/neonatal alloimmune thrombocytopenia (FNAIT), post-transfusion purpura and platelet-transfusion refractoriness (PTR). In Asian populations, anti-CD36 isoantibodies are more frequently found in PTR and FNAIT patients compared to alloantibodies against human platelet antigens. In this short review, we illustrate the relevance of CD36 by providing information on the frequency and molecular basis of CD36 deficiency, laboratory diagnostic tests of CD36 antigens/antibodies and treatment of immune-mediated thrombocytopenia caused by anti-CD36 antibodies.

Manuscript #8:

Author:

Xiuzhang Xu, Dawei Chen, Xin Ye, Wenjie Xia, Yaori Xu, Yangkai Chen, Yuan Shao, Jing Deng, Haoqiang Ding, Jing Liu, Jiali Wang, Heyu Ni, Yongshui Fu and Sentot Santoso.

Title:

Successful prenatal therapy of anti-CD36-mediated severe FNAIT by deglycosylated antibodies in a novel murine model

Journal:

Blood

Blood. 2021 Nov 4;138(18):1757-1767. doi: 10.1182/blood.2021011131. PMID: 34041523.

Impact Factor: 22.113

Summary of the Manuscript:

In publication "Successful prenatal therapy of anti-CD36-mediated severe FNAIT by deglycosylated antibodies in a novel murine model", we established a novel mouse FNAIT model to evaluate the efficacy of deg-anti-CD36 to treat FNAIT caused by maternal anti-CD36 antibodies and compared this treatment with IVIG. Through this model, we found that maternal anti-CD36 antibodies caused a high frequency of fetal death, associated with placental dysfunction. This deleterious effect could be diminished by the antenatal administration of IVIG and deg-mAb 32-106. Interestingly, treatment with deg-32-106 appears more beneficial considering the lower dose, later start of treatment, and therapy success.

3. Manuscripts

3.1. Studies on CD36 deficiency in South China: Two cases demonstrating the clinical impact of anti-CD36 antibodies

Studies on CD36 deficiency in South China: Two cases demonstrating the clinical impact of anti-CD36 antibodies

Xiuzhang Xu[1,2*]; Xin Ye[1*]; Wenjie Xia[1]; Jing Liu[1]; Haoqiang Ding[1]; Jing Deng[1]; Yangkai Chen[1]; Yuan Shao[1]; Jiali Wang[1]; Yongshui Fu[1]; Sentot Santoso[2]

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Summary

CD36 (also known as GPIV) deficiency is known to be responsible for the production of anti-Nak[a] antibodies in different clinical settings such as fetal/neonatal alloimmune thrombocytopenia (FNAIT), platelet transfusion refractoriness (PTR) and post-transfusion purpura (PTP). However, no data regarding the relevance of CD36 immunisation is currently available for China. In this study, healthy blood donors were typed for CD36 deficiency using flow cytometry. Nucleotide sequencing was performed to identify the molecular basis underlying the CD36 deficiency. Anti-Nak[a] antibodies in CD36-deficient individuals were analysed by ELISA and flow cytometry. By analysis of 998 healthy blood donors, 18 individuals failed to express CD36 on their platelets. In 5/12 individuals no CD36 expression was detected both on platelets and monocytes. This result suggested that the frequencies of type I CD36 deficiency (platelets and monocytes) and type II CD36 deficiency (platelets only) are approximately 0.5 and 1.3%, respectively. Nucleotide sequencing analysis of type I CD36 deficient individuals revealed eight different mutations; four of them were not described so far. However, 1228–1239*del* ATTGTGCCTATT and 329–330*del*AC appear to be the most common mutations related to type I CD36 deficiency in South Chinese population. Further analysis showed that 1/5 type I CD36 deficient individuals developed anti-Nak[a] antibodies. In addition, anti-Nak[a] antibodies could be identified in two cases of thrombocytopenia associated with FNAIT and PTR. In conclusion, more than 0.5% of CD36 type I-deficient individuals are at risk to be immunised through blood transfusion or pregnancy in China. Testing of anti-Nak[a] antibodies should be considered in FNAIT and PTR suspected cases. A registry of CD36-deficient donors should be established to allow treatment of immune-mediated bleeding disorders caused by anti-Nak[a] antibodies.

Keywords

CD36, platelet glycoprotein IV, anti-Nak[a], gene mutation, neonatal alloimmune thrombocytopenia, platelet transfusion refractoriness

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Thromb Haemost 2013; 110: 1199–1206

Introduction

CD36, also known as platelet glycoprotein IV (GPIV), is a highly glycosylated 88-kDa protein and is expressed widely in different cells such as platelets, monocytes, macrophages, endothelial and epithelial cells. CD36 belongs to the class B scavenger receptor family, which binds many ligands including, collagen, thrombospondin, erythrocytes parasitised with *Plasmodium falciparum,* and oxidised low-density protein (1, 2).

CD36 deficiency was first reported in a patient who developed platelet-transfusion refractoriness caused by anti-CD36 isoantibody (termed anti-Nak[a] antibody) after multiple platelet transfusions (3, 4).The frequency of CD36 deficiency on platelets has been reported in 4-8% of Africans, about 3-4% of Japanese, 2.4% of African Americans and 0.3% of Caucasians (3, 5-8). Two types of CD36 deficiency are known: type I lacking CD36 surface expression on platelets and monocytes, and type II lacking CD36 on platelets only (6). It has been suggested that individuals with type I deficiency may be at risk of developing anti-Nak[a] isoantibodies

after receiving transfusions or during pregnancy (6). Meanwhile, more than 20 mutations underlying type I CD36 deficiency have been described. In contrast, the molecular basis of type II CD36 deficiency is still unclear (9).

The impact of anti-Nak^a antibodies has been reported in several clinical conditions of immune-mediated thrombocytopenia including, fetal/neonatal alloimmune thrombocytopenia (FNAIT), post-transfusion purpura (PTP) and platelet-transfusion refractoriness (PTR) (8, 10-13). Recently, Nakajima et al. (14) reported that anti-Nak^a antibodies are also responsible for the development of transfusion-related acute lung injury (TRALI).

Currently, the relevance of CD36 in immune-mediated platelet disorders among Chinese is not known. Here, we studied the frequency of GPIV deficiency in healthy blood donors in South China and describe two cases of CD36 immunisation associated with FNAIT and PTR.

Materials and methods

Study population

Blood donors

Blood samples from healthy blood donors (n = 998) were collected in Guangzhou Blood Center, China, consisting of 78.86% men and 21.14% women with an average age of 32.5 ± 9.5 years (range, 18-60 years). According to geographical dividing line of China (Qinling Mountain-Huaihe River line), our cohort comprises 88.48% southern and 11.52% northern Chinese populations.

Case 1

A 30-year-old mother (Mo) with no history of blood transfusion or transplantation experienced four abortions. The last pregnancy ceased with foetal death at 27 weeks of gestation. Autopsy of the foetus showed oedema of foetal skin with bleeding spots in the lower extremities, severe intracranial haemorrhage and hydrops fetalis. Screening of antibodies against erythrocytes by Coombs test was negative.

Case 2

A 22-year-old male patient (Li) with T lymphoblastic cell tumour received induction chemotherapy. After several platelet transfusions, he failed to respond to apheresis random donor platelet transfusion (24 hours post-transfusion CCI < 2,600).

Isolation of platelets

Platelets were isolated from 10 ml EDTA anti-coagulated blood. After centrifugation at 120 g for 30 minutes (min), the top 3/4 of platelet-rich plasma (PRP) was isolated, and washed three times with PBS/EDTA buffer (Dulbecco's Phosphate Buffered Saline containing 10 mM EDTA, pH 7.0-7.2; Gibco BRL). Finally, washed platelets were suspended in PBS/EDTA buffer and adjusted to a concentration to 1.0×10^5 platelets/µl.

Flow cytometry analysis of CD36 expression on platelets and monocytes

Aliquots of 50 µl platelets suspension were incubated with 10 µl fluorescein isothiocyanate (FITC)-labelled anti-human CD36 monoclonal antibody (mab FA6-152; Immunotech, Marseille, France) for 30 min at room temperature. After washings, labelled platelets were suspended in 1 ml PBS/EDTA buffer and analysed by FACS Canto II system (BD Biosciences, San Jose, CA, USA). For the analysis of CD36 expression on monocytes, 100 µl of EDTA anti-coagulated blood were incubated with 10 µl FITC-labelled mab FA6-152 and phycoerythrin (PE)-labelled anti-CD14 (mab RMO52, Immunotech) at room temperature for 20 min. Subsequently, 500 µl lysis solution (Optilyse C; Immunotech) was added for 10 min at room temperature to remove erythrocytes from cell suspension. Remaining cells were then washed twice with 4 ml PBS/EDTA buffer, resuspended in 500 µl PBS/EDTA buffer and analysed as described above.

Analysis of anti-CD36 antibodies against platelets

Flow cytometry

Platelets were isolated from group O blood donors as described above. Fifty µl of platelets suspension were incubated with 50 µl test serum for 30 min at 37°C. After washings with 4 ml PBS/EDTA buffer, platelets were incubated with 50 µl of FITC-labelled rabbit anti-human IgG (dilution 1:50; DAKO, Hamburg, Germany) for 30 min at room temperature. After washings, labelled platelets were suspended in 500 µl PBS/EDTA buffer and analysed by flow cytometer as described above.

ELISA

Anti-Nak^a antibodies were identified by the use of a commercial ELISA Kit (PAKPLUS, GTI Diagnostics, Waukesha, WI, USA) as recommended by the manufacturer. Fifty µl appropriate diluted serums (1:3) were added to microtitre wells coated with different platelet glycoproteins (HLA class I, GPIIb/IIIa, GPIa/IIa, GPIb/IX and GPIV). Bound antibodies were detected with alkaline phosphatase labelled anti-human IgG (dilution 1:100). In some cases, antibodies specificities were determined by the use of antigen capture ELISA (MACE; GTI Diagnostics).

Immunoprecipitation

Washed platelets (10^9 cells) were incubated with 1 ml sulfo-NHS-LC biotin (1 mg/ml; Thermo Scientific, Rockford, IL, USA) for 30 min at room temperature. Labeled cells were then resuspended in 2 ml lysis buffer. After centrifugation at 10,000 x g for 10 min, cell lysates were precleared for 30 min with 50 µl of 20% protein G-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) in the

presence of 30 µl normal human serum for 30 min. Aliquots of 50 µl precleared cell lysates were incubated with 50 µl AB serum (as negative control), anti-HPA-1a (as positive control) and patient's serum overnight at 4°C. Immunocomplexes were then washed five times with 10 mM Tris HCl at pH 7.4. Bound proteins were released by boiling in SDS buffer for 5 min at 95°C. After centrifugation at 10,000 x g for 2 min, samples were analysed on 7.5% SDS-PAGE under nonreducing conditions. Proteins were then transferred to PVDF membrane by blotting procedure. Membrane were incubated with 8 µl streptavidin horseradish peroxidase (1:8,000 dilution; Amersham Life Sciences) for 30 min at room temperature and visualized by using an enhanced ECL chemiluminescence kit (Calbiochem, Darmstadt, Germany).

Preclearing experiment

Aliquots of 50 µl biotin-labelled platelet lysates were incubated with 10 µl mab against CD36 for 45 min at 4°C, and precipitated with protein G as described above. After centrifugation, cell supernatant was incubated again with anti-CD36 mab and precipitation was repeated. After three times precipitation, cell lysates were incubated with 30 µl patient's serum for overnight at 4°C. Immunocomplexes were then washed five times with washing buffer (10 mM Tris HCl, pH 7.4). Bound proteins were released by boiling in SDS buffer for 5 min at 95°C. After centrifugation at 10,000 g for 2 min, samples were analysed by SDS-PAGE and immunoblot, as described above.

Nucleotide sequencing analysis

Genomic DNA was extracted from EDTA anti-coagulated blood using a QIA Mini kit (Qiagen, Hilden, Germany) and subjected to sequencing from exon 3 to exon 15. Each exon (primers were shown in ►Table 1) including a part of flanking introns was amplified with CD36 specific primers (reference sequence: NG_008192; see ►Table 1) under following conditions: denaturation (30 seconds [sec], 95°C), annealing (30 sec, 57°C), extension (1 min, 72°C) for 35 cycles, followed by a final extension (5 min, 72°C). PCR products of CD36 gene were purified using MultiScreen-Filt96 plate (Millipore, Carrigtwohil Co., Cork, Ireland). Purified PCR products and CD36 constructs were sequenced with an ABI BigDye Terminator cycle sequencing kit and 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Amplification of CD36 transcript

Total RNA was isolated from platelets using peqGOLD RNAPure FLTM (PEQLAB Biotechnologie, GmbH, Erlangen, Germany). cDNA was synthesised using Ready-To-GoTMYou-Prime Frist–Strand Beads (GE Healthcare, Buckinghamshire, UK) with pd(N)6 primer in a final volume of 33 µl and was amplified with PCR GPIV-F sense primer (5'-GGTGCTTAACACTAATTCACCTCC-3') and GPIV-R antisense primer (5'-TTTTATTGTTT TCGATCTGCATGC-3'). PCR products were purified by gel extraction (Qiagen), and cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen, Karlsruhe, Germany). Subsequently, positive clones were screened by PCR, and amplified plasmid was purified using a QIAprep Miniprep kit (Qiagen).

Results

Frequency of CD36 deficiency in China

When we screened the expression of CD36 on platelets by flow cytometry, 18 out of 998 individuals were negative for CD36 expression indicating the frequency of CD36-deficient individuals is around 1.8%. In order to identify the frequency of type I CD36-deficient individuals, monocytes derived from CD36-negative platelets donors were analysed. Among 12 tested individuals, five showed no CD36 expression on monocytes as well (►Table 2A). This result suggested that the frequencies of type I CD36-deficient (negative on platelets and monocytes) and type II CD36-deficient (negative on platelets only) in our cohort are approximately 0.5 and 1.3%, respectively.

Table 1: PCR and sequencing primers for CD36 gene.

Primer name for CD36	Primer sequence (5'→3')	PCR product
Exon3-F	ACAGGTGCTTAACACTAA	189bp
Exon3-R	GAGAAGGCATATTCTTTG	
Exon4-F	TTTTATTCTGGCTGACTC	335bp
Exon4-R	ATGACATTTGCCAAGTAG	
Exon5-F	CCTTCTCGTTAGTTTGCTA	392bp
Exon5-R	GATAGGATTAATTACATGAG	
Exon6-F	TTGTATTAAGCTCAATATTAGC	350bp
Exon6-R	ATAAAATTATGCCTTGCC	
Exon7-F	AAGTAACATTTTCCCATAC	187bp
Exon7-R	ATGAATACTATTCCTGCT	
Exon8-F	TGCAATAAGATAAAAGGTTC	356bp
Exon8-R	AATTTTGTTGTGGGGATA	
Exon9-F	ATGGACTACACTGGAGGAG	400bp
Exon9-R	CTGGACTTGATCGTTAATAGAC	
Exon10-F	CAGAATGTAAGTTCAGGTT	268bp
Exon10-R	GACTGTGCTACTGAGGTT	
Exon11-F	TAGACATATTACTGCCTGAA	485bp
Exon11-R	AGGAAGAAATCGACCTAA	
Exon12-F	CCTTAAGTTACTACCTTCTC	201bp
Exon12-R	AATAACCATTTTCAAGAG	
Exon13-F	TATTTCAGTTCCCCGAGA	463bp
Exon13-R	TTTGTTCAATTGGATCAT	
Exon14-F	CTTGCCTTATAGATACTG	470bp
Exon14-R	TACTTTAGTGATCTGCGT	

Molecular analysis of CD36 deficiency

To characterise the molecular base underlying CD36 deficiency, the entire coding region of CD36 gene was amplified by PCR (►Table 1), and was analysed by a direct sequencing approach. In nine individuals no mutation was identified (►Table 2A). Among seven type II CD36 deficient individuals, no mutation was found in six subjects. In only one (Donor 18), deletion of 12 nucleotides (1228-1239*del*ATTGTGCCTATT in heterozygous form) located in exon 13 leading to four amino acids deletion (Ile-Val-Pro-Ile; AA 410-413) was found. This deletion was also found in other four donors (Donors 2, 4, 10, and 16). Three of them are found as type I CD36 deficiency. Other

Table 2A: CD36 mutations found in 18 Chinese blood donors.

Subjects	Age/Sex	CD 36 Plt/Mo	Type	Mutations of CD36 gene	Change in amino acid	Reference
1	24/F	neg/n.t	n.d	-	-	-
2	22/M	neg/n.t	n.d	1228–1239*del*ATTGTGCCTATT	deletion of Ile-Val-Pro-Ile	15,19
3	24/F	neg/neg	I	1200–5*inv*49bp§	exon 13 skipping	*This study*
				429+4*ins*g§	exon 6 skipping	*This study*
4	38/F	neg/neg	I	1228–1239*del*ATTGTGCCTATT;	deletion of Ile-Val-Pro-Ile;	15,19
				121–126*del*gCAAGTT§	unknown	*This study*
5	29/M	neg/n.t	n.d	329–330*del*AC ;	frameshift at AA 110;	17
				C1156T	Arg386Trp	21
6	50/F	neg/neg	I	329–330*del*AC ;	frameshift at AA 110;	17
				C220T§	Gln74Term	*This study*
7	19/M	neg/pos	II	-	-	-
8	19/M	neg/pos	II	-	-	-
9	28/M	neg/n.t	n.d	-	-	-
10	31/M	neg/neg	I	1228–1239*del*ATTGTGCCTATT;	deletion of Ile-Val-Pro-Ile;	15,19
				329–330*del*AC	frameshift at AA 110	17
11	33/M	neg/n.t	n.d	-	-	-
12	49/M	neg/pos	II	-	-	-
13	25/M	neg/pos	II	-	-	-
14	30/M	neg/n.t	n.d	429+4*ins*g§	exon 6 skipping	*This study*
15	20/F	neg/pos	II	-	-	-
16	23/F	neg/neg	I	1228–1239*del*ATTGTGCCTATT;	deletion of Ile-Val-Pro-Ile;	15,19
				329–330*del*AC	frameshift at AA 110	17
17	25/M	neg/pos	II	-	-	-
18	35/F	neg/pos	II	1228–1239*del*ATTGTGCCTATT	deletion of Ile-Val-Pro-Ile	15,19

GenebankNM: 000072; the first mRNA nucleotide encoding CD36 protein is +1; n.t: not tested; n.d: not defined; -: not found; inv: inversion; del: deletion; ins: insertion; AA: amino acid; § new mutations.

Table 2B: Type I CD36 deficiency associated with anti-Nak^a isoantibodies.

Subjects	Age/Sex	CD 36 Plt/Mo	Type	Antibody Clinic	Mutations of CD36 gene	Change in amino acid	Reference
Donor 6	50/F	neg/neg	I	anti-Nak^a none	329–330*del*AC;	frameshift at AA 110;	17
					C220T§	Gln74Term	*This study*
Mo	30/F	neg/neg	I	anti-Nak^a FNAIT	C380T;	Ser127Leu;	22
					429+4*ins*g§	exon 6 skipping	*This study*
Li	22/M	neg/neg	I	anti-Nak^a PTR	Homozygous 329–330*del*AC	frameshift at AA 110	17

GenebankNM: 000072; the first mRNA nucleotide encoding CD36 protein is +1; *del*: deletion; *ins*: insertion; AA: amino acid; § new mutations.

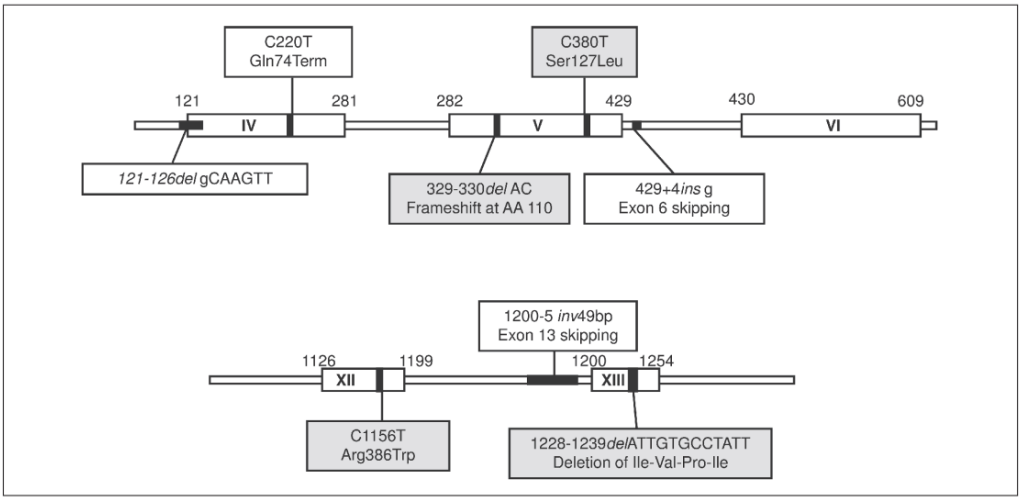

Figure 1: CD36 mutations found in Chinese blood donors. Mutations located in the region between exon IV – VI (upper panel) and exons XII – XIII (bottom panel) are shown. Grey boxes represent known mutations, white boxes new mutations. Nucleotides substitution, deletion (del.), insertion (ins.) and inversion (inv.) are indicated.

mutations associated with type I CD36 deficiency were found including 429+4*ins*g (Donors 3 and 14), 1200-5*inv*49bp (Donor 3), 121-126*del*gCAAGTT (Donor 4), C220T (Donor 6) and 329-330*del*AC (Donor 6, 10, 16) (►Figure 1 and ►Table 2A). 4/8 mutations (white boxes) have not been described so far. However, the most common mutations related to type I CD36 deficiency in China seem to be 1228-1239 *del*ATTGTGCCTATT and 329-330 *del*AC (►Table 2A). To study the molecular bases of the new mutations, we amplified cDNA derived from GPIV-defective platelets and cloned into pcDNA3.1/V5-TOPO vector. In Donor 6, analysis of the entire coding region of CD36 gene showed a compound heterozygous mutation, C220T and 329-330*del*AC located in exon 4, and exon 5, respectively. The nonsense mutation C220T led to premature stop codon (Gln74Term), whereas the two-nucleotide deletion, 329-330*del*AC, caused frame shift at amino acid residue 110 as previously reported by Kashiwagi et al. (17) (data not shown). In Donor 14, insertion of one nucleotide at fourth position of intron 5 (429+4*ins*g) was found, which corresponds to a loss of exon 6. Sequencing analysis of Donor 3 showed a compound heterozygous mutation, 49bp inversion of intron 12 (1200-5*inv*49bp) induced exon 13 skipping and 429+4*ins*g insertion as found in Donor 14. Since mRNA from Donor 4 was not available, the molecular base 121-126*del*gCAAGTT located on the border of exon 4 is currently unclear.

Antibodies against CD36 in blood donors and patients

All sera derived from blood donors with type I and type II CD36 deficiency (n = 18) were tested for the presence of anti-Nak^a antibodies by solid phase ELISA (PAKPLUS, GTI Diagnostics). Only one serum (Donor 6) showed specific positive reaction with CD36 (►Figure 2). This serum originated from a type I CD36-deficient female donor (Donor 6) (see above), who was most probably immunised during her pregnancy.

In addition, in two patients (Mo and Li) anti-Nak^a antibodies against CD36 (►Figure 2) were identified. In case 1 (Mo), FNAIT was suspected. Nucleotide sequencing analysis of maternal genomic DNA showed a point mutation (C380T) located in exon 5 of CD36 gene, which leads to missense amino acid substitution Ser127Leu, and insertion of one nucleotide (429+4*ins*g) at fourth position of intron 5 leading to exon 6 skipping. It was suspected these mutations are directly responsible for the absence of CD36 expression both on maternal monocytes and platelets (►Table 2B). Indeed, analysis of maternal platelets and monocytes by flow cytometry showed no CD36 expression on the cell surface (►Figure 3A). In contrast, normal reactions were detected with paternal platelets and monocytes. Cross match analysis between maternal sera with paternal platelets showed strong reaction in MACE (optical density [OD] 1.348) when compared to negative control (OD 0.149; cut-off 0.300). These results indicated that anti-Nak^a antibodies developed by a type I CD36-deficient mother during the pregnancy reacted with CD36 expressed on foetal platelets leading to FNAIT. These results could be further confirmed by immunoprecipitation analysis. As shown in ►Figure 3B, maternal serum (Mo) precipitated CD36 from normal donor with an apparent molecular weight of ~95 kDa. This reaction was abolished when CD36 were precleared with mab against CD36 prior to immunoprecipitation with Mo serum.

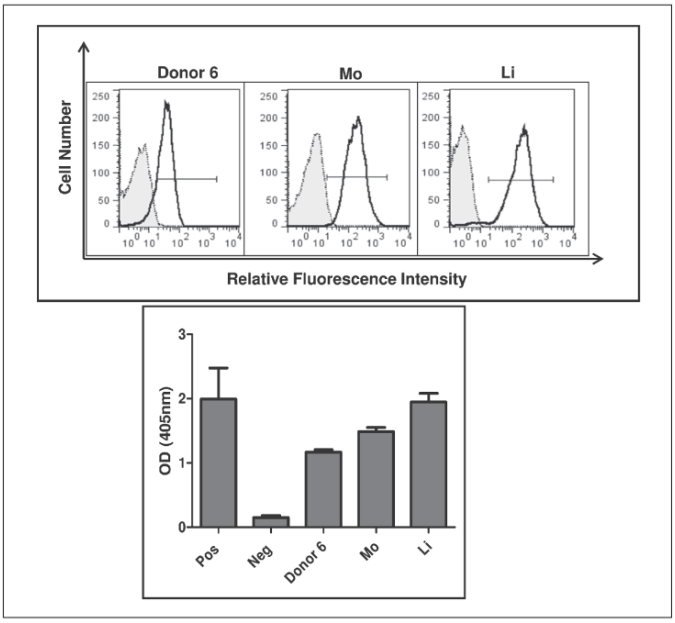

Figure 2: Analysis of anti-Nak^a antibodies by flow cytometry and ELISA. Upper panel: normal platelets were incubated with serum of Donor 6, serum Mo, or serum Li as indicated (white fluorogramms). After washings, bound antibodies were detected with FITC-labelled specific for human IgG and analysed by flow cytometry. Serum from healthy donor was used as control (grey). Bottom panel: Anti-Nak^a antibodies in serum of Donor 6, serum Mo, or serum Li were analysed by sandwich ELISA using GTI PAKPLUS as recommended by the manufacturer. Optical density was measured at 405 nm.

In case 2 (Li), PTR was suspected. Analysis of Li serum showed strong reaction with CD36 (►Figure 2). When Li platelets and monocytes were analysed by flow cytometry, no CD36 expression was found (data not shown). This type I CD36 defect could be ascribed to homozygous 329-330*del*AC which leads to frame shift at amino acid 110 as described above.

Discussion

In this study, we aimed to determine the frequency of type I and type II CD36 deficiencies among healthy blood donors in South China.

Four major CD36 gene mutations, including C268T, 329-330*del*AC, 949*ins*A, and 1228-1239*del*ATTGTGCCTATT, have been currently reported (9). These four mutations cover almost 90% of CD36 genetic defects characterised so far. Studies in Japanese and Korean populations showed high occurrence of C268T (Pro90Ser) mutation. This mutation was claimed to represent the most common mutation (~50%) among Asians (15, 16).

Interestingly, our current study showed that Pro90Ser mutation did not represent the most common cause of type I CD36 deficiency in South China. No individual carrying Pro90Ser was detected in this study. However, similar frequency of the type I CD36 deficiency (>0.5%) was found in our cohort and in Japanese population (6). In contrast, we found fewer individuals carrying CD36 negative platelets (1.8% vs 4.0%).

The most common mutations found in our cohort are 329-330*del*AC and 1228-1239*del*ATTGTGCCTATT (►Table 2A). The impact of these mutations on CD36 gene expression has been reported by Kashiwagi et al. (15, 17). The 329-330*del*AC dinucleotide deletion (also known as 539-540*del*AC) located in exon 5 results in frame shift with a translation stop at position ^{396}TGA (also known as ^{606}TGA) (17). The deletion of 12bp (also known as 1438-1449 *del*ATTGTGCCTATT) located in the middle of exon 13 leads to four amino acid deletion (Ile-Val-Pro-Ile) at positions 410-413 impairs the transportation of CD36 precursors from the endoplasmic reticulum to Golgi apparatus (15).

In this study, we could identify new CD36 mutations, C220T, 429+4*ins*g and 1200-5inv49bp) associated with type I CD36-deficient individuals. Analysis of CD36 transcript showed termination of CD36 at Gln74, skipping of exon 6 and exon 13, respectively. Several skippings of CD36 exons associated with CD36 deficiency have been described (11, 15, 18-19). Expression analysis of 1438-1449del associated with exon 9 skipping caused maturation and transport defects of CD36 precursor form. Further study is necessary to unravel the mechanism(s) of the exon 6 and exon 13 skipping found in our CD36-deficient individuals.

It has been showed that type II CD36-deficient individual could develop occasionally anti-Nak^a antibodies (5). In our cohort, however, no anti-Nak^a antibodies were found in type II CD36-deficient donors. Anti-Nak^a antibodies just were found in a female blood donor with type I CD36 deficiency. Molecular analysis showed a compound heterozygous mutation (C220T and 329-330*del*AC) in

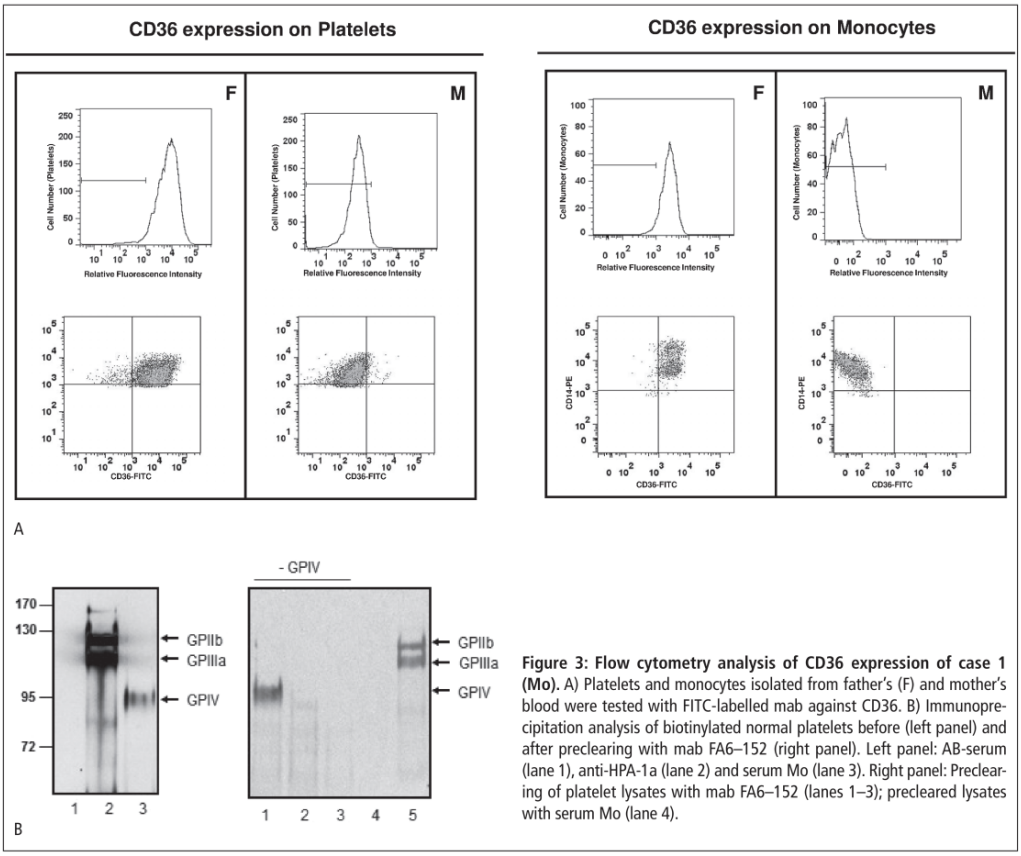

Figure 3: Flow cytometry analysis of CD36 expression of case 1 (Mo). A) Platelets and monocytes isolated from father's (F) and mother's blood were tested with FITC-labelled mab against CD36. B) Immunoprecipitation analysis of biotinylated normal platelets before (left panel) and after preclearing with mab FA6–152 (right panel). Left panel: AB-serum (lane 1), anti-HPA-1a (lane 2) and serum Mo (lane 3). Right panel: Preclearing of platelet lysates with mab FA6–152 (lanes 1–3); precleared lysates with serum Mo (lane 4).

exon 4, and exon 5, respectively. The mutation C220T leads to premature stop codon ^{220}TAG (Gln74*Term*), and the 329-330*del*AC causes frame shift at amino acid residue 110 (see above).

Curtis et al. reported the presence of anti-Nak^a antibodies in two African-American mothers with FNAIT, who were homozygous for the T1264G mutation (10). This mutation introduces premature stop codon in exon 10 leading to the absence of CD36 on maternal platelets. In South-East Asian, single cases of FNAIT have been reported in Thai and Japanese populations (11, 20). Analysis of the maternal CD36 transcript showed a compound heterozygous mutation associated with skipping of exon 4 and 9 that led to type I CD36 deficiency, and consequently anti-Nak^a antibody formation during the pregnancy (11). In addition, anti-Nak^a antibodies associated with hydrops fetalis in two siblings have been described (21). These antibodies showed suppressive effects on the CFU-E colony formation of CD36$^+$ cells. Analysis of the CD36 gene of the mother revealed a compound heterozygous mutation, a novel C1366T (also known as C1156T; see ▶Table 2A) mutation in exon 12, corresponding to Arg386Trp, and a 12bp deletion (1438-1449 *del*ATTGTGCCTATT; see above).

In this study, we found a case FNAIT with severe intracranial hemorrhage in the fetus of a type I CD36-deficient multi gravida mother, who has developed anti-Nak^a antibodies during pregnancies. Analysis of maternal DNA showed a compound heterozygous mutation: one missense amino acid substitution Ser127Leu and one nucleotide (429+4*ins*g) insertion in intron 5. However, the exact molecular mechanism(s) how these mutations cause CD36 defects are unclear. The Ser127Leu mutation was first described as a minor-allele in Thai patients with malaria (22). Interestingly, the 429+4*ins*g leading to exon 6 skipping were found in two normal donors (▶Table 2A). Recently, Hori et al. showed that mutation in intron (1248+5g>a) of *OXCT1* gene can affect donor splice leading to exons skipping in fibroblast of patients with a typical ketolytic

What is known about this topic?

- CD36 (GPIV)-deficient individuals (type I and type II) exist in African and Asian populations. CD36-deficient individuals could develop antibodies during pregnancies and multiple platelet transfusion (case reports).
- CD36 deficiencies are caused by different mutations on the CD36 gene including nucleotide substitutions, deletions and insertions.
- However, the frequency of GPIV-deficient and its clinical impact in immune mediated thrombocytopenia is not known, especially in Chinese populations.

What does this paper add?

- This paper reports the first information regarding the frequency of CD36-deficient individuals in China, its molecular bases and its role in immune thrombocytopenia in a large cohort.
- This result suggests that antibodies against GPIV play an important role in the mechanism of immune thrombocytopenia among Asian populations.

defect caused by Succinyl-CoA transferase deficiency (SCOT disease) (23).

Several studies have demonstrated the role of anti-Nak^a antibodies in PTR (10). In most of the cases (87.5%) transfusion with CD36-deficient platelets resulted in satisfactory response. In this study, we could identify a case of PTR associated with strong anti-Nak^a antibodies in a patient receiving multiple transfusions of random platelets. Further analysis documented homozygous 329-330*del*AC underlying the type I deficiency in this patient.

In conclusion, our results show that CD36 deficiency exists in Chinese population, and CD36 immunisation can occur in these individuals. Based on these observations, screening for anti-Nak^a antibodies should be considered in suspected immune-mediated thrombocytopenia. A donor registry of CD36-deficient individuals should be established to supply patients with these disorders.

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Conflicts of interest

None declared.

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3.2. Two cases of platelet transfusion refractoriness and one case of possible FNAIT caused by antibodies against CD36 in China

TRANSFUSION MEDICINE
Official Journal of the British Blood Transfusion Society
Transfusion Medicine | LETTER TO THE EDITOR

Two cases of platelet transfusion refractoriness and one case of possible FNAIT caused by antibodies against CD36 in China

Dear Sir,

CD36 (also known as GPIV) is expressed on platelets, monocytes, red cells as well as on endothelial cells (Nakajima *et al.*, 2008). The majority of CD36 deficiencies are type II, whose platelets, but not monocytes, failed to express CD36 on the cell surface. A minority of CD36-deficient individuals are type I, neither platelets nor monocytes do express CD36 (Taketani *et al.*, 2008). Individuals with type I deficiency could develop isoantibodies (called anti-Nak^a) against CD36 (Kashiwagi *et al.*, 2001; Curtis *et al.*, 2002). The frequencies of type I and type II CD36 deficiencies in South China are ~0·5 and 1·3%, respectively (Xu *et al.*, 2013) (Table 1).

Here, we reported two patients who suffered platelet transfusion refractoriness (PTR) and one possible FNAIT case due to CD36 immunisation. Patient 1, an 11-year-old male with history of transfusion received chemotherapy for acute leukaemia. Patient 2, a 35-year-old female with history of pregnancies and transfusions underwent chemotherapy for myelodysplastic syndromes (MDS). Both patients did not respond to apheresis random donor and subsequent HLA-compatible platelet transfusions. Flow cytometry analysis of platelets and monocytes from patient 1 using FITC-labelled mAb FA6-152 against CD36 showed marked reduction of CD36 surface expression when compared with CD36-positive platelets and monocytes derived from a healthy blood donor (platelets: 5·07 vs 97·4%; monocytes: 2·28 vs 95·1%). Similar result was obtained with patient 2 (platelets 3·90%, monocytes 4·96%), indicating that both patients are CD36 type I deficient. Antibody screening by using solid phase assay (PakPLUS kit, GEN-PROBE Incorporated, Waukesha, WI, USA) showed that both sera reacted exclusively with GPIV, but not with GPIIb/IIIa, GPIb/IX, GPIa/IIa and HLA class I antigens. The absence of anti-HLA class I antibodies in these sera were confirmed by extensive analysis using the Lifecode Class I (Immucor, Stamford, USA) HLA class I antibodies screening. Accordingly, sera from both patients reacted with platelets from three different CD36-positive donors (patient 1: 88·1 ± 2·65%; patient 2: 96·2 ± 1·90%) but not with platelets from CD36-negative donors ($n = 3$) by flow cytometry (patient 1: 4·2 ± 1·06%; patient 2: 4·67 ± 0·40%). By DNA sequencing analysis heterozygous 329-330*del*AC in exon 5 was found in patient 1. In patient 2, a single homozygous A1163T mutation in exon 12 leading to Gln388Leu mutation was detected. In comparison to transfusions with non-compatible apheresis platelets (CD36 positive), significant improvement of corrected count increment (CCI) was obtained after transfusions with compatible platelets (CD36 negative) (see Fig. 1).

In case 3, 35-year-old female with no history of blood transfusion and transplantation experienced five abortions. Flow cytometry analysis of platelets and monocytes showed both marked reduction of CD36 surface expression when compared with control (platelets: 4·60 vs 98·4%; monocytes: 2·82 vs 96·3%). Antibody screening by using PakPLUS kit showed that the sera reacted with GPIV and HLA class I antigens, but not with GPIIb/IIIa, GPIb/IX and GPIa/IIa. Panel reactive antibodies (PRA) was 14% and anti-HLA-B*40 and HLA-B*27 antibodies were identified. The maternal serum reacted with platelets from CD36-positive (90·2 ± 3·60%) but not with platelets from CD36-negative donors (4·53 ± 1·07%) by flow cytometry ($n = 3$). By DNA sequencing analysis homozygous 329-330*del*AC in exon 5 leading to frame shift at AA 110 was found in this patient.

All results could be confirmed by sequencing analysis of complementary DNA (cDNA). Further study should be conducted to understand the molecular mechanism of CD36 deficiency associated with these mutations.

In summary, the three cases of immune thrombocytopenia described here indicated further the clinical significance of anti-Nak^a antibodies in our population and substantiate the necessity of laboratory diagnostics for the identification of these antibodies and underline the important of donor register of CD36-negative donors for the treatment of these patients.

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W. X. and X. Y. wrote this paper, managed and analysis the results. X. X., J. L., J. D., J. W. and H. L. performed the enzyme-linked immunosorbent assay (ELISA) and flow cytometry and collected information from hospital. Y. F. reviewed the manuscript. S. S. read and wrote the manuscript. H. D., Y. S. and Y. C. performed the sequencing analysis.
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Table 1. CD36 deficiencies and anti-Naka antibodies found in Guangzhou, China

	Subjects	Clinical information	Age/Sex	CD36 Plt/Mo	Type	Antibody	Diagnosis	Mutations of CD36 gene	Change in amino acid
Xu *et al.*, 2013	Donor (GZ-3183)	Donor	50/F	neg/neg	I	Anti-Nak^a	None	329-330*del*AC; C220T	Frame shift at AA 110; Gln74Term
	Patient 1	T lymphoblastic cell tumour	22/M	neg/neg	I	Anti-Nak^a	PTR	Homozygous 329-330*del*AC	Frame shift at AA 110
	Patient 2	Abortion four times	30/F	neg/neg	I	Anti-Nak^a	FNAIT	C380T; 429+4*ins*g	Ser127Leu; Exon 6 skipping
This study	Patient 3	Acute leukaemia	11/M	neg/neg	I	Anti-Nak^a	PTR	Heterozygous 329-330delAC	Frame shift at AA 110
	Patient 4	MDS	35/F	neg/neg	I	Anti-Nak^a	PTR	Homozygous A1163T	Gln388Leu
	Patient 5	Abortion five times	35/F	neg/neg	I	Anti-Nak^a	FNAIT	Homozygous 329-330*del*AC	Frame shift at AA 110

F, female; FNAIT, Foetal/Neonatal Alloimmune Thrombocytopenia; M, male; MDS, myelodysplastic syndrome; Mo, monocytes; neg, negative. Plt, platelets; PTR, Platelet Transfusion Refractoriness.

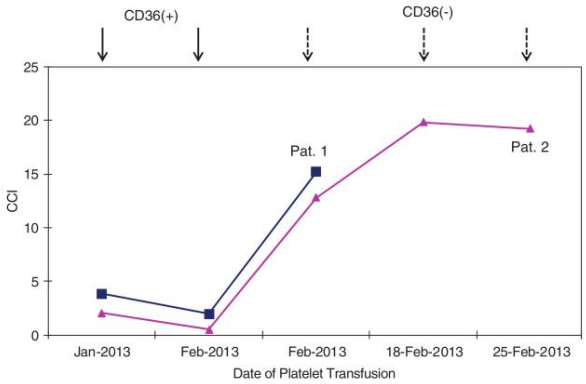

Fig. 1. Effects of platelet transfusion of CD36 (+) or CD36 (−) platelets. CCI values are obtained with transfusions of CD36 (+) and (−) apheresis platelets in patient 1 and patient 2.

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CONFLICT OF INTEREST

The authors have no competing interests.

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3.3. Successful management of a hydropic fetus with severe anemia and thrombocytopenia caused by anti-CD36 antibody

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CASE REPORT

Successful management of a hydropic fetus with severe anemia and thrombocytopenia caused by anti-CD36 antibody

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Abstract Cases of CD36 deficiency are not rare in Asian populations, foetal and neonatal alloimmune thrombocytopenia (FNAIT) caused by anti-CD36 *iso*antibodies appears more frequent than other HPA alloantibodies. However, little is known about the treatment of anti-CD36 mediated FNAIT in this region. A Chinese male foetus, whose mother had a history of multiple intrauterine foetal demise and/or hydrops, was diagnosed with severe FNAIT at 27 weeks of gestational age. Immunological analysis revealed total absence of CD36 on platelets and monocytes from mother, caused by a 329–330*del*AC mutation of the CD36 gene. Anti-CD36 and anti-HLA class I antibodies were detected in the maternal serum, whereas only anti-CD36 *iso*antibodies were detectable in the foetal blood sample. Serial intrauterine transfusions with red blood cells (RBC) and platelets from a CD36*null* donor were performed to improve the severe anaemia and thrombocytopenia. The baby (2250 g; Apgar scores 10) was delivered vaginally at 32 weeks of gestation with normal haemoglobin (186 g/L) but low platelet count (48×10^9/L). After 2 days the platelet count rose to 121×10^9/L. This report suggests that intrauterine transfusions with compatible RBC and CD36*null* platelets are useful in preventing the deleterious clinical effects of anti-CD36-mediated severe FNAIT.

Keywords Foetal and neonatal alloimmune thrombocytopenia · Anti-CD36 antibody · Foetal anaemia and hydrops · Intrauterine transfusion

Introduction

Foetal and neonatal alloimmune thrombocytopenia (FNAIT), occurring in 1/800–1/1000 live births, is the most common cause of severe thrombocytopenia and intracranial haemorrhage (ICH) in foetus and term newborns [1, 2]. FNAIT is caused by maternal antibodies which recognize paternal-derived antigen on foetal platelets leading to platelet destruction. In Caucasians, more than 75% of FNAIT cases are induced by alloantibodies against human platelet antigen (HPA)-1a [3, 4], whereas the most common antibodies related to FNAIT for Japanese are anti-HPA-4b alloantibodies [5]. Both alloantibody specificities, however, have not been reported in Chinese population so far. In contrast, anti-CD36 *iso*antibodies, developed in type I CD36 deficient mothers are frequently reported as the cause of FNAIT in China [6, 7].

CD36 (also known as GPIV), is a highly glycosylated 88-kDa protein and is expressed widely on human cells including platelets, monocytes, macrophages, erythroid precursors and endothelial cells [8]. There are two types of CD36 deficiency; type I with total absence of CD36 expression on platelets and monocytes and type II lacking of CD36 on platelets but not on monocytes [9]. CD36 deficiency on

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platelets is more frequent in Asians (about 3–11%) and Africans (about 8%) than in white people (<0.4%) [7].

Anti-CD36 antibodies (originally named anti-Nak^a) were first described in a case of platelet transfusion refractoriness [10, 11]. Meanwhile, the clinical relevance of anti-CD36 antibodies has been reported in other immune mediated disorders including thrombotic thrombocytopenic purpura, adverse transfusion reactions and FNAIT [12–14].

The clinical pictures of anti-CD36 mediated FNAIT are heterogeneous ranging from widespread petechial haemorrhages, gastrointestinal bleeding, severe anaemia and thrombocytopenia and hydrops fetalis [14–17]. Recently, we reported two cases of FNAIT with recurrent abortions and hydrops [6, 18].

In comparison to anti-HPA alloantibodies, little is known about the management of FNAIT caused by anti-CD36 *iso*antibodies. Here, we present a successful management of a life-threatening case of FNAIT case with hydrops fetalis, serious anaemia and thrombocytopenia caused by anti-CD36 *iso*antibodies.

Case report

A Chinese male foetus was found with ascites, pericardial effusion, and cardiomegaly and hydropic placenta by ultrasound at 26 weeks of gestational age, and these clinical symptoms became severe at 27 weeks of gestational age (Fig. 1a). Doppler assessment of the middle cerebral artery revealed a peak systolic velocity (MCA-PSV) is 2.1 multiples of median (MOM). Umbilical cord centesis showed foetal anaemia (haemoglobin 84 g/L, haematocrit 25.1%) at 23 weeks of gestational age, whereas the reticulocytes (0.0385×10^{12}/L) and platelet count (192×10^9/L) were normal. However, at 27 weeks of gestational age foetal anaemia (haemoglobin 48 g/L, haematocrit 16.6%) became severe; the reticulocytes (0.1417×10^{12}/L) showed reactive proliferation and thrombocytopenia (platelet 16×10^9/L) was found. Parvovirus and cytomegalovirus infections, maternal syphilis, thalassemia, fetomaternal haemorrhage had been ruled out. Furthermore, immunization against red blood cells (RBC) was excluded by negative direct antiglobulin test (DAT) and indirect antiglobulin test (IAT) using DG Gel Coombs (GRIFOLS).

His mother, a 36-year-old Chinese woman, gravida 3 para 1, had a history of abortion at the first pregnancy and intrauterine foetal deaths with foetal hydrops occurred during 6–8 months in the following five pregnancies. Blood tests of his mother for cytomegalovirus, parvovirus, irregular and autoantibody were negative. Interestingly, anti-CD36 *iso*antibodies were detectable in the maternal serum indicating a severe case of FNAIT associated with anti-CD36 *iso*antibodies.

Materials and methods

The platelet antibodies in sera collected from the mother and umbilical cord were detected using a commercial ELISA Kit (PAKPLUS, Immucor GTI Diagnostics Inc., Waukesha, USA). Aliquots of 50 µL sera (1:3 dilutions) were added to microtiter wells coated with different platelet glycoproteins or HLA class I antigens. Bound antibodies were detected with alkaline-phosphatase-conjugated anti-human IgG and the appropriate substrate. The characterization of CD36 on protein as well as on DNA level was performed as previously described [18].

Results

Figure 2 shows the results of antibody containing in sera of maternal and umbilical cord at 14, 23 and 27 weeks of gestation. Increasing amounts of HLA antibodies against B*27:08, B*45:01, B*27:05, B*49:01, B*15:12, B*07:02, B*15:01, B*41:01, B*44:03, B*73:01, B*44:02, B*35:01

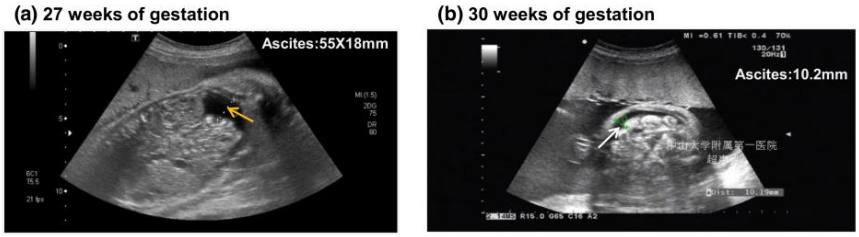

Fig. 1 Ascites was measured in diameter of fluid in the abdominal cavity by ultrasound. **a** 55 mm diameter of ascites was found at 27 weeks of gestation (*arrow*). **b** The diameter of fluid (10.2 mm) at 30 weeks of gestation shows significant reduction of ascites in the transversal view (*arrow*)

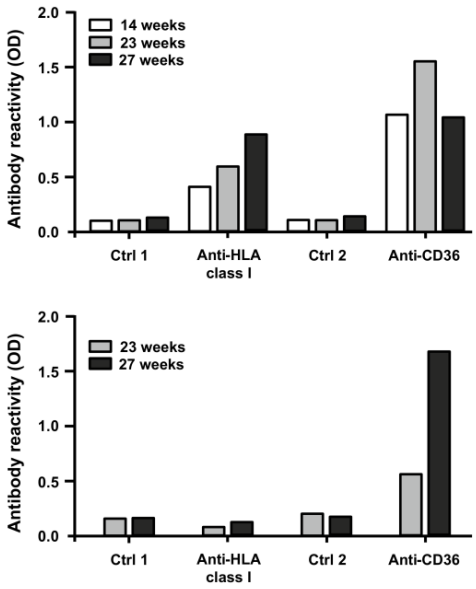

Fig. 2 Detection of anti-CD36 antibodies and anti-HLA class I by ELISA. Platelet reactive antibodies were screened by a commercial ELISA Kit (PAKPLUS). In maternal serum (*upper panel*), antibodies against CD36 and HLA class I were found at 14, 23 and 27 weeks of gestation age, respectively as indicated. In serum of umbilical cord (*bottom panel*) only anti-CD36 antibodies was identified at 23 and 27 weeks of gestation age. Controls (Ctrl) 1 and 2 represent the negative control of anti-HLA class I and anti-CD36, respectively

and B*07:03 were found in maternal serum using LIFECODES LSA (Gen-Probe). However, these antibody specificities could not be found in umbilical cord blood sample, and inconsistent with the HLA-genotype of foetus (A*02:03, A*24:02; B*40:01, B*54:01; C*01:02, C*07:02; DRB1*08:03, DRB1*16:02). This observation indicated that these HLA class I antibodies did not play a role in the pathomechanism of thrombocytopenia in this case. Interestingly, although the amount of anti-CD36 *iso*antibodies in maternal sera decreased from 23 to 27 weeks of gestation, significant increase of anti-CD36 *iso*antibodies was detected in the umbilical cord blood sample. Flow cytometry analysis showed the normal CD36 expression of father and foetus on platelets (MFI 240; MFI 20.0) as well as on monocytes (MFI 480; MFI 60.1). In contrast, total absence of CD36 was observed on the maternal platelets (MFI 2.87) and monocytes (MFI 1.14) (Fig. 3), demonstrating type I CD36 deficiency. This is in accordance with the molecular biological analysis. Two nucleotides deletion AC at position 329–330 located in exon 5 of the CD36 gene of the mother was found. As expected, normal CD36 gene (wild-type) was found in the father. Accordingly, the foetus is heterozygous carrying both the mutant and the wild-type allele (Fig. 4). All together, these results strongly indicated that anti-CD36 *iso*antibodies developed by CD36*null* phenotype mother, passed through placenta and induced severe FNAIT conditions with hydrops fetalis and serious anaemia.

From 27 until 29 weeks of gestation, a total of three intrauterine transfusions with RBC were performed to improve the severe anaemia. The haemoglobin value increased rapidly (113 g/L), and the ultrasound assessment showed the diameter of fluid in the abdominal cavity of fetus was decreased markedly (Fig. 1B). However, the platelet count (11×10^9/L) remained low (Table 1). At 30 weeks of gestation, 4.81×10^{10} CD36*null* leuko-depleted, non-irradiated platelets were transfused to the foetus and the platelet count rose to 177×10^9/L. In addition, the mother (body weight, 50 kg) received prednisone for 11 days (10 mg, bid). Due to the persistent elevation of MCA-PSV (1.8–2.5 MOM), RBC and platelets from a CD36*null* donor were given 3 days later. The post-transfusion platelet count and haemoglobin were 105×10^9/L and 114 g/L, respectively. Foetal hydrops could not be detected by the subsequent ultrasound (data not shown). During this time the mother refused the prenatal treatment with intravenous immunoglobulin (IVIG).

At 32 weeks of gestation, the male baby (2250 g) was delivered vaginally due to premature rupture of membranes with Apgar scores of 10 (after 5 min). At birth, the haemoglobin was normal (186 g/L), but the platelet count was low (48×10^9/L). Although diffuse petechial haemorrhages in lower limbs and chest were observed shortly after birth, intracranial haemorrhage (ICH) was not detected by ultrasounds as well as by computed tomography (CT) scan. After 2 days the platelet count rose to 121×10^9/L. The child was discharged from the hospital on day 30. No neurologic abnormality was observed until today (9 months old).

Discussion

We report a severe case of FNAIT complicated with foetal anaemia and hydrops due to anti-CD36 *iso*antibodies, which represents a rare clinical condition in this disorder. Usually the clinical features of FNAIT are associated with the occurrence of petechiae, hematomas and ICH [19]. Only anti-CD36 *iso*antibodies, but not specific anti-HLA class I antibodies could be found in the foetal blood sample indicating that anti-CD36 *iso*antibodies were directly responsible for the clinical complications observed in this FNAIT case.

Curtis et al. described five FNAIT cases caused by anti-CD36, but neither prenatal nor postnatal anaemia was observed [15]. In two of these infants, the platelet counts rose to the normal range within 2 weeks without any

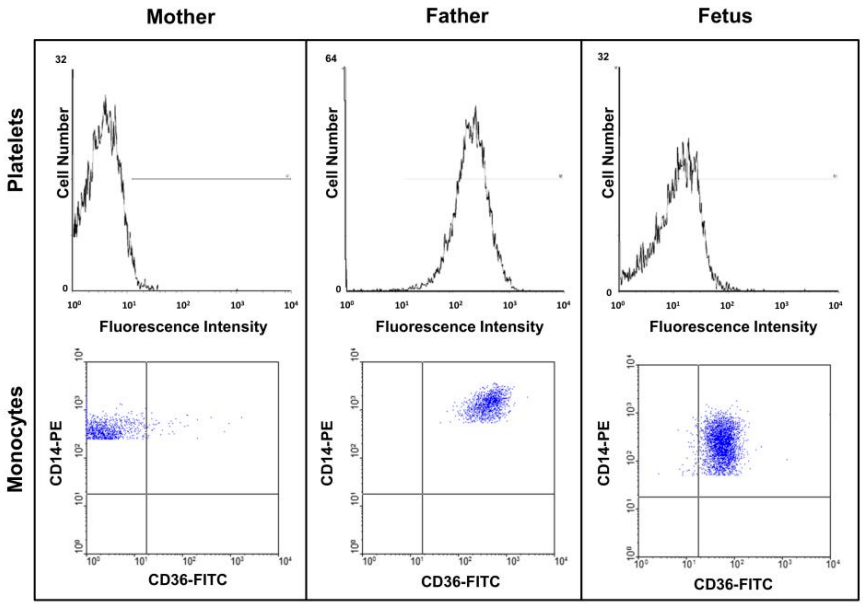

Fig. 3 Flow cytometry analysis of CD36 expression on platelets and monocytes. Platelets and monocytes of mother, father and foetus were measured with FITC labelled mab against CD36 and PE-labelled mab against CD14. Anti-CD36 reacted with platelets (*top panel*) from the father and the foetus, but none with the mother. Similarly, only monocytes (CD14 positive) of the father and the foetus reacted with anti-CD36 antibodies (*bottom panel*)

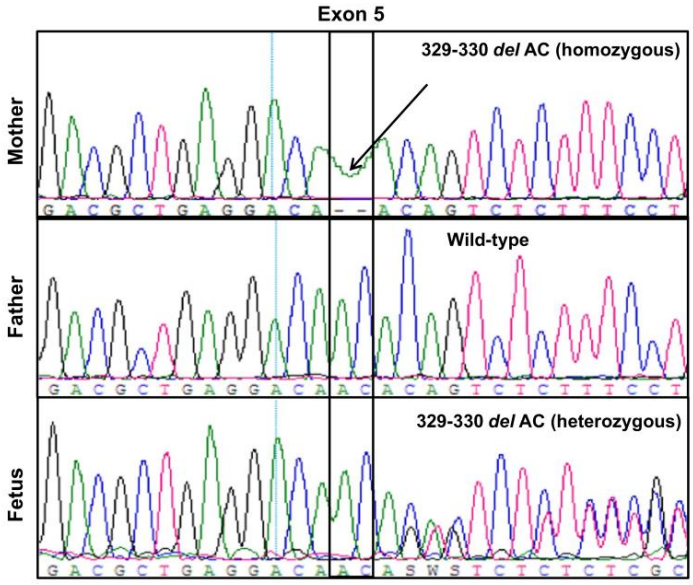

Fig. 4 Sequencing analysis of exon 5 of CD36 gene. The two nucleotides deletions AC at position 329–330 (*arrow*) in mother (homozygous state) and in foetus (heterozygous state) in comparison to the father (wild-type) are presented

Table 1 Clinical characteristics before and after five intrauterine interventions

No.	GA (weeks + days)	FBS	Intrauterine transfusion		Before intrauterine transfusion			After intrauterine transfusion		
			RBC (mL)	PLT (mL)	Hb (g/L)	Hct (%)	PLT (10^9/L)	Hb (g/L)	Hct (%)	PLT (10^9/L)
1	23 + 3	√	–	–	84	25.1	192	–	–	–
2	27 + 1	√	–	–	48	16.6	16	–	–	–
3	28 + 1	√	21	–	39	12.8	12	51	16.2	10
4	28 + 3	√	60	–	34	10.9	18	75	23.8	14
5	29 + 1	√	92	–	61	18.4	7	113	33.5	11
6	30	√	–	67	85	26.2	20	75	24	177
7	30 + 3	√	85	34	71	21.4	57	114	33.4	105

No number of cordocentesis or intrauterine transfusion, *GA* gestational age, *FBS* fetal blood sample by cordocentesis, *Hb* hemoglobin, *Hct* hematocrit, *RBC* red blood cells, *PLT* platelets

specific treatment. Recently, Okajima et al. [17] described a case of two siblings with hydrops fetalis due to anti-CD36. Foetal anaemia was observed in the elder sister. She was managed by intraperitoneal transfusion with RBC and delivered at 30 weeks of gestation. The younger sister was delivered at 29 weeks of gestation without intrauterine intervention.

In our patient, intrauterine transfusions with compatible RBC seem to be of benefit to manage severe foetal anaemia and the development of hydrop fetalis. Actually, platelet transfusion prior to RBC administration is recommended in severe thrombocytopenia (platelet count $<50 \times 10^9$/L) to prevent haemorrhage [20]. In our case, transfusion with CD36*null* platelets did not be administered until 30 weeks of gestation for two reasons. First, compatible platelet donor was not formerly available. Second, comparing to foetal thrombocytopenia, hydrops fetalis and anaemia threatened the foetal life and may result in foetal demise so that immediate red cell transfusion should be performed as the first priority. Transfusion with CD36*null* platelets after 30 weeks of gestation resulted in significance increase of foetal platelet counts. Fortunately, signs of ICH, intrauterine growth restriction or other structural abnormalities were not found by prenatal ultrasound or postnatal computer tomography.

Although ICH is regarded as the most severe complication of FNAIT [21], the mechanism has not been fully understood. Recently, Yougbaré et al. [22] demonstrated that anti-β3 antibodies developed in immunized β3 knock-out mice mothers could induce ICH in pups in a murine model of FNAIT. More recently, Santoso and co-workers found significant association between certain types of anti-HPA-1a alloantibodies with the development of ICH. This antibody type bound strongly to αvβ3 integrin expressed on endothelial cells and inhibited thereby the interaction between αvβ3 and its ligand vitronectin leading to endothelial cell apoptosis (anoikis) and consequently impaired angiogenesis [23]. These observations underline the important of anti-endothelial antibodies as important trigger of ICH. The question whether anti-CD36 *iso*antibodies may impair endothelial function(s) leading to foetal hydrops is intriguing.

Nowadays, high risk cases of FNAIT cases caused by anti-HPA-1a alloantibodies are commonly treated with intravenous immunoglobulin (IVIG) and corticosteroid [24]. Currently, little is known about the benefit of IVIG for the treatment of FNAIT caused by anti-CD36 *iso*antibodies. In our case, this clinical approach could not be approved, because no compliance could be achieved.

Taking together, although anti-CD36 *iso*antibodies turn out to be the most frequent platelet antibodies found in Asia and can cause life-threatening FNAIT, this serious disorder is still precious little considered. In this study, we present the careful elaboration of the case, the laboratory diagnostic as well as the successful management of severe case of FNAIT caused by anti-CD36 antibodies. Since hydrops fetalis and anaemia rather than thrombocytopenia seem to threatening for the foetal life, immediate RBC transfusion should be recommended as the first priority.

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Compliance with ethical standards

Conflict of interest All authors declare that there is no conflict of interest.

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3.4 Hydrops fetalis associated with anti-CD36 antibodies in fetal and neonatal alloimmune thrombocytopenia: Possible underlying mechanism

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ORIGINAL ARTICLE

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Hydrops fetalis associated with anti-CD36 antibodies in fetal and neonatal alloimmune thrombocytopenia: Possible underlying mechanism

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Abstract

Objectives: In the present study, we asked whether anti-CD36 antibodies impair the maturation of erythropoietic stem cells to mature red blood cells (RBCs), leading to anaemia and hydrops fetalis (HF).

Background: Recent studies have shown the importance of anti-CD36 antibodies in the development of Fetal/Neonatal Alloimmune Thrombocytopenia (FNAIT). In comparison to other types of antibody-mediated FNAIT, anti-CD36 antibodies are frequently associated with anaemia and HF. As mature RBCs do not express CD36, the reason for this phenomenon is currently not fully understood.

Material and methods: A case of FNAIT with signs of HF was characterised in this study. Maternal anti-CD36 antibodies were isolated by an absorption/elution approach. We cultured haematopoietic stem cells (HSCs) with purified anti-CD36 antibodies, and the formation of burst-forming unit-erythroid and colony-forming unit-erythroid (CFU-E/BFU-E) cells was analysed. Apoptosis of HSCs was also investigated.

Results: Analysis of the mother showed type-1 CD36 deficiency. Anti-CD36 antibodies were found in maternal serum, as well as on fetal platelets, by ELISA, and the specificity of these antibodies was further substantiated by flow cytometry. In comparison to control IgG, incubation of HSCs with purified anti-CD36 antibodies led to a significant reduction in CFU-E/BFU-E cell formation, and this result was associated with an increased number of apoptotic CD34+ erythroid/myeloid precursor cells. Administration of intrauterine transfusion with washed RBCs was effective in improving fetal anaemia.

Conclusions: Anti-CD36 antibodies may cause anaemia and trigger HF through apoptosis of CD34+ erythroid/myeloid precursor cells. However, the contribution of other cells must also be taken into account.

KEYWORDS
anti-CD36, fetal anaemia, FNAIT, haematopoietic stem cells, hydrops fetalis

1 | INTRODUCTION

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a blood-related disease caused by maternal antibodies (abs) that destroy fetal

† Yongbin Wu and Dawei Chen authors contributed equally to this work.

platelets. The major risk to FNAIT is severe bleeding, especially intracranial haemorrhage.[1] In the Caucasian population, approximately 80% of maternal abs react with human platelet antigen-1a (HPA-1a).[2] However, in Asian populations, the occurrence of FNAIT is mainly associated with anti-CD36 abs (originally named anti-Nak^a)[3-7].

CD36 is a highly glycosylated transmembrane protein and is widely expressed in platelets, monocytes, macrophages, nucleated red blood cells and endothelial cells. CD36 acts as a multifunctional receptor that can interact with collagens, thrombospondin, malaria-infected red blood cells (RBCs), oxidised low-density lipoproteins and long-chain fatty acids—demonstrating involvement in the mechanisms of various disorders.[8,9] Two different types of CD36 deficiency are known: type I is characterised by the absence of CD36 surface expression on both platelets and monocytes, whereas type II lacks CD36 expression on platelets but expresses CD36 on monocytes.[10] Type-I mothers can develop anti-CD36 abs due to immunisation with fetal cells that carry CD36 antigen, which leads to clinical pictures of FNAIT—including petechial haemorrhages and severe thrombocytopenia.[3,11-14]

In addition, several cases of FNAIT accompanied by severe anaemia and hydrops fetalis (HF) have been reported.[3,6,7] We recently reported a severe case of FNAIT with HF that was associated with anti-CD36 abs and that was successfully managed by intra-uterine transfusions with RBCs and CD36-negative platelets.[6]

Investigators have reported that anti-CD36 antibodies reacted strongly with erythroid progenitor cells but not with mature fetal erythrocytes and reticulocytes,[11,12] indicating that anti-CD36-mediated HF differs from other conditions where abs against RBC antigens (such as Rh) are found.[13] In the case of Rh, maternal anti-Rh antibodies reacted directly with fetal erythrocytes, causing haemolysis of RBCs and resulting in severe anaemia that led to hypoxia and acidosis; this subsequently damaged capillary epithelium, thus allowing loss of fluid in the extracellular compartment and consequently inducing fetal oedema.[13]

A previous study showed that serum from individuals with FNAIT contained anti-CD36 abs that suppressed the formation of CFU-E colony formation, indicating that serum containing anti-CD36 abs impaired haematopoiesis of RBCs.[4] However, whether other IgG abs that may exist in the serum sample triggered this phenomenon could not be excluded.

In the present study, we report a case of FNAIT caused by anti-CD36 abs developed by a type-1 deficient mother who manifested a 2-nucleotide deletion (329-330 *del*AC). Incubation of purified maternal anti-CD36 IgG abs inhibited burst-forming unit-erythroid and colony-forming unit-erythroid (CFU-E/BFU-E) cell formation derived from CD34+ haematopoietic stem cells (HSCs), which was most likely due to cellular apoptosis.

2 | MATERIAL AND METHODS

2.1 | Case report

FNAIT was suspected in a Chinese mother with a history of recurrent spontaneous abortions. Viral and bacterial infections were excluded. Analysis of maternal platelets and monocytes by flow cytometry (Figure 1A) showed the absence of CD36 expression, indicating type-1 deficiency. This assumption was proven by nucleotide sequencing of the CD36 gene, where we identified the deletion of two nucleotides in exon 5 (329-330 *del*AC) in a homozygous state (Figure 1B). Irregular autoantibody screening against RBCs in the maternal serum was negative by direct and indirect anti-globulin tests. Based on these findings, we suspected anti-CD36-mediated FNAIT.

Analysis of umbilical cord blood showed fetal anaemia (haemoglobin, 96 g L^{-1}; haematocrit, 28%) at an early gestational age (23-24 weeks), whereasthe platelet count was slightly reduced (84×10^9 L^{-1}). Ultrasonography at 24 weeks of gestational age showed no signs of oedema, but we detected an increase in the middle cerebral arterial peak systolic velocity (MCA-PSV: MoM, 1.60) in the fetus. However, 5 days after 26 weeks of gestational age, fetal conditions deteriorated slightly (haemoglobin, 8 7 g L^{-1}; haematocrit, 25.2%), and the platelet count remained low (89×10^9 L^{-1}). Pericardial effusion and scant ascites were detected by ultrasonography, and a further increase in MCA-PSV was observed (MoM, 1.65). We therefore initiated intra-uterine transfusions with washed RBCs to improve the fetal anaemia (Table 1). At 36 + 4 weeks of gestation, a healthy female baby (2600 g) was delivered with an Apgar score of 10. The baby's haemoglobin (152 g L^{-1}), HCT (46%), and platelet count (253×10^9 L^{-1}) were normal at birth.

2.2 | Stably transfected HEK293 cell lines

Non-transfected HEK293 cells and CD36-transfected HEK293 cells established in a previous study[15] were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Paisley, UK) containing 10% fetal bovine serum (FBS, Gibco). CD36 expression on the surface of these cells was evaluated by flow cytometry using fluorescein-isothiocyanate (FITC)-labelled monoclonal antibody (mAb) against CD36 (FA6.152; Beckman Coulter, Los Angeles) on a flow cytometer (FACSCanto II, Becton and Dickinson, Franklin Lakes).

2.3 | Screening of platelet antibodies

Platelet abs in maternal serum, fetal serum and in the eluate from the fetal platelets derived from umbilical cord blood were screened using a commercial ELISA Kit (PAKPLUS, Immucor GTI Diagnostics, Waukesha) as previously described.[6] Briefly, microtitre wells coated with different platelet glycoproteins (GPIa/IIa, GPIb/IX, GPIIb/IIIa and GPIV) or HLA class I antigens were incubated with aliquots of 50 μL of serum (at a 1:3 dilution). After washings, bound abs were detected with enzyme-labelled secondary abs and substrate. The reaction was read with using an ELISA plate reader (Multiskan FC 51119000, Thermo Scientific, Waltham) at 405 nm.

2.4 | Identification of the CD36 antigen on platelets, monocytes and RBCs

We analysed CD36 antigens on platelets and monocytes by flow cytometry as previously described.[14] To analyse CD36 expression on

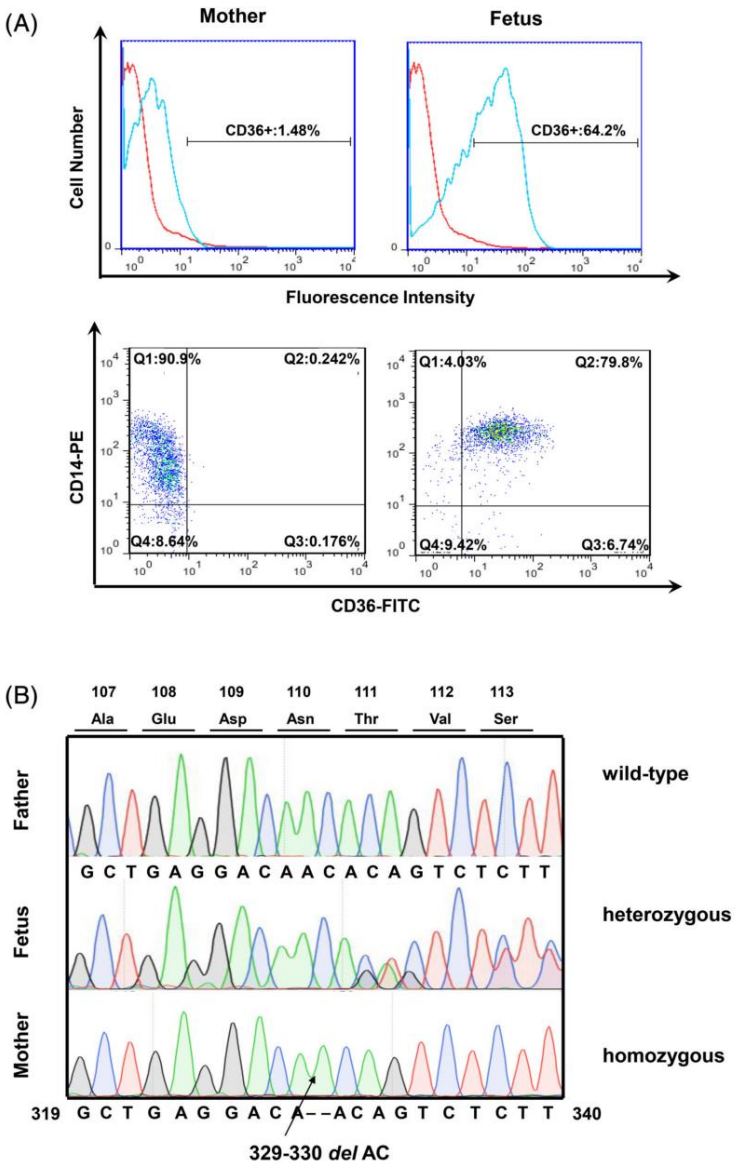

FIGURE 1 Characterisation of type-I CD36 deficiency. A, Flow cytometric analysis of CD36 expression on platelets and monocytes. The expression of CD36 antigen on platelets and monocytes from the mother was measured by anti-CD36-FITC- and anti-CD14-PE-labelled mAbs. Fetal platelets, but not maternal platelets, reacted with anti-CD36 (*upper panel*), and only fetal monocytes (CD14+) reacted with anti-CD36 antibody (lower panel). B, Sequencing analysis of exon 5 of the CD36 gene. Mother (homozygous) and fetus (heterozygous) showed two nucleotide deletions, AC, at positions 329-330 (arrow) in comparison to the father (wild-type)

fetal RBCs, 5 mL of umbilical cord blood in EDTA was centrifuged at 3000 rpm for 5 minutes. Aliquots of 50 μL of RBCs were then collected and incubated with 10 μL of FITC-CD36 mAb (Beckman Coulter) for 30 minutes at room temperature. After washings, we suspended the labelled RBCs in 0.5 mL of PBS/EDTA buffer and analysed them by flow cytometry (FACSCanto II, BD).

We performed DNA sequencing of the CD36 gene as previously described.[14]

TABLE 1 Clinical characteristics before and after two intra-uterine interventions

			Intra-uterine transfusion	Before intra-uterine transfusion		After intra-uterine transfusion	
No.	GA (weeks + day)	FBS	RBC (mL)	Hb (g L^{-1})	Hct (%)	Hb (g L^{-1})	Hct (%)
1	24 + 1	√	–	96	28.0	–	–
2	26 + 5	√	–	87	25.2	–	–
3	27	√	30	81	24.1	105	30.5
4	29 + 2	√	45	92	26.7	122	34.9

Note: No.: Sequence of treatment; 1, 2: only cordocentesis, 3: the first intra-uterine transfusion, 4: the second intra-uterine transfusion.
Abbreviations: FBS, fetal blood sample by cordocentesis; GA, gestational age; Hb, haemoglobin, Hct, haematocrit; RBC, red blood cells.

2.5 | Identification of anti-CD36 antibodies

Maternal serum was tested with CD36-positive platelets from group-O blood donors by flow cytometry to confirm the presence of anti-CD36 abs. Briefly, 50 μL aliquots of maternal serum or serum from healthy donors were incubated with a 50 μL platelet suspension for 30 minutes at 37°C. After two washings with 4 mL of PBS/EDTA buffer, sensitised cells were stained with FITC-conjugated anti-human IgG (at a dilution of 1:50; DAKO, Hamburg, Germany) for 30 minutes at 4°C, washed and analysed by flow cytometry as described above.

2.6 | Purification of IgG and anti-CD36 antibodies from serum

IgG was purified from maternal serum or from donated healthy blood by using a Melon Gel IgG Spin Purification Kit as recommended by the manufacturer (Thermo Scientific). We concentrated the diluted and purified IgG using Slide-A-Lyzer Dialysis Cassettes (10K MWCO; Thermo Scientific) or used the IgG directly for further experiments (see below). Diluted IgG was absorbed consecutively with non-transfected and CD36-transfected HEK293 cells to isolate anti-CD36-specific abs. Briefly, 100 μL of diluted IgG was incubated with 10^5 washed non-transfected HEK293 cells for 30 minutes at 37°C. After centrifugation (10 000*g* for 5 minutes), the supernatant was transferred into a new tube containing 10^5 washed CD36-transfected HEK293 cells for 30 minutes at 37°C. After washing twice with PBS-BSA (bovine serum albumin) buffer, bound abs were eluted with 1 M glycine (pH 2.7) elution buffer and subsequently neutralised with 15 μL of 1 M Tris-buffer (pH 9.0) as previously described.[16] Abs were extensively dialysed against DPBS overnight at 4°C, and we measured the IgG concentration with a photometer (Implen, Munich, Germany). Purified abs were then used in subsequent experiments (see below).

2.7 | Generation of HSCs

We selected healthy, mature placenta without obstetric complications at the First Affiliated Hospital of Sun Yat-Sen University. This study was approved by the medical ethics committee of Guangzhou Blood Center (GZBC2019-38), and informed consent was signed by the women (GZBC201903-00). Twenty ml of EDTA-anticoagulated umbilical cord blood was isolated during delivery and stored at 4°C until use. Blood was layered carefully onto a Ficoll gradient (1.077 g mL^{-1}) in a volume ratio of 1:1 to 1:2 and centrifuged at 800 g for 30 minutes. Mononuclear cells were isolated and washed twice using 1 mL of IMDM medium (STEMCELL, Vancouver, Canada) and centrifuged at 400 g for 5 minutes. Aliquots of 10^5 cells were labelled with 25 μL of FITC-labelled mAb against CD34 (Ebioscience, San Diego) and subsequently sorted by flow cytometry (MoFlo Astrios EQ; Beckman Coulter).[17] Purified CD34+ cells were cultured in serum-free stem cell medium (StemSpan SEFM II, STEMCELL) for 5 to 7 days and then analysed by flow cytometry to confirm purity. After washing twice with DPBS (Hyclone, South Logan) containing 0.1% BSA (DPBS-BSA, Biofroxx, Einhausen, Germany) and centrifuging at 400 g for 5 minutes, 1 to 5×10^5 cells were suspended in 500 μL of DPBS-BSA, stained with 25 μL of FITC-labelled mAb specific for CD34 and analysed by flow cytometry as described above.

2.8 | CFU-E/BFU-E cell clonogenicity assay

We inoculated aliquots of sorted HSCs (10^4) in 1 mL of culture medium (MethoCult H4435 erythroid-oriented medium, STEMCELL) in each well of 6-well culture plates (Corning, New York) and cultured them (37°C, 5% CO_2) in the presence of PBS (background), purified IgG (negative control) or IgG anti-CD36 abs derived from maternal serum at a concentration of 2 μg mL^{-1} for 14 days. The numbers of CFU-E/BFU-E clones in every well (totalling 9.6 cm^2) were all counted blindly by microscopic analysis by two independent individuals.

2.9 | Apoptosis assay

Aliquots of 10^4 sorted HSCs in 1 mL of culture medium (MethoCult H4435 erythroid-oriented medium, STEMCELL) were inoculated in each well of 6-well culture plates (Corning) and cultured (37°C, 5% CO_2) with PBS (background), IgG from normal individual as controls or with purified anti-CD36 abs (2 μg mL^{-1}) for 24 hours. We evaluated apoptosis with an Annexin V-FITC/PI kit (MultiSciences, Hangzhou, China). Briefly, 1 to 5×10^5 cells were collected, washed with DPBS at 400 g for 5 minutes and resuspended in 500 μL of binding buffer

(1×). After adding 5 μL of FITC-labelled annexin V and 10 μL of propidium iodide, we analysed the numbers of apoptotic cells by flow cytometry as shown above (FACSCanto II).

2.10 | Statistical analysis

All experiments were performed at least thrice, and results are expressed as mean ± SD, unless otherwise stated. GraphPad Prism 5.0 software (GraphPad Software) was used for statistical analysis. One-way analysis of variance (ANOVA) was used to analyse the data, and post-hoc comparisons using the Tukey test were made. *P* values of <.05 were considered statistically significant.

3 | RESULTS

In Figure 1A, we describe the analysis of CD36 antigen in both the mother and fetus. When platelets and monocytes from the mother were tested with an anti-CD36 mAb by flow cytometry, we found no reaction, indicating a type-1 CD36 deficiency; in contrast, fetal platelets and fetal monocytes showed positive reactions. When we sequenced the nucleotides of the coding region of the CD36 gene, we detected a two-nucleotide deletion (329-330 *del*AC) located in exon 5 in the maternal CD36 gene (Figure 1B). These deletions lead to a premature stop codon at amino acid 110, underlying type-1 CD36 deficiency.[18]

Antibody screening against HPA, CD36 and HLA class I antigens showed only positive reactions with CD36 and HLA class I abs. However, other HPA alloantibodies were undetectable. In addition, screening of alloantibodies against RBCs was negative, indicating that the mother only developed anti-CD36 and anti-HLA class I abs during pregnancy (at 23 and 26 weeks of gestation). Analysis of umbilical cord blood samples showed no reaction with CD36 at 23, 26 and 28 weeks of gestation; however, anti-CD36 abs bound to fetal platelets could be detected after elution (Figures 2 and S1, left panel). These results indicated that anti-CD36 abs played a role in the development of HF in this case of FNAIT.

HLA typing showed that the mother carried HLA A*24.02, A*33.03; HLA B*54.01, B*58.01; and C*0102, C*0302 and that the fetus inherited HLA A*11.01, A*33.03; HLA B*15.01, B*58.01; and C*0102, C*0302. Anti-HLA B*15:01 abs were only weakly detected in maternal serum, and we obtained negative results when we analysed those antibodies that bound to fetal platelets for HLA specificity (data not shown).

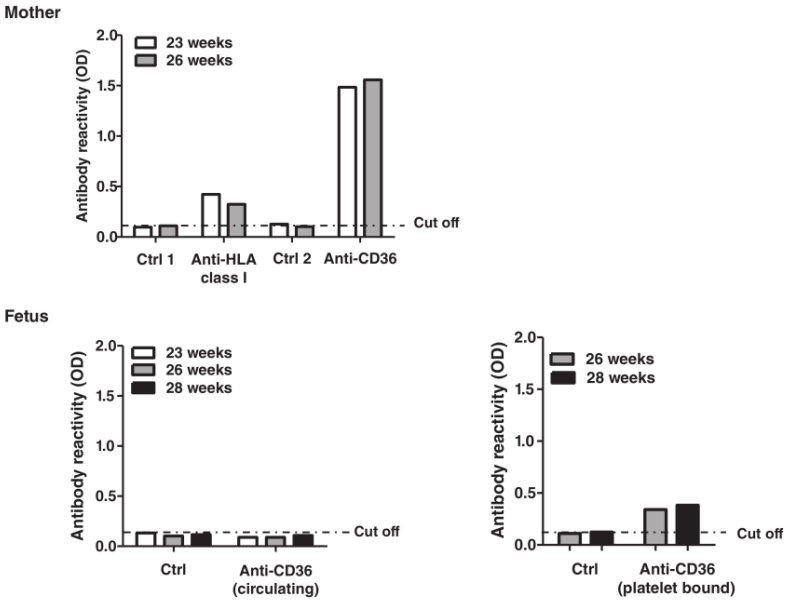

FIGURE 2 Detection of anti-CD36 and anti-HLA class I antibodies by ELISA. A, Platelet-reactive antibodies were screened using a commercial ELISA Kit (PAKPLUS). In maternal serum, antibodies against CD36 and HLA class I antigens were found at 23 and 26 weeks of gestation age as indicated (*top panel*). In contrast, no anti-HLA and anti-CD36 antibodies were detected in serum from the fetus (bottom, *left panel*); however, anti-CD36 bound to fetal platelets were identified after elution (bottom, *right panel*). Ctrl represents the negative controls for anti-HLA class I and anti-CD36 antibodies, respectively

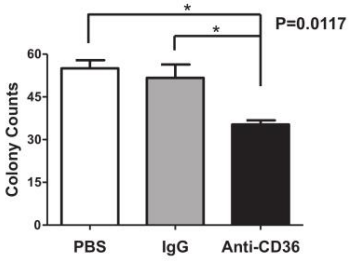

FIGURE 3 Analysis of CFU-E/BFU-E cell colonies treated with anti-CD36 antibodies. HSCs were co-incubated either with (1) PBS, (2) purified IgG (2 μg mL^{-1}) or (3) purified anti-CD36 IgG (2 μg mL^{-1}) in 6-well plates for 14 days. The numbers of CFU-E/BFU-E cell clones were then counted by microscopic analysis. The experiment was repeated thrice (mean ± SD). We analysed the data statistically with one-way analysis of variance (ANOVA) and made post-hoc comparisons using the Tukey test. *$P < .05$

Although CD36 expression was initially reported only on erythroblasts and not on mature RBCs, recent evidence showed that CD36 expression was detected on mature RBCs under trauma-haemorrhagic shock syndrome.[19] However, in our case, analysis of fetal RBCs isolated from cord blood with FITC-labelled mAb against CD36 showed a negative result (Figure S1, right panel). These results suggested that anaemia and HF found in this case may not due to the haemolysis of RBCs as fetal mature RBCs could not bind to anti-CD36 abs.

To evaluate our hypothesis further, we investigated the role of anti-CD36 abs on erythropoiesis, and we isolated the IgG fraction from maternal serum and subsequently purified specific anti-CD36 abs by absorption with non-transfected HEK293 cells prior to incubation with CD36-transfected HEK293 cells. Bound anti-CD36 abs were then eluted and used for further tests.

When HSCs isolated from MNCs derived from umbilical cord blood were sorted using fluorescence-labelled anti-CD34 mAb by flow cytometry, we observed that approximately 1.41% (1.36% ± 0.68%, n = 3) of CD34+ cells could be isolated and expanded further

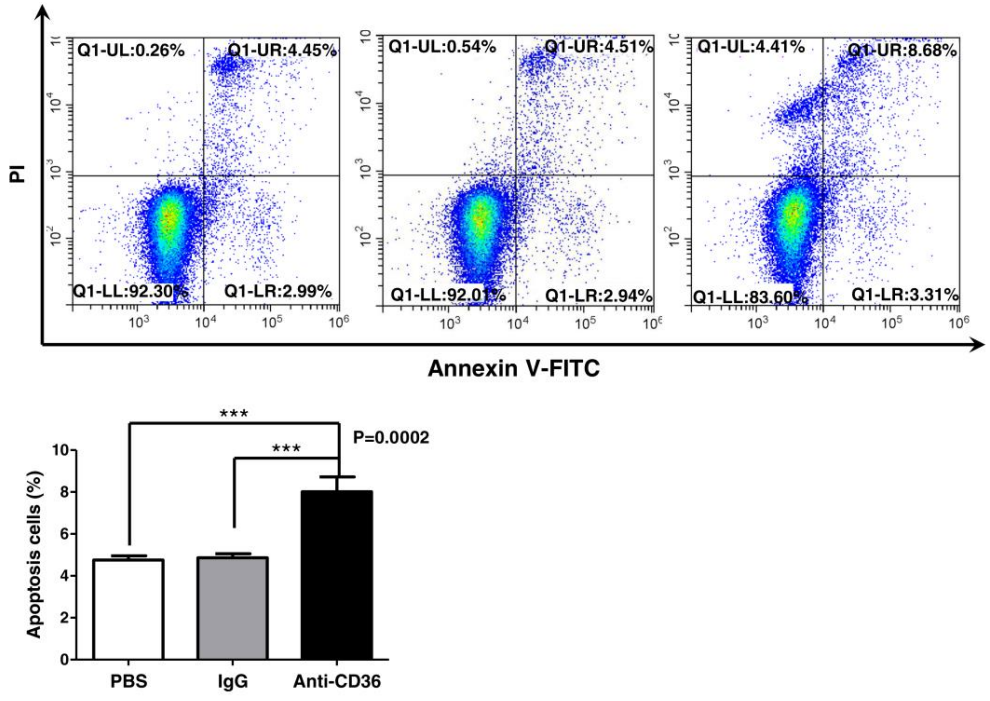

FIGURE 4 Flow cytometric analysis of apoptotic HSCs treated with anti-CD36. We incubated HSCs with either PBS, purified IgG (2 μg mL^{-1}) or purified anti-CD36 antibodies (2 μg mL^{-1}) for 24 hours. After staining with annexin V and propidium iodide, cells were analysed with flow cytometry. Double-stained cells were considered apoptotic. The upper panel shows representative results, and the lower panel shows the summarised data of three time-independent experiments. We analysed the data statistically with one-way analysis of variance (ANOVA) and the Tukey test for multiple comparisons. ***$P < .001$

TABLE 2 FNAIT cases associated with anti-CD36 antibodies

Cases	Platelets (10^3 μ L^{-1})[a]	Haemoglobin (g dL^{-1})[a]	Clinical symptoms	Mutations of CD36 gene	Reference
1	15	11.9	Gastrointestinal haemorrhage, severe anaemia, thrombocytopenia	—	[3]
2	40	—	Unremarkable	Homozygous T1264G	[20]
3	60	—	Unremarkable	Homozygous exons1-3 *del*	
4	<10	—	Petechial haemorrhages	Homozygous T1264G	
5	50	—	Unremarkable	—	
6	47	4.5	Hydrops	Heterozygous C1366T and 1438-1449*del*12bp	[4]
7	108	3.9	Hydrops	Heterozygous C1366T and 1438-1449*del*12bp	
8	106	18.2	Thrombocytopenia with cephalhaematoma	Heterozygous exon4 skip and exon 9 skip	[7]
9	16	4.8	Hydrops, severe anaemia and thrombocytopenia	Homozygous 329-330*del*AC	[6]
10	84	9.6	Hydrops, anaemia	Homozygous 329-330*del*AC	Current report

Note: —, data not available.
[a]Platelets and haemoglobin were tested at birth (cases 1-8), 27 + 1 weeks of gestation (case 9) and 24 + 1 weeks of gestation (case 10).

(Figure S2). After two to three passages (5-7 days), 84.09% (83.21% ± 3.08%, n = 3) of HSCs were CD34+, and 30.39% (31.02% ± 1.77%, n = 3) were stained as CD36-positive cells (Figure S3).

These cells were subsequently cultured with purified anti-CD36 abs, normal IgG as control (concentration, 2 g mL^{-1}) and PBS as background, and we counted the numbers of CFU-E/BFU-E cells after 14 days (Figure 3). In comparison to non-treated (55.00 ± 5.00, n = 3) and IgG-treated HSCs (51.67 ± 8.15, n = 3), we observed a significant reduction in CFU-E/BFU-E cell formation (35.33 ± 2.05, n = 3, P = .0117).

To further clarify this phenomenon, we performed an apoptosis assay (Figure 4), and in comparison to the controls, we counted a higher percentage of apoptotic cells that were double-stained with annexin V and PI when HSCs were incubated with purified anti-CD36 abs for 24 hours (8.01% ± 0.71%, n = 3). In contrast, only 4.86% ± 0.20% (n = 3) of cells were apoptotic when we incubated them with purified IgG alone, as well as 4.75% ± 0.20% (n = 3) with PBS (background).

4 | DISCUSSION

FNAIT is relatively rare bleeding disorder, although the clinical consequences may be severe. A fetus/newborn that is thrombocytopenic due to FNAIT is at risk of intra-cranial haemorrhage (ICH) that may result in lifelong disability or death. In FNAIT, anti-CD36 abs cause not only thrombocytopenia and ICH, but they also cause recurrent early fetal loss and hydrops. Several cases of FNAIT caused by anti-CD36 abs associated with HF (Table 2) have been reported; however, little is known regarding the pathological mechanism underlying the induction of HF by these abs.

HF is a clinical condition in which excessive fluid accumulation in the extravascular compartment of the fetus leads to widespread oedema. Immune HF is commonly associated with removal and destruction of fetal Rh(+) RBCs by maternal anti-RhD abs, which leads to anaemia and subsequently causes cardiac decompensation and hydrops. However, the pathophysiology of HF is decidedly more complex.[21]

CD36 is found on late erythroblasts but not on mature RBCs and reticulocytes.[11] Therefore, the mechanism of anti-CD36 antibody-induced HF must be different from classical HF caused by anti-RhD abs. Investigators have previously reported that protein-A purified IgG from maternal serum containing anti-CD36 abs suppressed CFU-E colony formation, indicating that anti-CD36 abs can induce anaemia by affecting haematopoietic stem (progenitor) cells.[4] However, a possible role(s) for other IgG abs present in maternal serum cannot be excluded.

In our study, we characterised a case of FNAIT with HF associated with anti-CD36 abs. To further prove our hypothesis that anti-CD36 abs affect HSCs, we used purified anti-CD36 abs that we isolated by an absorption/elution approach using CD36-transfected HEK293 cells. In accordance with the previous observation, our results showed that purified anti-CD36 abs significantly suppressed CFU-E/BFU-E cell formation.

It has been reported that CD34+ HSCs undergo apoptosis when incubated with anti-Fas antibody and serum from an aplastic anaemia patient.[22] Likewise, we found a significantly increased number of apoptotic CD34+ HSCs when they were incubated with purified anti-CD36 abs.

Thus, although the exact mechanism underlying the interaction of anti-CD36 abs with haematopoietic stem cells remains unclear, apoptosis of the progenitor cells by anti-CD36 abs is likely the primary reason for fetal anaemia and consequent HF.

Little is currently known with respect to antenatal treatment of anti-CD36-mediated FNAIT. As IVIG treatment was declined by our patient, we conducted intra-uterine transfusions to prevent severe anaemia and mild thrombocytopenia. As anaemia appeared to be a primary component in the development of HF in our case, we performed the intra-uterine transfusions twice with RBCs alone (after 27 and 29 gestational weeks; see Table 1). Administration of intra-uterine transfusion with washed RBCs initially improved fetal anaemia (Table 1). At 36 + 4 weeks of gestation, a healthy female baby (2600 g) was delivered, with an Apgar score of 10, and her haemoglobin (152 g L^{-1}), HCT (46%) and platelet count (253 x 10^9 L^{-1}) were normal at birth.

In conclusion, our findings provide additional information regarding the mechanism underlying the association of HF with anti-CD36 abs. In the present case, intra-uterine transfusions with RBCs alone alleviated fetal anaemia and improved fetal survival. However, a role for anti-CD36-induced thrombocytopenia in HF requires further elucidation.

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Y.W. performed the research, analysed the data and wrote the manuscript. D.C. analysed the data and wrote the manuscript. Y.F. designed the study together with X.Y. and C.L., X.X. and W.X. helped perform the research. S.S., W.X. and Y.F. helped interpret the data and reviewed the manuscript.

CONFLICT OF INTEREST

The authors have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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3.5. Improvement of anti-CD36 antibody detection via monoclonal antibody immobilization of platelet antigens assay by using selected monoclonal antibodies

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Improvement of Anti-CD36 Antibody Detection via Monoclonal Antibody Immobilization of Platelet Antigens Assay by Using Selected Monoclonal Antibodies

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Antibodies against human CD36 are responsible for several immune-mediated disorders. The detection of anti-CD36 antibodies using the standard monoclonal antibody (mAb) immobilization of platelet antigens (MAIPA) assay is hampered by a high frequency of false-negative results, most likely due to competitive inhibition of the mAb used as the capture antibody. We generated a panel of mouse mAbs against CD36 and seven hybridomas (GZ-3, GZ-13, GZ-70, GZ-143, GZ-413, GZ-507, and GZ-608), which were selected for MAIPA assays, as they reacted with mouse and human CD36. Fourteen anti-CD36 sera were assayed; all of which showed a positive reaction in a PakPlus (Immucor GTI Diagnostics, Inc., Waukesha, WI, USA) ELISA-based screening (optical density: 0.257–2.292). When the reference anti-CD36 mAb FA6-152 was used in the MAIPA assay, only 6/14 (42.9%) sera displayed a positive reaction. In contrast, anti-CD36 antibodies were detected in 13/14 (92.9%) sera when GZ-70 and GZ-608 mAbs were used. This significant improvement resulted in the identification of anti-CD36 antibodies by an antigen capture assay. Since patient's platelets possibly carrying rare native antigens are used, this method will facilitate the identification of new platelet antibodies against CD36 that are involved in immune-mediated thrombocytopenia and other diseases, such as transfusion-related acute lung injury.

Key Words: CD36, Monoclonal antibodies, Antigen capture assays

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CD36 is expressed in a variety of cells, including platelets, monocytes, macrophages, and endothelial and epithelial cells [1, 2]. There are two types of CD36 deficiencies: type I, which lacks CD36 on both platelets and monocytes, and type II, which lacks CD36 on platelets alone. Only type I individuals are at risk of developing isoantibodies against CD36 [3]. The prevalence of type I varies among different ethnic groups; it is ~3.0% in the African population, 1.0% in the Japanese population, and ~0.5% in the Chinese population [4-6]. Anti-CD36 isoantibodies (known as anti-Nak[a]) can cause different immune-mediated bleeding disorders, including platelet transfusion refractoriness (PTR), fetal neonatal alloimmune thrombocytopenia (FNAIT), and posttransfusion purpura (PTP) [7-9]. Transfusion-related acute lung injury (TRALI) associated with anti-CD36 antibodies (Abs) has also been reported [10].

Various methods have been developed for anti-CD36 Ab de-

tection, including flow cytometry and antigen capture assays using platelets, such as the monoclonal antibody (mAb) immobilization of platelet antigens (MAIPA) assay, or stably transfected cell lines as the target [4, 6, 11-13]. Additionally, a solid-phase ELISA using purified CD36 is commercially available (PakPlus; Immucor GTI Diagnostics, Inc., Waukesha, WI, USA). In contrast to protein-based methods, antigen capture assays allow the testing of recipient serum with donor platelets carrying native, individual CD36 antigens and *vice versa* (crossmatch). However, these assays depend on the use of a capture mAb against CD36 [14]. Due to competitive inhibition of CD36 mAb by human anti-CD36 Abs in patients, the rate of false-negative reactions is high [11, 13]. To address this, we developed a panel of mAbs against CD36 and selected noncompetitive mAbs for anti-CD36 Ab detection by an antigen capture assay.

Male CD36$^{-/-}$ mice (B6.129S1-*Cd36*^{tm1Mfe}/J; Jackson Laboratory, Bar Harbor, ME, USA) were immunized with 5×10^6 HEK293T cells expressing mouse CD36 intraperitoneally three times on days 1, 8, 15; a booster was administered once on day 19. Splenocytes from the immunized mice were fused with SP2/0-Ag14 mouse myeloma cells (American Type Culture Collection, Manassas, VA, USA). Hybridomas were selected on hypoxanthine-aminopterin-thymidine medium (Life Technologies, Darmstadt, Germany) and screened by flow cytometry (BD FACSCanto II; BD Biosciences, San Jose, CA, USA) using HEK293T cells expressing mouse or human CD36. The study was conducted from July 2014 to May 2020 in the Animal Center of Sun Yat-Sen University, Guangzhou, China, following approval by the University's Animal Ethical and Welfare Committee (IACUC-2014-0303).

Seven out of 25 clones that produced strong anti-CD36 Abs (termed GZ-3, GZ-13, GZ-70, GZ-143, GZ-413, GZ-507, and GZ-608) were selected. Flow cytometry results showed that all

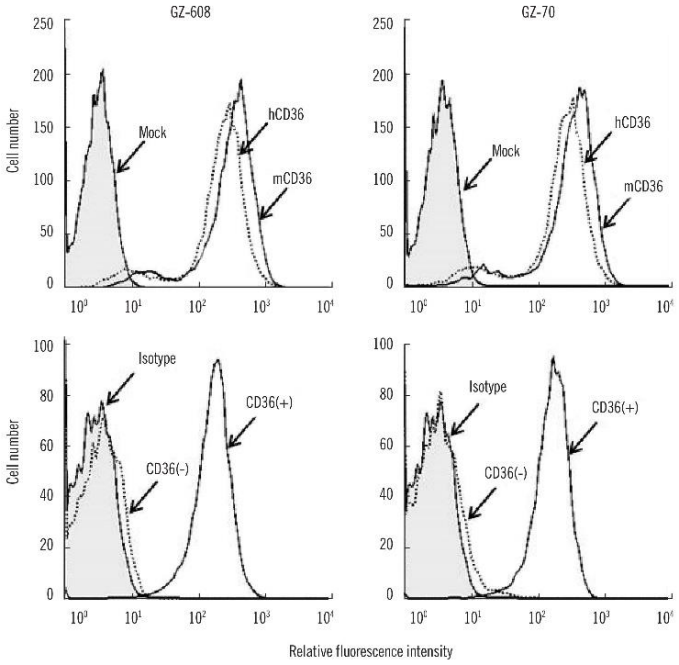

Fig. 1. Flow cytometry analysis of monoclonal antibodies (mAbs) against CD36 using transfected HEK293T cells and platelets. Upper panels: mAbs (GZ-608 and GZ-70) were incubated with mock, hCD36, and mCD36. Bound antibodies were detected using fluorescence-conjugated goat anti-mouse IgG and analyzed using flow cytometry. Lower panels: CD36$^-$ platelets from a healthy blood donor and CD36$^-$ platelets from a CD36-deficient donor were incubated with GZ-608 or GZ-70 mAb. "Mock" stands for C220T variant CD36 construct-transfected HEK293T cells, "hCD36" for human CD36-transfected HEK293T cells, "mCD36" for mouse CD36-transfected HEK293T cells, and "Isotype" for mouse IgG1.

seven mAbs reacted with both mouse and human CD36 expressed on HEK293T cells, underscoring the high sequence similarity (85.0%) between mouse and human CD36 [15]. In the control experiment, no reaction was observed with HEK293T cells transfected with a CD36 vector that carries the C220T variant, which leads to a premature stop codon [6]. These results were confirmed by flow cytometry using normal platelets and platelets from CD36-negative individuals. Fig. 1 shows the flow cytometry results for GZ-70 and GZ-608 mAbs.

Fourteen anti-CD36 sera from eight patients with PTR, four with FNAIT, and two healthy donors were first tested using the PakPlus ELISA kit. Informed consent was obtained from all individuals. These sera were analyzed by MAIPA assays using the reference anti-CD36 mAb FA6-152 and our mAbs against CD36. Briefly, 4×10^7 platelets were incubated with 25 µL of serum at 37°C for 30 minutes. After washing, 10 µL of anti-CD36 mAb (20 µg/mL) was added, and the samples were incubated at 37°C for 30 minutes. Then, the platelets were washed and solubilized, and bound anti-CD36 Abs were detected using 100 µL of *o*-phenylenediamine (Dako, Glostrup, Denmark) as a substrate [16]. After 15 minutes, the reaction was stopped with 2.5 N H_2SO_4, and the optical density at 492 nm was measured using an ELISA reader (Multiskan FC; Thermo Scientific, Shanghai, China).

Fig. 2A shows the reactivity of one anti-CD36 serum (serum number 8; Table 1) with different anti-CD36 mAbs. Only 3/7 mAbs (GZ-13, GZ-70, and GZ-608) were capable of detecting the anti-

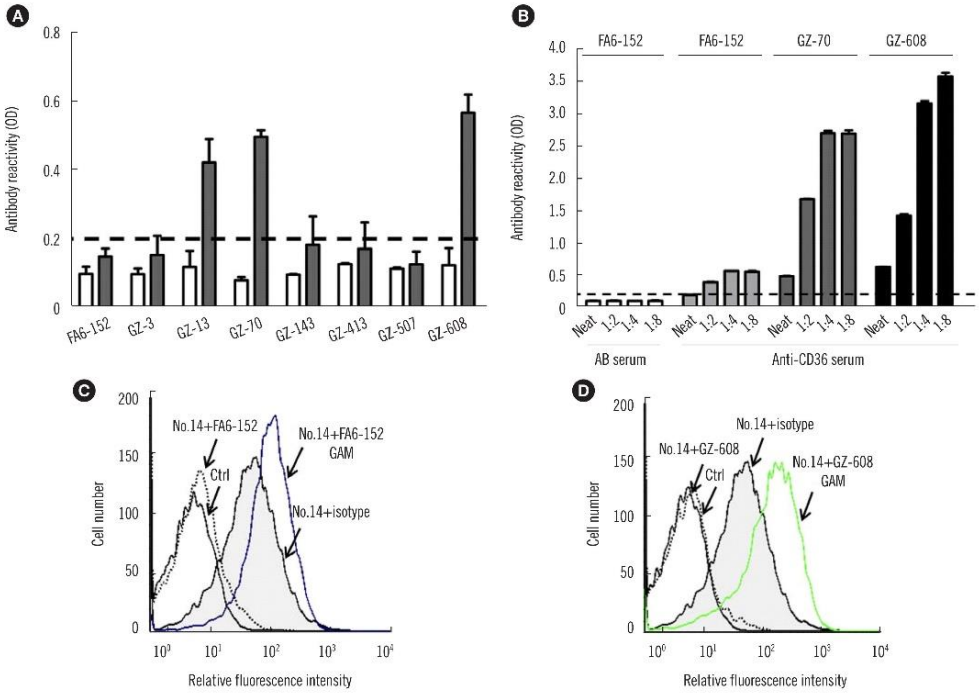

Fig. 2. Characteristics of anti-CD36 serum in MAIPA and binding assays using different capture mAbs. (A) Platelets were first incubated with anti-CD36 serum (serum number 8, gray columns) and AB serum (white columns) and then with eight anti-CD36 mAbs (20 µg/mL) as indicated and analyzed using the MAIPA assay. (B) Platelets were incubated with anti-CD36 serum at different dilutions (neat, 1:2, 1:4, and 1:8) and the anti-CD36 mAbs FA6-152, GZ-70, and GZ-608 (20 µg/mL), as indicated. The cut-off for each assay was determined by analyzing AB sera from healthy blood donors (N=8, white columns). The reaction was considered positive when the result was >0.200 (cut-off; mean value±3 SDs; N=8; dotted line). (C, D) Effects of the mAbs FA6-152 and GZ-608 on the binding of serum number 14 to $CD36^-$ platelets as determined using flow cytometry. "Isotype" stands for mouse IgG1, "Ctrl" for AB serum+mAb, and "GAM" for fluorescence-labeled goat anti-mouse IgG antibody.
Abbreviations: OD, optical density; MAIPA, monoclonal antibody immobilization of platelet antigens; mAbs, monoclonal antibodies.

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CD36 Abs present in this serum (Fig. 2A). False-negative results were obtained for five mAbs, including FA6-152.

The three reactive mAbs, GZ-70, GZ-608, and FA6-152, were used to analyze our serum cohort (N=14). Anti-CD36 Abs were detected in only 6/14 (42.9%) of the sera tested with FA6-152 mAb. In contrast, anti-CD36 Abs were detected in 13/14 (92.9%) sera using GZ-70 or GZ-608 mAb (Table 1). An additional serum cohort comprising four sera from patients with FNAIT (N=1) and TRALI (N=3) was tested using our assays [10, 17]; all four sera showed positive reactions (Supplemental Data Table S1).

To exclude other factors that may decrease MAIPA assay sensitivity, such as the prozone effect (i.e., an unfavorable ratio between human and mouse anti-CD36 Abs), serum numbers 8, 11, and 12 (Table 1) were retested at different dilutions (neat, 1:2, 1:4, 1:8). A weak positive reaction was detected in serum number 8 with FA6-152 mAb after dilutions. Significantly increased reactivity was found with GZ-70 and GZ-608 mAbs after dilutions (Fig. 2B). In contrast, decreased reactivity was detected with serum numbers 11 and 12 (data not shown). These results showed that not only the choice of mAb but also dilution of the test serum can increase MAIPA assay sensitivity.

Interestingly, a negative result was obtained for serum number 14 (Table 1). To clarify this phenomenon, the direct inhibitory effect of anti-CD36 mAbs on anti-CD36 Ab binding to platelets was analyzed. Fifty microliters of CD36-positive platelets (40×10^5 cells) was incubated with 25 μL of serum (at 1:16 dilution) at 37°C for 30 minutes and then with 25 μL of anti-CD36 mAb (20 μg/mL). After washing with EDTA/phosphate-buffered saline (PBS) (Dulbecco's PBS containing 10 mM EDTA, pH 7.0–7.2; Gibco BRL, Waltham, MA, USA), 50 μL of fluorescein-conjugated goat anti-human IgG or goat anti-mouse IgG (Jackson, West Grove, PA, USA) was added, and the samples were incubated for another 30 minutes. After washing, bound platelets were suspended in 0.5 mL of EDTA/PBS and analyzed by flow cytometry. The binding of anti-CD36 Abs in serum number 14 to platelets was disrupted upon incubation with mAb FA6-152 (Fig. 2C); similar results were found with GZ-608 (Fig. 2D) and mAb GZ-70 (data not shown) mAbs. These results indicated that serum number 14 contained lower-avidity anti-CD36 Abs

Table 1. Reactivity of anti-CD36 sera in solid-phase ELISA (PakPlus) and/or MAIPA assays using three mAbs against CD36 (FA6-152, GZ-608, and GZ-70)

Anti-CD36 serum number	Age (yr)/ sex	Diagnosis	Optical density			
			PakPlus	mAb FA6-152	mAb GZ-608	mAb GZ-70
1	44/M	Healthy donor	0.989	0.293	0.667	0.516
2	50/F	Healthy donor	1.138	0.438	1.003	0.733
3	50/M	Hypercholesterolemia	0.663	*0.168*	0.655	0.540
4	69/F	Anemia	0.791	*0.200*	0.797	0.341
5	41/M	Thoracic aortic aneurysm	0.569	*0.145*	0.255	0.290
6	22/M	T-lymphoblastic cell tumor	1.812	1.615	3.143	2.654
7	35/F	Myelodysplastic syndromes	0.923	0.431	0.754	0.696
8	35/F	FNAIT (abortion)	0.835	*0.189*	0.624	0.481
9	30/F	FNAIT (abortion)	1.551	*0.020*	0.550	0.440
10	36/F	FNAIT (thrombocytopenia)	0.990	*0.200*	0.355	0.375
11	46/M	Alcoholic cirrhosis	2.292	0.340	0.384	0.367
12	44/F	Acute leukemia	0.745	*0.185*	0.582	0.411
13	35/F	FNAIT (thrombocytopenia)	0.257	0.289	0.543	0.633
14	56/M	Myelodysplastic syndrome	0.827	*0.199*	*0.143*	*0.158*
		Cut-off*	>0.160	>0.200	>0.200	>0.200
			14/14 (100.0%)	6/14 (42.9%)	13/14 (92.9%)	13/14 (92.9%)

*The cut-off for each assay was determined by analyzing AB sera from healthy blood donors (N=8). The reaction was considered positive when the result was greater than the cut-off value (mean value±3 SDs).
Negative reactions are indicated in italics.
Abbreviations: MAIPA, monoclonal antibody immobilization of platelet antigens; mAbs, monoclonal antibodies; FNAIT, fetal/neonatal alloimmune thrombocytopenia.

that can be displaced by higher-avidity anti-CD36 mAbs that bind to the same or similar epitopes; these results were particularly obtained due to a rigorous MAIPA washing procedure.

Most anti-CD36 mAbs, including FA6-152, OKM5, and 10/5, bind to epitopes within the domain that comprises amino acid residues 155–183 and prevents anti-CD36 Abs in serum samples from binding [15, 18]. To avoid this, the use of a MAIPA panel containing different mAbs is recommended, but which mAbs should be included in the panel has not been defined.

MAbs against human CD36 have been produced through the immunization of mice with human fetal erythrocytes (FA6-152), human monocytes (OKM-5), or human platelets (10/5) [15, 18-20]. We generated mAbs against mouse CD36 through immunization of CD36-deficient mice with mouse CD36-transfected HEK293T cells and selected those mAbs that cross-reacted with human CD36. Through this strategy, we presumably obtained mAbs that do not recognize the highly immunogenic region(s) of human CD36 (residues 155–183), which harbor the major epitopes of human anti-CD36 Abs. Hence, in contrast to FA6-152, some of our mAbs (e.g., GZ-70 and GZ-608) had a significantly higher MAIPA reactivity (42.9% vs. 92.9%), indicating that these capture Abs did not compete with anti-CD36 Abs present in the sera. The anti-CD36 mAb 13/10 binds to another domain that spans residues 30–76. This unique mAb did not inhibit the binding of human anti-CD36 Abs [15, 18].

Although the existence of an immunodominant region in CD36 (residues 155–183) has been reported, little is known about the exact location and diversity of epitopes recognized by polyclonal anti-CD36 Abs. That three mAbs showed differential inhibition suggests that at least two antigenic determinants exist. Additional information about the structure of the exact binding site of different anti-CD36 mAbs may help to prevent competitive inhibition.

The combination of antigen phenotyping (e.g., by flow cytometry) and Ab detection (e.g., by PakPlus) is reliable, convenient, and sufficient for the detection of isoantibodies against CD36. Given the existence of numerous naturally occurring single-nucleotide polymorphisms (SNPs) and the immunogenicity of CD36, individuals with normal (wild-type) CD36 expression may develop alloantibodies against CD36 due to alloimmunization with donor platelets carrying certain SNPs in certain cases, such as PTR and FNAIT. The existence of such cases can only be identified by crossmatch analysis (e.g., recipient serum vs. donor platelets), especially through glycoprotein specific immunoassay use.

In summary, improving MAIPA assays by applying selected noncompetitive mAb(s) will facilitate the screening of anti-CD36 Abs and the identification of new platelet antigens/Abs in patients with immune-mediated thrombocytopenia and other related disorders. However, further study in a larger cohort is necessary to evaluate the sensitivity of the MAIPA assay for the detection of anti-CD36 Abs.

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AUTHOR CONTRIBUTIONS

Fu Y and Santoso S designed the study and reviewed the data. Xu X, Chen D, Ye X, Xia W, Shao Y, Chen Y, Deng J, Ding H, Liu J, and Xu Y contributed to data collection and analysis. Xu X and Santoso S performed the data analysis and wrote the manuscript. All authors have read and approved the final manuscript.

CONFLICTS OF INTEREST

All authors declare that there are no conflicts of interest.

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Supplemental Data Table S1. Detection of anti-CD36 serum samples reported in other cohorts using MAIPA assays with the mAbs FA6-152, GZ-608, and GZ-70

Anti-CD36 serum number	Diagnosis	Optical density			Reference
		FA6-152	GZ-608	GZ-70	
15	FNAIT	*0.194*	0.503	0.554	[17]
16	TRALI	0.701	0.871	0.918	[10]
17	TRALI	1.203	1.755	1.389	[10]
18	TRALI	0.957	2.231	2.498	[10]
	Cut-off*	>0.200	>0.200	>0.200	

*The cut-off for each assay was determined by analyzing AB sera from healthy blood donors (N=8). The reaction was considered positive when the result was greater than the cut-off value (mean value±3 SDs).
Negative reactions are indicated in italics.
Abbreviations: MAIPA, monoclonal antibody immobilization of platelet antigens; mAbs, monoclonal antibodies; FNAIT, fetal/neonatal alloimmune thrombocytopenia; TRALI, Tranfusion-related acute lung injury.

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3.6. Fetal/neonatal alloimmune thrombocytopenia due to anti-CD36 antibodies: antibody evaluations by CD36-transfected cell lines

ORIGINAL RESEARCH

Fetal/neonatal alloimmune thrombocytopenia due to anti-CD36 antibodies: antibody evaluations by CD36-transfected cell lines

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BACKGROUND: Isoantibodies against CD36 (platelet glycoprotein 4), developed in Type I CD36-deficient mothers are frequently reported as the cause of fetal/neonatal alloimmune thrombocytopenia in the Asian population. Therefore, further detailed characterization of anti-CD36–mediated fetal/neonatal alloimmune thrombocytopenia is warranted. Here, we report the characterization of a patient with fetal/neonatal alloimmune thrombocytopenia in a Taiwanese family caused by anti-CD36 isoantibodies using a novel antigen-capture method.
STUDY DESIGN AND METHODS: Platelets and monocytes were analyzed for CD36 expression by flow cytometry. Sequencing analysis of the CD36 gene was performed to identify the mutation underlying the CD36 deficiency. Stable transfected human embryonic kidney HEK293 cells expressing recombinant CD36 were established. These cells were used for the characterization of anti-CD36 isoantibodies by flow cytometry, immunoprecipitation, and antigen-capture assay.
RESULTS: Flow cytometry analysis revealed a total absence of CD36 on both platelets and monocytes of the mother (Type I CD36-deficient) caused by heterozygous deletions of the CD36 gene (332_333delCA and c.1254 + 6_1254 + 11delTATTTG). Analysis of maternal serum with CD36-transfected HEK293 cells by flow cytometry, immunoprecipitation, and antigen-capture assay demonstrated the presence of anti-CD36 isoantibodies in maternal serum. Interestingly, this antibody could not be detected by the monoclonal antibody immobilization of platelet antigens assay when anti-CD36 monoclonal antibody (clone FA6-152) was used as the capture antibody.
CONCLUSION: This case reemphasizes the role of anti-CD36 isoantibodies on the pathomechanism of fetal/neonatal alloimmune thrombocytopenia. The fact that the monoclonal antibody immobilization of platelet antigens assay does not seem to be reliable for the identification of all anti-CD36 antibodies indicates that screening of anti-CD36 isoantibodies by a monoclonal antibody-independent method, as presented here, should be considered.

Fetal/neonatal alloimmune thrombocytopenia (FNAIT), which occurs in from 1 of 800 to 1 of 1000 live births, is the most common cause of severe thrombocytopenia and intracranial hemorrhage in the fetus and in term newborns.[1,2] FNAIT is caused by maternal antibodies, which recognize paternal-derived alloantigens on fetal platelets, leading to platelet destruction. In Caucasians, more than 75% of FNAIT cases are induced by alloantibodies against human platelet antigen-1a (HPA-1a),[3,4] whereas the most common antibodies causing FNAIT in Japanese are anti-HPA-4b alloantibodies.[5] However, to date, this alloantibody specificity has not been found in Taiwan or China. In contrast, isoantibodies against CD36 (platelet glycoprotein 4) developed

ABBREVIATIONS: FNAIT = fetal/neonatal alloimmune thrombocytopenia; HLA = human leukocyte antigen; HPA = human platelet antigen; MAIPA = monoclonal antibody immobilization of platelet antigens.

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in Type I CD36-deficient mothers are frequently reported as the cause of FNAIT in Chinese populations.[6]

CD36 is not found exclusively on platelets but is also present on other blood cells, including platelets, monocytes, macrophages, erythroid precursors, as well as on endothelial cells.[7] There are two types of CD36 deficiency; Type I, with a total absence of CD36 expression on both platelets and monocytes, and Type II, which lacks CD36 on platelets but not on monocytes.[8] CD36 deficiencies are more frequent in Asians (approximately 3%-11%) and Africans (approximately 7%-8%) than in Caucasians (<0.3%).[6]

The clinical importance of anti-CD36 isoantibodies in the development of FNAIT has been reported in different populations.[9-13] Currently, the antigen-capture assay represents the gold standard for detecting platelet antibodies.[14] With this assay, platelet antigens captured by mouse monoclonal antibody are used as the target for platelet antibodies. Unfortunately, this approach does not seem to be reliable for the identification of all anti-CD36 isoantibodies.

In the current study, we describe one case of anti-CD36–mediated FNAIT and establish a novel antigen-capture assay using CD36-transfected mammalian cells to identify the presence of anti-CD36 isoantibodies in the maternal serum.

CASE REPORT

The mother was a 26-year-old Taiwanese woman without any history of blood transfusion. Her obstetric history was uneventful, and this was her second pregnancy. The first pregnancy ended in an abortion. The neonate was found to have developed thrombocytopenia with a platelet count of 36,000/μL at 4 days of age. However, neither petechial bleeding nor anemia (hemoglobin 16.7 g) was observed. The birth weight was 3500 g. The baby was hospitalized without a final diagnosis of neonatal infection, whereas the neonatal hyperbilirubinemia, without any evidence of neonatal hemolytic disease, was recovered soon by phototherapy. Bacterial blood and urine were culture negative. A brain echogram showed no abnormalities. Based on this clinical picture, FNAIT was suspected, and intravenous immunoglobulin 1 g/kg was given at 8 days of age. Subsequently, the platelet count rose to 45,000/μL. The platelet count was 160,000/μL 14 days after birth, when the infant was discharged from hospital.

MATERIALS AND METHODS

Genotyping of HPAs

Genomic DNA was isolated by using a blood kit (QIAamp; Qiagen) according to the manufacturer's instructions, with the blood sample collected in an acid citrate dextrose tube. Genotyping of HPA-1, HPA-2, HPA-3, HPA-4, HPA-5, and HPA-15 was performed by single-specific primer polymerase chain reaction (PCR), as previously described.[15,16]

Analysis of maternal serum by crossmatch

Crossmatch was performed with the solid-phase red blood cell (RBC) adherence test, as previously described.[17] Briefly, platelets adhering to a sensitized polystyrene microtiter plate were incubated with serum under a low-ionic-strength condition. After washing, anti-immunoglobulin G (IgG)-coated indicator RBCs were added to the wells, and the plate was subsequently centrifuged. A confluent monolayer of RBCs indicated a positive reaction, whereas a defined RBC button in the center of the well indicated a negative result.

Flow cytometric analysis of CD36 expression on platelets and monocytes

CD36 expression on monocytes and platelets was measured in whole blood by using three-color flow cytometry, as previously described.[18] Briefly, 200 μL ethylene diamine tetra acetic acid (EDTA)-anticoagulated blood was incubated at room temperature for 30 minutes with 4 μL fluorescence-labeled monoclonal antibodies (Biolegend): allophycocyanin-conjugated anti-human CD36 (clone 5-271), fluorescein isothiocyanate (FITC)-labeled anti-human CD61 (clone VI-PL2), and phycoerythrin-conjugated anti-human CD14 (clone HCD14). After the addition of lysis solution (BD Biosciences), labeled cells were washed with 2 mL 10 mM EDTA/phosphate-buffered saline (PBS) and then fixed with 500 μL 1% paraformaldehyde in PBS/EDTA buffer. CD36 expression on platelets (CD61 positive) and monocytes (CD14 positive) was evaluated on a FACSCalibur cytometer using CellQuest acquisition and analysis software (BD Biosciences).

Nucleotide sequencing analysis of CD36 gene

Genomic DNA was extracted from EDTA-anticoagulated whole blood using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. In total, 12 PCRs were performed to amplify the entire coding region of the CD36 gene (Exons 3-14; National Center for Biotechnology Information identifier NM_000072.3). Aliquots of 30 to 100 ng DNA were amplified with 2 μM primers and 0.4 mM deoxynucleoside triphosphate using Simple Red DNA polymerase in a total volume of 12 μL (ABgene). The PCR cycle conditions comprised a pre-PCR denaturing step at 95°C for 2 minutes, followed by 35 cycles (denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 60 seconds), with a final extension of 7 minutes at 72°C. PCR products were analyzed in 2% agarose gel electrophoresis. After treatment with a pre-sequencing kit (USB), PCR products were directly

sequenced using the Big Dye Terminator version 3.1 cycle-sequencing kit (Applied Biosystems) in both directions on an automated ABI 3730 sequencer (Applied Biosystems) and analyzed using CodonCode Aligner software (Codon Code Corporation). The PCR products were cloned using TA-cloning protocol to confirm the mutations. Four positive clones were sequenced and analyzed in both directions.

Isolation of residual platelet mRNA (messager)

Platelets were isolated from 20-mL EDTA-anticoagulated blood using RNA-free tubes. After centrifugation at 120 × *g* for 30 minutes, the top three-quarters of platelet-rich plasma was isolated and washed three times using Phillips buffer (96.5 mM NaCl; 87.5 mM glucose; 1.1 mM EDTA; and 8.5 mM Tris, pH, 7.4). Subsequently, washed platelets were suspended in 250 μL sterile PBS. Total RNA was extracted using peqGOLD RNAPure (PEQLAB Biotechnologie). Full-length complementary DNA (cDNA) was synthesized using Ready-To-Go You-Prime First-Strand Beads and random hexamer as primer in a final volume of 33 μL (GE Healthcare).

Generation of stable CD36-transfected cell lines

Full-length CD36 cDNA from platelets was amplified by PCR using forward primer (5′-GGTGCTTAACACTAATTCACCTCC-3′) and reverse primer (5′-TTTTATTGTTTTCGATCTGCATGC-3′) under the following conditions: denaturation (30 sec at 95°C), annealing (50 sec, 54°C), and extension (2 min, 72°C) for 32 cycles, followed by a final extension (10 min, 72°C). The PCR products were purified from 1% agarose gels using a gel-extraction kit (Qiagen) and were ligated into pcDNA3.1/V5-His-TOPO vector with a T/A cloning strategy, as recommended by the manufacturer (Invitrogen), and then transformed into TOP10 competent *Escherichia coli* (Invitrogen). Positive clones were screened by PCR using CD36 forward primer and bovine growth hormone plasmid reverse primer (5′-TAGAAGGCACAGTCGAGG-3′) under the same PCR conditions described above. Subsequently, amplified plasmid from a selective positive clone was purified with the QIAprep Miniprep Kit (Qiagen) and validated by nucleotide sequencing analysis on a 3130 genetic analyzer (Applied Biosystems) before transfection.

Aliquots of 4×10^5 human embryonic kidney cells (HEK293T; American Tissue Culture Collection) in 1 mL medium were transfected with 1 μg plasmid using Effectene, as recommended by the manufacturer (Qiagen). After 3 days, the transfected cells were selected in culture medium containing Geneticin (800 μg/mL; Biochrom). Then, transfected cells that expressed high CD36 density were selected by flow cytometry (FACSAria; Becton Dickinson) and were further cultured or stored in liquid nitrogen as aliquots.

Characterization of anti-CD36 isoantibodies by three different methods

Flow cytometry

Fifty-microliter aliquots of platelet suspension (10^5/μL) from Blood Group O donors were incubated with 50 μL serum for 30 minutes at 37°C. After washing twice with 4 mL PBS/EDTA buffer, platelets were incubated with 50 μL of FITC-labeled rabbit anti-human IgG (dilution 1:50; Dako) for 30 minutes at room temperature. After washing, labeled platelets were suspended in 500 μL PBS/EDTA buffer and analyzed by flow cytometer (FACSCalibur; Becton Dickinson). For the analysis of isoantibodies with CD36-transfected HEK293T cells, aliquots of 2.5×10^5 cells were incubated with 20 μL serum for 30 minutes at 4°C. After washing, 50 μL FITC-labeled secondary antibodies (Dako) were added for 30 minutes at 4°C and were measured as described above. Nontransfected HEK293 cells were used as a negative control.

Immunoprecipitation

Immunoprecipitation was performed with biotinylated, CD36-transfected cells. Briefly, aliquots of 10^6 transfected cells or nontransfected HEK293 cells (as a control) were incubated with 50 μL of maternal serum for 1 hour at 37°C. After washing twice, the cells were solubilized in lysis buffer and subsequently centrifuged at 15,000 × *g* for 30 minutes. Cell lysates were then incubated with protein G magnetic beads (Invitrogen) at 4°C overnight. Beads were washed three times with washing buffer and then eluted with sodium dodecyl sulfate buffer. After boiling at 95°C for 5 minutes, the samples were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels under reducing conditions. Proteins were then transferred to a polyvinylidene fluoride membrane by a blotting procedure, stained with monoclonal antibody against V5 peptide (Invitrogen), and visualized by using an enhanced chemiluminescence kit (Calbiochem).

Antigen-capture assay with platelets and transfected HEK293 cells

The monoclonal antibody immobilization of platelet antigens (MAIPA) assay with platelets was performed as previously described.[14] Briefly, platelets (2×10^7) were incubated with 50 μL of serum for 30 minutes at 37°C. After washing, monoclonal antibody FA6-152 (20 μg/mL; Hycult Biotech) was added for 30 minutes at 37°C. Subsequently, platelets were washed and solubilized in 100 μL of 10 mM Tris-buffered saline (TBS) containing 0.5% Triton-X100 for 30 minutes at 4°C. After centrifugation at 15,000 × *g* for 30 minutes at 4°C, 50 μL supernatant was diluted with 200 μL TBS buffer (TBS with 0.5% Triton-X100; 0.05% Tween 20; and 0.5 mM $CaCl_2$, pH 7.4). Next, 100 μL aliquots of diluted supernatant were added to a microtiter well coated with goat anti-mouse IgG (Dianova)

for 90 minutes at 4°C. Then, unbound antigen-antibody complex was removed, and peroxidase-labeled goat anti-human IgG (Dianova) was added. Finally, a reaction was developed by adding 100 μL of o-Phenylenediamine (Dako), stopped after 15 minutes with 2.5 N H_2SO_4, and measured at 492 nm using an enzyme-linked immunosorbent assay reader (Tecan). In addition, the MAIPA assay was performed to analyze the presence of common anti-HPA antibodies in the maternal serum. Maternal serum was incubated with a panel of HPA typed platelets and bound antigens were captured by monoclonal antibodies against human leukocyte antigen (HLA) Class I, glycoprotein Ia (GPIa)/IIa, GPIb/IX, GPIIb/IIIa, and CD109.[14]

Antibody analysis with transfected HEK293 cells was performed as follows: Aliquots of 10^5 cells were incubated with 25 μL of serum and processed as described above. Aliquots of 70 μL cell lysates were diluted with 180 μL washing buffer, and 100 μL of diluted cell lysates was transferred to microtiter wells that were precoated with anti-V5 antibody (Invitrogen). Unbound antigen-antibody complex was removed by washing and detected with an enzyme-labeled secondary antibody, as described above.

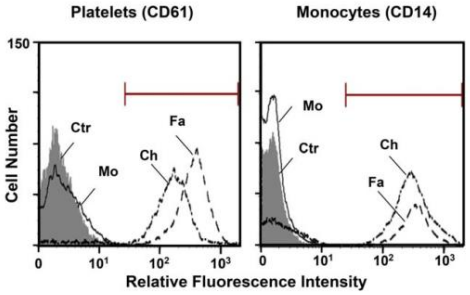

Fig. 1. Flow cytometry analysis of platelets and monocytes derived from mother (Mo), father (Fa), and child (Ch) using allophycocyanin fluorescence-labeled anti-CD36. Isotype control (Ctr) was run as control. Platelets and monocytes were gated by the use of platelets and monocytes antibodies (anti-CD61 and anti-CD14, respectively). Bar represents positive reaction. [Color figure can be viewed at wileyonlinelibrary.com]

RESULTS

Characterization of CD36*null* deficient mother

Flow cytometry analysis revealed normal CD36 expression on platelets and on monocytes from both the father and the newborn. In contrast, total absence of CD36 surface expression was observed on both platelets and monocytes from the mother (Fig. 1). This result indicated that the mother possessed congenital Type I CD36 deficiency (CD36*null*). This observation is in accordance with our molecular biological analysis. The mother carries two heterozygous mutations of the CD36 gene; two nucleotide deletions (CA at position 332-333 in exon 5 [CD36: c.332_333delCA]) in one allele and six nucleotide deletions (TATTTG in intron 13 [CD36:c.1254 + 6_1254 + 11del-TATTTG]) in another allele (Fig. S1, available as supporting information in the online version of this paper). No other mutations were found, indicating that both deletions were responsible for the total absence of CD36 glycoprotein on platelets and monocytes.

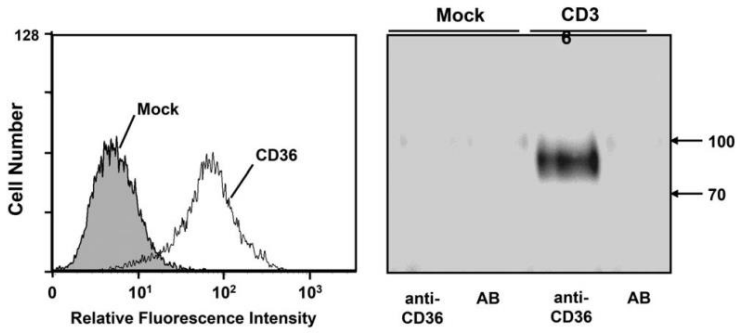

Fig. 2. Characterization of maternal antibodies using CD36 stable transfected HEK293 cells by flow cytometry (left) and by immunoprecipitation (right). *Left*: Maternal serum was incubated with nontransfected HEK293 cells (Mock) and CD36-transfected HEK cells (CD36). Bound antibodies were detected with fluorescence-labeled secondary antibody and analyzed by flow cytometry. *Right*: Biotin-labeled Mock and CD36-transfected cells were incubated with AB serum as a control (AB) or with maternal antibodies (anti-CD36). After washing, cells were lysed and precipitated with protein G-coupled beads. Precipitates were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels under nonreduced conditions and visualized by immunoblotting using an enzyme-labeled streptavidin and chemiluminescence substrate system.

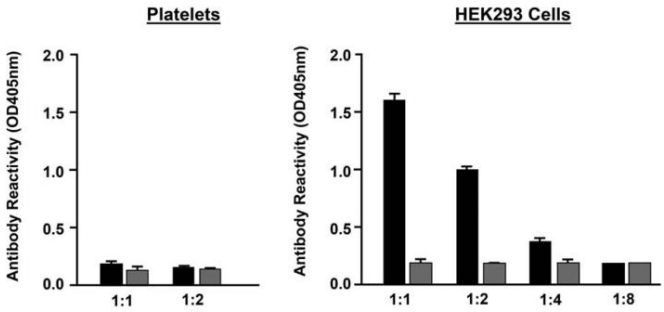

Fig. 3. Analysis of maternal antibodies with platelets and CD36 stable transfected HEK293 cells by antigen-capture assay. *Left*: Maternal serum (black) or AB serum (gray) at different dilutions (1:1 and 1:2) was incubated with platelets and monoclonal antibody against CD36. After washing and lysis, platelets cell lysates were incubated with anti-mouse IgG coated onto microtiter wells. Bound antibody-antibody complex was then detected with an enzyme-labeled secondary antibody and substrate system (see Materials and Methods). *Right*: Maternal serum (black) or AB serum (gray) at different dilutions (1:1 to 1:8) was incubated with CD36-transfected HEK293 cells. After washing and lysis, platelets cell lysates were incubated with monoclonal antibody against V5 coated on microtiter wells. Bound antibody-antibody complex was then detected with an enzyme-labeled secondary antibody and substrate system (see Materials and Methods).

Characterization of anti-CD36 isoantibodies developed by immunized mothers

To prove the presence of anti-CD36 antibodies, maternal serum was tested by flow cytometry using CD36-transfected HEK293 cells. As shown in Fig. 2 (left), maternal serum reacted with transfected, but not nontransfected, HEK239 cells (Mock). These results were confirmed by immunoprecipitation analysis (Fig. 2, right). Compared with normal serum, maternal serum precipitated CD36 glycoprotein with an apparent molecular mass of approximately 90 kDa. This band could not be detected with nontransfected HEK293 cells.

Furthermore, maternal serum was tested in a modified antigen-capture assay using platelets and CD36-transfected HEK293 cells (Fig. 3). Compared with the current MAIPA assay approach, this assay does not utilize monoclonal antibody against CD36 as capture antibody. Because the CD36 recombinant protein expressed on these cells exhibits V5 peptide, immobilization using antibody against this peptide is feasible. Interestingly, no reaction was obtained with platelets when monoclonal antibody FA6-152 against CD36 was applied as the capture antibody in MAIPA (Fig. 3, left). In contrast, a strong positive reaction was detected using CD36 antigen derived from transfected cells and captured by monoclonal antibody against V5 peptide (Fig. 3, right). This phenomenon was not caused by a prozone-effect, as shown by titration analysis. In contrast, a negative reaction was observed when monoclonal antibody FA6-152 was applied (data not shown).

To exclude the involvement of platelet-specific alloantibodies against HPAs, maternal serum was screened with HPA typed platelets by MAIPA (Fig. S2, available as supporting information in the online version of this paper). Negative reactions were found, indicating that maternal serum did not contain anti-HPA-1, HPA-2, HPA-3, HPA-5, or HPA-15 alloantibodies or anti-HLA Class I antibodies. This result is in concordance with the HPA genotyping and demonstrates no HPA incompatibility between mother and child (Table S1, available as supporting information in the online version of this paper). However, crossmatch analysis between maternal serum and paternal platelets using the solid-phase RBC adherence test showed a positive reaction (data not shown).

Taken together, these findings demonstrate that the anti-CD36 isoantibodies present in this mother were responsible for this FNAIT case.

DISCUSSION

In Taiwan, recent studies have demonstrated that the HPA incompatibility between fetus and mother leading to the production of maternal anti-HPA alloantibodies is mostly in the HPA-3, HPA-5, and HPA-15 systems.[19,20] More recently, Lo and coworkers reported that 0.6% of Taiwanese fail to express CD36 on both platelets and monocytes (Type I CD36 deficient; CD36*null*).[21] It is known that CD36*null* mothers can produce anti-CD36 isoantibodies during pregnancy, leading to the destruction of fetal platelets, known as FNAIT syndrome.[22]

Here, we report for the first time a clinical case of FNAIT in Taiwan caused by anti-CD36 isoantibodies developed by a CD36*null* mother. The total absence of CD36 expression on monocytes as well as platelets in this mother could be attributed to heterozygous deletions:

332_333del CA and 1254 + 6_1254 + 11delTATTTG in Exon 5 and Intron 13, respectively. The dinucleotide deletion 329-330delAC represents the most frequent mutation found in Taiwanese and Chinese populations[13,21,23] leading to frameshift at Position 110.[24] The mutation of six nucleotide deletions (1254 + 6_1254 + 11delTATTTG) seems to be a rare mutation among the Taiwanese population (allele frequency = 0.00078).[21] Lo and colleagues have also reported one Taiwanese individual who had low CD36 expression on platelets. This individual was heterozygous, carrying both wild-type and mutant alleles. This deletion is located in the 5′ region of Intron 13, three nucleotides away from the splicing site. Theoretically, this deletion may alter the splicing site, leading to a premature stop codon and the consequent absence of Exon 14.[25]

Subsequently, stable, transfected HEK293T cells expressing CD36 were established to characterize the antibody specificity. Flow cytometry as well as immunoprecipitation analysis clearly revealed the presence of anti-CD36 isoantibodies in the maternal serum. However, a negative result was obtained when the antigen-capture assay (MAIPA) was performed with platelets using monoclonal antibody FA6-152 as the capture antibody. A previous study indicated that the monoclonal antibody FA6-152 and many other functional monoclonal antibodies similar to human anti-CD36 isoantibodies react with the same epitope(s), which are located on residues 155 through 183 of the CD36 glycoprotein. Consequently, binding of monoclonal antibody FA6-152 on target cells prevents the binding of human anti-CD36 isoantibodies to CD36 that is defined by competitive inhibition.[26] To avoid this problem, antibodies reactive with the V5 peptide that were tagged onto the C-terminal part of our CD36 recombinant protein were used. Because these antibodies do not bind directly to the extracellular domain of CD36, competitive inhibition with any human anti-CD36 isoantibodies is prevented. Indeed, anti-CD36 isoantibodies present in the maternal serum were strongly detected by this approach.

Saw and colleagues demonstrated that the use of different monoclonal antibodies directed against different epitopes on CD36 antigen could reduce the false-negative reaction observed by MAIPA.[27] Currently, little is known about the diversity of epitopes recognized by maternal antibodies. Thus, defining a superior panel of anti-CD36 monoclonal antibodies that do not completely compete with all maternal antibodies is a difficult task. Currently, a monoclonal antibody-independent enzyme-linked immunosorbent assay using purified CD36 protein is commercially available. However, the fact that purified antigens immobilized in solid phase may undergo epitope changes that lead to false-negative or false-positive results cannot be completely excluded. Indeed, the use of transfected cell lines for routine serological analysis implies several drawbacks, including cell culture facilities and the high cost of cryopreservation. However, these problems probably could be solved by the use of lyophilized transfected cells.[28]

Interestingly, variable clinical pictures of FNAIT mediated by anti-CD36 isoantibodies, including mild and severe thrombocytopenia, hydrops fetalis, and recurrent abortions, have been observed.[9-13,29] However, the precise mechanism of this phenomenon is not known.

In the future, analysis of anti-CD36-containing sera by the use of monoclonal antibody-dependent assays (e.g., with anti-CD36 monoclonal antibody) and monoclonal antibody-independent assays (e.g., with anti-V5 isoantibodies, as presented here) may allow better characterization of the different epitopes recognized by human anti-CD36 isoantibodies. This knowledge may help us to understand the variable clinical pictures of FNAIT caused by anti-CD36 isoantibodies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Fig. S1. Nucleotide sequencing analysis of CD36 gene of the CD36 defective mother (mutant) and CD36 normal individual (wild-type). Left panel: Two nucleotide deletions CA (underlined in red) in one allele at positions of 332-333 in exon 5 region (black underlined) and right panel: Six nucleotide deletion TATTTG (red underlined) in another allele at positions 1254 + 6 in intron 13 region (black underlined) are presented.

Fig. S2. Analysis of maternal isoantibodies with HPA typed platelets by MAIPA capture assay. Maternal serum (grey) or positive controls (black, see below) were incubated with heterozygous HPA typed platelets (from left to right: HPA-1a/b, HPA-2a/b, HPA-3a/b, HPA-5a/b and HPA-15a/b) together with the respective monoclonal antibodies (from left to right: anti-GPIIb/IIIa, anti-GPIb/IX, anti-GPIIb/IIIa, anti-GPIa/IIa, anti-CD109 and anti-HLA class I). After washing and lysis, platelets cells lysates were incubated with anti-mouse IgG coated onto microtiter wells. Bound antibody-antibody complex was then detected with enzyme labeled secondary antibody and substrate system (as above). Sera containing anti-HPA-1a, anti-HPA-2a, anti-HPA-3a, anti-HPA-5b, anti-HPA-15a and anti-HLA class I was used as positive controls.

Table S1. HPA genotypes of the mother, child and father.

3.7. Role of CD36 in immune-mediated thrombocytopenia in Asian populations

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CONGRESS REVIEW

Role of CD36 in immune-mediated thrombocytopenia in Asian populations

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It has been suggested that type I cluster of differentiation 36 (CD36)-deficient individuals are at risk of developing anti-CD36 isoantibodies (also known as anti-Nak[a]) after receiving platelet transfusions or during pregnancy. These antibodies are responsible for several immune thrombocytopenic disorders including fetal/neonatal alloimmune thrombocytopenia (FNAIT), post-transfusion purpura and platelet-transfusion refractoriness (PTR). In Asian populations, anti-CD36 isoantibodies are more frequently found in PTR and FNAIT patients compared to alloantibodies against human platelet antigens. In this short review, we illustrate the relevance of CD36 by providing information on the frequency and molecular basis of CD36 deficiency, laboratory diagnostic tests of CD36 antigens/antibodies and treatment of immune-mediated thrombocytopenia caused by anti-CD36 antibodies.

Key words: CD36, CD36 deficiency, CD36 isoantibodies, immune-mediated thrombocytopenia.

Introduction

Cluster of differentiation 36 (CD36) is an 88 kDa glycoprotein that is widely expressed on a variety of cells such as monocytes, macrophages, platelets, endothelial cells and epithelial cells [1]. It belongs to the class B scavenger receptor family, which binds many different ligands including thrombospondin, oxidized phospholipids, oxidized low-density lipoprotein (oxLDL) and long-chain fatty acids. Furthermore, CD36 also binds *Plasmodium falciparum*-infected erythrocytes, *Staphylococcus* and *Mycobacterium* bacterial components [2]. Accordingly, it is involved in diverse physiological and pathological processes including thrombosis/haemostasis, atherogenesis, the innate immune defence and diabetes [3–6]. Recent studies have demonstrated that the binding of oxLDL to platelet CD36 promotes platelet signalling, resulting in hyperactive platelets [7]. These lipid-laden platelets can trigger endothelial activation, inhibit endothelial regeneration [8] and promote monocyte extravasation [9]. Hence, CD36 plays an important role in the progression of atherosclerosis.

CD36 deficiency is divided into two subgroups: type I in which neither platelets nor monocytes express CD36 and type II in which only platelets lack CD36 expression [10]. Until recently, anti-CD36 antibodies could only be detected in immunized type I-deficient individuals but not in type II deficiency. Anti-CD36 antibodies cause increased platelet destruction, which leads to the immune-mediated thrombocytopenia observed in cases of fetal/neonatal alloimmune thrombocytopenia (FNAIT), platelet-transfusion refractoriness (PTR) and post-transfusion purpura (PTP) [11–14]. In rare cases, anti-CD36 antibodies can also cause platelet activation associated with non-haemolytic transfusion reactions (NHTRs) and haemolytic uremic syndrome [15,16]. Interestingly, transfusion-related acute lung injury (TRALI) associated with anti-CD36 antibodies was recently reported [17].

In Caucasians, alloimmunization against diallelic human platelet alloantigen (HPA) systems such as HPA-1, HPA-2, HPA-3, HPA-5 and HPA-15 cause alloimmune-mediated thrombocytopenia [18]. Among them, anti-HPA-1a alloantibodies are the most important. Based on analysis of the allelic distribution of the HPA-1 system,

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alloimmunization against HPA-1a is expected to be an extremely rare event in Asian populations. Indeed, no cases of alloimmune thrombocytopenia mediated by anti-HPA-1a antibodies were found in China between 2008 and 2015 [19]. However, recent data have indicated that anti-CD36 antibodies are frequently the cause of immune-mediated thrombocytopenia in Chinese populations [19, 20]. To date, immunization against CD36 has not been reported in Caucasians. However, PTR cases linked to anti-CD36 antibodies have been reported among non-Caucasians living in Canada [21], underscoring the value of national and international co-operation in the management of patients with thrombocytopenia caused by anti-CD36 antibodies.

Incidence of CD36 deficiency in Asian populations

Population studies have shown that the incidence of CD36 deficiency varies among different ethnic groups. CD36 deficiency is very rare in Caucasians (<0·3%) and is more frequent in African and Asian populations (4–8%) [10, 22, 23]. Table 1 shows the frequency of CD36 deficiency in different Asian populations. The highest frequency of CD36-deficient individuals (type I and type II) was reported in Japan (8·15%). In China, the frequency of CD36 deficiency was about 3·56% (1·80–4·27%), similar to that in Korea (3·64%). Interestingly, a higher incidence of CD36 deficiency was found in the Chinese *Zhuang* ethnic group living in Guangxi province (5·76%), and a lower frequency was found in Malaysia (2·0%), Iran (1·89%), Taiwan (1·56%) and Thailand (0·62%). These observations indicate the decreasing frequency of CD36 deficiency from North to South in the eastern hemisphere, although only small cohorts were investigated in some studies. Currently, only limited studies exist on the frequency of type I CD36 deficiency. In China, the frequency of type I-deficient individuals in *Han* and *Zhuang* ethnic groups was about 0·69% (36/5248) and 1·3% (18/1389) lower than that in Japan (1·50%; 2/135), respectively. The study in China was performed in a large cohort, indicating a significant difference among ethnic groups. However, analyses of type I CD36 deficiency in a large cohort have not been conducted in other Asian populations.

Molecular basis of CD36 deficiency in Asian populations

The CD36 gene is located on chromosome 7q11·2 and is encoded by 15 exons, but only exons 3 to 14 encode the CD36 protein [24]. More than 30 mutations in the coding region of CD36 gene responsible for type I CD36 deficiency have been described [25]. Homozygous or compound heterozygous mutations in the CD36 gene associated with the total absence of CD36 surface expression on platelets and monocytes have been discovered. **Table** 2 shows 29 mutations located in exons and introns of the CD36 gene

Table 1 Frequency of CD36 deficiency in Asian populations

Population	Number of individuals	Type I + Type II (%)	Type I (%)	References
Chinese				
Guangzhou	998	1·80% (18/998)	>0·5% (>5/998)[a]	Xu X *et al.*, 2013 [27]
Zhejiang	192	3·65% (7/192)	0·0% (0/192)	Xu X *et al.*, 2014 [35]
Shanghai	1022	2·15% (22/1022)	0·2% (2/1022)	Li R *et al.*, 2014 [25]
Guangxi	4425	4·27% (189/4425)	>1·06% (>47/4425)[a]	
Han	3036	3·59% (109/3036)	>0·96% (>29/3036)[a]	Zhong Z *et al.*, 2014 [36]
Zhuang	1389	5·76% (80/1389)	>1·30% (>18/1389)[a]	Zhong Z *et al.*, 2014 [36]
Total	6637	3·56% (236/6637)	>0·81% (>54/6637)[a]	
Japanese	135	8·15% (11/135)	1·5% (2/135)	Masuda Y *et al.*, 2015 [37]
Indonesian	168	4·17% (7/168)	NT	Santoso S *et al.*, 1993 [38]
Malaysian	200	2·00% (4/200)	NT	Xia W *et al.*, 2016 [39]
Iranian	53	1·89% (1/53)	1·89% (1/53)	Wang J *et al.*, 2017 [40]
Taiwanese	640	1·56% (10/640)	0·63% (4/640)	Lo SC *et al.*, 2016 [41]
Thai	162	0·62% (1/162)	NT	Xia W *et al.*, 2016 [39]
Koreans	55	3·64% (2/55)	NT	Xia W *et al.*, 2016 [39]

NT, not tested.

[a]: not all type I and type II individuals (CD36 negative on platelets) presented in the third column were tested for the absence of CD36 on monocytes to determine type I deficiency (CD36 negative on platelets and monocytes). Therefore, the percentage of type I deficiency was estimated more than the numbers presented here.

Table 2 Genetic variation of CD36 responsible for CD36 deficiency in Asian populations

Exon number	mRNA nucleotides[a]	Amino acid position	Nucleotide change	Amino acid change	References
3	−89 to +120	1–40	G14A (14 G>A)	Arg5Gln	Li R *et al.*,2014 [25]
4	121–281	41–94	C268T(268 C>T)	Pro90Ser	Kashiwagi H *et al.*, 1993 [42]
			121–126 *del* gCAAGTT	unknown	Xu X *et al.*, 2013 [27]
			C220T(220 C>T)	Gln74Term	Xu X *et al.*, 2013 [27]
			198-205 *del* GATCTTTG	frameshift at AA66	Xia W *et al.*, 2016 [39]
5	282–429	94–143	329-330 *del* AC	frameshift at AA 110	Kashiwagi H *et al.*,1994 [43]
			C371T	Pro124Leu	Li R *et al.*,2014 [25]
			C380T	Ser127Leu	Omi K *et al.*,2003 [44]
			T410C	Val137Ala	Imai M *et al.*,2002 [45]
			429 + 4*ins*g	exon 6 skipping	Xu X *et al.*, 2013 [27]
6	430-609	144-203	560*ins*T	frameshift at AA 187	Tanaka T *et al.*,2001 [46]
			T538C	Trp180Arg	Li L *et al.*, 2016 [47]
7	610–701	204–234	619–624 *del* ACTGCA/*ins*AAAAC	frameshift at AA 207	Kashiwagi H *et al.*,2001 [26]
8	702–748	234–250	T737A	Ile246Asn	Xia W *et al.*, 2016 [39]
9	749–818	250–273	T760C	Phe254Leu	Hanawa H *et al.*,2002 [48]
10	819–1006	273–336	949*ins*A	frameshift at AA 317	Kashiwagi H *et al.*, 1996 [49]
12	1126–1199	376–400	*Del* tttagAT	skipping exon 12	Tanaka T *et al.*,2001 [46]
			G1150C+1155*del*A	Ala384Pro+frameshift at AA 385	Atiman T *et al.*,2000 [50][b]
			C1156T	Arg386Trp	Okajima *et al.*, 2006 [31]
			A1163T	Gln388Leu	Li R *et al.*,2014 [25]
13	1200–1254	400–418	*Del*tattacagAG	skipping exon 13	Tanaka T *et al.*,2001 [46]
			1200-5*inv*49 bp	exon13 skipping	Xu X *et al.*, 2013 [27]
			*dupl.*1204-1246	frameshift at AA 416	Tanaka T *et al.*,2001 [46]
			1218-1224 *del*GAGGAAC	frameshift at AA 406	Kashiwagi H *et al.*, 2001 [26]
			1228–1239 *del*ATTGTGCCTATT	deletion of Ile-Val-Pro-Ile410-413	Tanaka T *et al.*,2001/Kashiwagi H *et al.*,2001 [26,46]
			A1226G	Tyr409Cys	Li R *et al.*,2014 [25]
			A1237C	Ile413Leu	Hanawa H *et al.*,2002 [48]
14	1255–1419	419–472	1343–1344 *ins*TCTT	frameshift at AA 448	Li R *et al.*,2014 [25]
			1332–1335 *ins*TGAT	frameshift at AA444	Xia W *et al.*, 2016 [39]

[a]GenBank NM001001548; The first mRNA nucleotide encoding CD36 protein is +1.
[b]Found also in Guangzhou Blood Centre.

associated with type I and type II deficiency characterized in Asian populations. Three common mutations, C268T, 949*ins*A and 329–330 *del*AC have been identified in Japanese individuals [26], of which the C268T nucleotide substitution is the most frequent mutation (>50%). In contrast, 329–330 *del*AC and 1228–1239 *del*ATTGTGCCTATT are the most common mutations in Chinese individuals [27]. Because of these heterogeneous mutations, a practicable genotyping approach for identifying individuals with CD36 deficiency has been difficult to develop.

Detection of CD36 isoantibodies in Asian laboratories

During the last two decades, different approaches have been developed to detect platelet-specific alloantibodies including in-house assays such as monoclonal antibody-specific immobilization of platelet antigens (MAIPA), the platelet suspension immunofluorescence test (PSIFT) and commercial assays such as the solid-phase assay (PakPlus) and Luminex bead-based platelet antibody detection assay (Pak Lx) [28]. Although these methods can be used to detect CD36 antibodies, they have some drawbacks. False-negative results often occur in MAIPA due to competitive inhibition between capture mouse monoclonal antibodies and human anti-CD36 antibodies. PSIFT is a simple and fast flow cytometry-based method; however, it requires fresh CD36-deficient platelets as a negative control to exclude the presence of other platelet-reactive antibodies in the serum samples (e.g., anti-HPA or anti-HLA antibodies). Commercial kits such as PakPlus and Pak Lx are expensive and require special instruments, and false-negative results caused by the use of purified

protein cannot be ruled out. In recent approaches, transfected cell lines stably expressing CD36 antigen have been introduced for the detection of CD36 antibodies [29,30]. Another approach for detecting CD36 antibodies has been to express recombinant CD36 harbouring V5-peptide (GKPIPNPLLGLDST) on the surface of mammalian HEK293 cells for the MAIPA [30]. In this method, CD36-transfected cells are incubated with the serum sample and then lysed, after which human anti-CD36 antibodies bound to recombinant CD36 are immobilized on a microtitre plate pre-coated with anti-V5 antibodies. Bound human antibodies are detected with enzyme-labelled secondary antibodies and corresponding substrate. This improvement allows the detection of anti-CD36 antibodies by MAIPA without the use of capture monoclonal antibody, thereby preventing false-negative reactions caused by competitive inhibition. Finally, a CD36-transfected K562 cell line has been established for the detection of anti-CD36 antibodies by flow cytometry [29]. Compared to the MAIPA approach, however, a flow cytometry-based method cannot completely exclude false-positive reactions that may occur between antibodies and other platelet proteins besides CD36 antigen. Comparable studies should be performed in the near future to identify a suitable method for routine laboratory diagnostic tests of anti-CD36 antibodies.

Immune-mediated thrombocytopenia caused by CD36 antibodies

Immune-mediated thrombocytopenia is responsible for approximately 15% of thrombocytopenia cases and occurs due to alloantibodies against platelet antigens including ABO, HLA class I and HPA. However, only limited data are available for CD36. In Table 3, cases of immune thrombocytopenia mediated by CD36 antibodies reported in Asia are summarized. The majority of patients (16/18) were immunized either by transfusions/transplantation (12 cases) or pregnancy (four cases). In China, most cases of immune-mediated thrombocytopenia caused by CD36 antibodies occur in the Guangxi province, which has a higher frequency of CD36-deficient residents than other provinces. The clinical presentation of FNAIT caused by anti-CD36 antibodies is heterogeneous, ranging from widespread petechial haemorrhages and thrombocytopenia to severe anaemia and hydrops fetalis [11,31,32]. Because the reason for this phenomenon is currently unknown, a treatment strategy cannot be easily developed. We recently reported a case of FNAIT with severe anaemia (haemoglobin 48 g/l, haematocrit 16·6%) and severe thrombocytopenia (platelet $16x10^9$/l) found at gestational age 27 weeks [33]. This case was successfully managed by intrauterine transfusions with red blood cells and platelets from

Table 3 CD36-type I deficiency and immune thrombocytopenia in Asian populations

Subjects	Age	Sex	Area	Population	Diagnosis	No. of transfusions	No. of pregnancies	Cases	References
Patient 1	22	M	Hainan	Chinese	T-lymphoblastic tumour	Multiple	N/A	PTR	Xu X *et al.*, 2013 [27]
Patient 2	30	F	Guangxi	Chinese	Pregnancy	0	4	FNAIT	Xu X *et al.*, 2013 [27]
Patient 3	11	M	Hainan	Chinese	Acute leukaemia	Multiple	N/A	PTR	Xia W *et al.*, 2014 [34]
Patient 4	35	F	Guangzhou	Chinese	Myelodysplastic syndromes	Multiple	3	PTR	Xia W *et al.*, 2014 [34]
Patient 5	35	F	Guangxi	Chinese	Pregnancy	1	7	FNAIT	Xia *et al.*, 2014 Xu *et al.*, 2017 [33,34]
Patient 6	46	M	Guangzhou	Chinese	Alcohol cirrhosis	Multiple	N/A	PTR	[a]
Patient 7	5	M	Guangzhou	Chinese	Acute leukaemia	1	N/A	PTR	[a]
Patient 8	7	M	Guangxi	Chinese	Comminuted fractures	Multiple	N/A	PTR	Yin X *et al.*, 2011 [51]
Patient 9	26	F	Guangxi	Chinese	Acute leukaemia	2	1	PTR	Wu G *et al.*, 2014 [52]
Patient 10	21	F	Guangxi	Chinese	Pregnancy with bleeding	4	2	PTR	Wu G *et al.*, 2014 [52]
Patient 11	63	F	Guangxi	Chinese	Acute leukaemia	Multiple	2	PTR	Wu G *et al.*, 2014 [52]
Patient 12	[b]	F	Thailand	Thai	Pregnancy	0	1	FNAIT	Kankirawatana S, *et al.*, 2001 [11]
Patient 13	36	F	Japan	Japanese	Acute myeloid leukaemia	Multiple	0	PTR	Ikeda H, *et al.*, 1989 [53]
Patient 14	19	M	Japan	Japanese	Seminoma	Multiple	N/A	PTR	Fujino H, *et al.*, 2001 [13]
Patient 15	47	M	Japan	Japanese	HBV-related liver cirrhosis	Liver transplant	N/A	PTR	Ogata T, *et al.*, 2005 [54]
Patient 16	[b]	F	Japan	Japanese	Pregnant	[b]	3	FNAIT	Taketani T *et al.*, 2008 [32]
Patient 17	36	F	Japan	Japanese	Pregnant	0	2	FNAIT	Okajima S *et al.*, 2006 [31]
Patient 18	26	F	Taiwan	Taiwanese	Pregnant	0	2	FANIT	Lin M *et al.*, 2017 [30]

[a]Unpublished case which found in Guangzhou Blood Centre.

[b]Unknown; HBV, Hepatitis B virus; Multiple, the number of transfusions is more than four times.

CD36-defective donors. In addition, intravenous IgG therapy (400 mg/kg/days or 1 g/kg) seemed to be effective for the treatment of some FNAIT cases by gradually increasing the platelet count of the newborns [30,32]. Transfusion with CD36-deficient platelets is the method of choice for preventing bleeding in patients with PTR caused by anti-CD36 antibodies [13,34].

Conclusions

This study provides a better approximation of CD36 immunization risk in Asian populations. Current data strongly indicate that anti-CD36 antibodies play important roles in the pathomechanisms underlying immune-mediated thrombocytopenia in Asian populations, especially PTR and FNAIT. In the past two decades, significant progress has been made on the identification of anti-CD36 antibodies, allowing suitable laboratory diagnostic tests for anti-CD36-induced disorders. Therefore, testing anti-CD36 antibodies should be considered in immune-mediated thrombocytopenia and as well as other diseases such as TRALI and NHTR. Furthermore, a registry of blood donors with CD36 deficiency should be established to improve bleeding disorders caused by anti-CD36 antibodies. Currently, a large cohort of CD36-negative platelet donors is available in Guangzhou blood centre, and CD36 phenotyping of platelets donors is currently an ongoing process in different blood centres in China and other countries in Southeast Asia. However, there is still little known about the mechanisms underlying the effects of anti-CD36 antibodies on different cell types. Knowledge about this mechanism may help us to understand the diverse clinical presentation of anti-CD36 immune-mediated thrombocytopenia, facilitating the development of a treatment strategy.

Disclosure

The authors have no conflicts of interest to declare.

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3.8. Successful prenatal therapy of anti-CD36-mediated severe FNAIT by deglycosylated antibodies in a novel murine model

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Successful prenatal therapy for anti-CD36-mediated severe FNAIT by deglycosylated antibodies in a novel murine model

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KEY POINTS

- A murine model of anti-CD36–mediated FNAIT showed frequent fetal death in immunized mothers that could be prevented by IVIG and deg-mAb.
- Therapy with deg-mAb against CD36 is more beneficial than IVIG based on the dose, the success rate of therapy, and the start of application.

Recent studies have shown that maternal anti-CD36 antibodies represent a frequent cause of fetal/neonatal alloimmune thrombocytopenia (FNAIT) in Asian and African populations. However, little is known about the pathomechanism and antenatal treatment of anti-CD36–mediated FNAIT. Here, we established a novel animal model to examine the clinical features of pups from immunized *Cd36*$^{-/-}$ female mice after breeding with wild-type male mice. Mild thrombocytopenia was observed, but high pup mortality was also documented (40.26%). Administration of intravenous immunoglobulin (IVIG) (1 g/kg) on days 7, 12, and 17 to immunized *Cd36*$^{-/-}$ mothers after breeding reduced fetal death (12.70%). However, delaying the IVIG administration series on days 10, 15, and 20 did not reduce fetal death (40.00%). In contrast, injection of deglycosylated anti-CD36 (deg-anti-CD36) polyclonal antibodies (5 mg/kg) on days 10, 15, and 20 significantly reduced fetal death (5.26%). Subsequently, monoclonal antibodies (mAbs) against mouse CD36 were developed, and one clone producing high-affinity anti-CD36 (termed 32-106) effectively inhibited maternal antibody binding and was therefore selected. Using the same approach of deg-anti-CD36, the administration of deg-32-106 significantly reduced fetal death (2.17%). Furthermore, immunized *Cd36*$^{-/-}$ mothers exhibited placental deficiency. Accordingly, maternal anti-CD36 antibodies inhibited angiogenesis of placenta endothelial cells, which could be restored by deg-32-106. In summary, maternal anti-CD36 antibodies caused a high frequency of fetal death in our animal model, associated with placental dysfunction. This deleterious effect could be diminished by the antenatal administration of IVIG and deg-mAb 32-106. Interestingly, treatment with deg-32-106 seems more beneficial considering the lower dose, later start of treatment, and therapy success.

Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal antibodies that cross through the placenta via neonatal Fc receptor (FcRn)-mediated transport during pregnancy. This action can lead to the clearance of fetal platelets and endothelial dysfunction, which results in bleeding complications.[1-3] In White populations, antibodies against human platelet antigen-1a (HPA-1a) are responsible for ~80% of FNAIT cases.[4] The incidence of severe FNAIT is ~1:2500 newborns,[5] in which intracranial hemorrhage (ICH) occurs in ~20% of these cases, leading to fetal death or persistent neurologic sequelae.[6] However, FNAIT caused by anti–HPA-1a antibodies has not been well recognized in other populations.

The data indicate that antibodies against CD36 represent an important risk factor for FNAIT in Asian and African populations.[7-10] CD36 is a receptor for thrombospondin-1, a free fatty acid, and a scavenger receptor for different danger signals. Accordingly, CD36 is implicated in inflammatory response, atherosclerosis, thrombosis, and metabolic disorders.[11] Individuals lacking CD36 on both platelets and monocytes (type I) are at risk of developing anti-CD36 antibodies (known as anti-Nak^{a}) after receiving a platelet transfusion or during pregnancy.[12,13] These antibodies may cause immune-mediated thrombocytopenia and repeated early fetal loss.[14-16] Immunization in type II individuals (lacking CD36 on platelets only but not on monocytes) has not been described. Type I CD36 deficiency is extremely rare in White

subjects but more common in African subjects (~2%) and relatively frequent in Asian populations (>0.5%).[7,9,17-19]

The clinical manifestation of FNAIT caused by anti-CD36 antibodies is heterogeneous, ranging from widespread petechial hemorrhages to thrombocytopenia, miscarriages, hydrops fetalis, and ICH. Compared with anti–HPA-1a, anti-CD36 antibodies cause more common hydrops and recurrent miscarriages.[16,20,21] The reason for this phenomenon is unknown. Therefore, the current approach for treating FNAIT caused by anti–HPA-1a may not be adaptable for the disease caused by anti-CD36 antibodies.

The current clinical approach for managing FNAIT relies on preventing fetal bleeding complications through antenatal therapy because the prophylaxis is currently unavailable.[22] Presently, noninvasive maternal treatment with intravenous immunoglobulin (IVIG) represents the antenatal therapy's first line.[1] However, such strategies may inhibit the transfer of immune-protective maternal immunoglobulin G (IgG), which might cause an increased risk of infection during pregnancy and the first weeks following birth.[23] An attractive alternative strategy would be to administer nondestructive IgG that shares the pathogenic antibodies' specificity and retains the ability to be transported across the placenta. Ghevaert et al[24,25] introduced such a strategy using human recombinant single-chain fragment variable anti–HPA-1a antibodies with a modified Fc part. These modified recombinant anti–HPA-1a antibodies could pass the placenta and ease FNAIT in immunized mothers.

The therapeutic potential of deglycosylated IgG (deg-IgG) antibodies for autoimmunity treatment has been widely recognized.[26] Removal of N-glycan (linked to asparagines 297), located on the Fc part, leads to a significant reduction of IgG binding with the FcγRs expressed on macrophages and its ability to activate complement C1q. Interestingly, these deg-IgG antibodies could still be transported from the maternal circulation to the fetus via FcRn.[27] Accordingly, our previous in vivo study in mice showed that deglycosylated–monoclonal antibody (deg-mAb) specific for HPA-1a could pass through the placenta and prevent the clearance of fetal platelets mediated by maternal anti–HPA-1a antibodies. This observation indicates that the use of epitope-specific antibodies for the antenatal therapy of severe FNAIT is feasible.[28]

The current study established a novel mouse FNAIT model to evaluate the efficacy of deglycosylated anti-CD36 (deg-anti-CD36) to treat FNAIT caused by maternal anti-CD36 antibodies and compared this treatment with IVIG.

Materials and methods

Intravenous immunoglobulin (IVIG)

Human IVIG was purchased from Jiangxi Boya Bio-Pharmaceutical, Jiangxi, China.

Mice

Wild-type (WT) C57BL/6J mice were provided by the Animal Centre of Sun Yat-Sen University, China. *Cd36*$^{-/-}$ mice were purchased from The Jackson Laboratory (B6.129S1-Cd36^{tm1Mfe}/J). The Animal Care Committee approved this study (IACUC-2014-0303).

Immunization of *Cd36*$^{-/-}$ mice and detection of anti-CD36 antibodies

Platelets were prepared as previously described.[29] *Cd36*$^{-/-}$ female mice (6-8 weeks old) were immunized 3 times with 10^8 WT platelets intraperitoneally at weekly intervals. After immunization, 5 μL of serum was collected and incubated with 100 μL of EDTA blood (1:100) from WT mice for 30 minutes and washed with phosphate-buffered saline/1% bovine serum albumin. Then, 50 μL of fluorescein isothiocyanate–conjugated anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 30 minutes. After red blood cell lysis (BD Biosciences, Shanghai, China), cells were suspended in 0.5 mL phosphate-buffered saline/1% bovine serum albumin and then analyzed by flow cytometry (FACS Canto II; BD Biosciences).

Counting of pups' platelets

Platelets from the pups were counted as previously described.[29] Briefly, 50 μL of counting beads (Life Technologies, Carlsbad, CA) was added to determine the platelet counts by using flow cytometry. Counting of 2,000 beads represented the standard.

Generation and characterization of mAbs against CD36

Cd36$^{-/-}$ mice were immunized as previously described.[3,29] Splenocytes were harvested and fused with SP2/0-Ag14 mouse myeloma cells (ATCC, Manassas, VA) with the help of polyethylene glycol using a standard protocol.[30] Hybridomas were screened by using mouse platelets according to flow cytometry. IgG subclasses were determined by a Pierce Rapid Isotyping Kit (Thermo Fisher Scientific, Waltham, MA). Seventeen hybridoma clones producing anti-CD36 could be generated. One clone (labeled 32-106) producing a high-affinity IgG antibody that effectively blocked maternal anti-CD36 binding was selected.

Deglycosylation of anti-CD36 antibodies

IgG was purified and digested as previously described.[28] Aliquots of 1 μL of native and deg-mAb (0.6 mg/mL) were analyzed by flow cytometry using mouse IgG2a as isotype control (eBioscience, San Diego, CA).

Competitive inhibition between maternal and mAb against CD36 in vitro

One milligram of IgG derived from immunized *Cd36*$^{-/-}$ mice was labeled with fluorescein-EX (Invitrogen, Carlsbad, CA). Unlabeled deg-32-106 (5-50 ng) was added to 100 μL of EDTA- anticoagulated mice blood at room temperature for 30 minutes. After washing, 8 μL of fluorescein-conjugated IgG (1.73 mg/mL) was added for 30 minutes. Red blood cells were lysed and the samples were analyzed by flow cytometry.

Competitive inhibition between maternal and mAb against CD36 in vivo

Deg-32-106 (100 μg) was labeled with Alexa Fluor dye (AF-647; Invitrogen). Subsequently, Alexa Fluor–labeled deg-32-106 was intravenously injected into WT female mice (1 mg/kg). After 10 minutes, fluorescein-EX conjugated maternal IgG containing anti-CD36 was administered (200 mg/kg). The binding of both antibodies on platelets was analyzed by flow cytometry at 30 and 60 minutes. A single administration of labeled deg-32-106 or maternal anti-CD36 was run as a control.

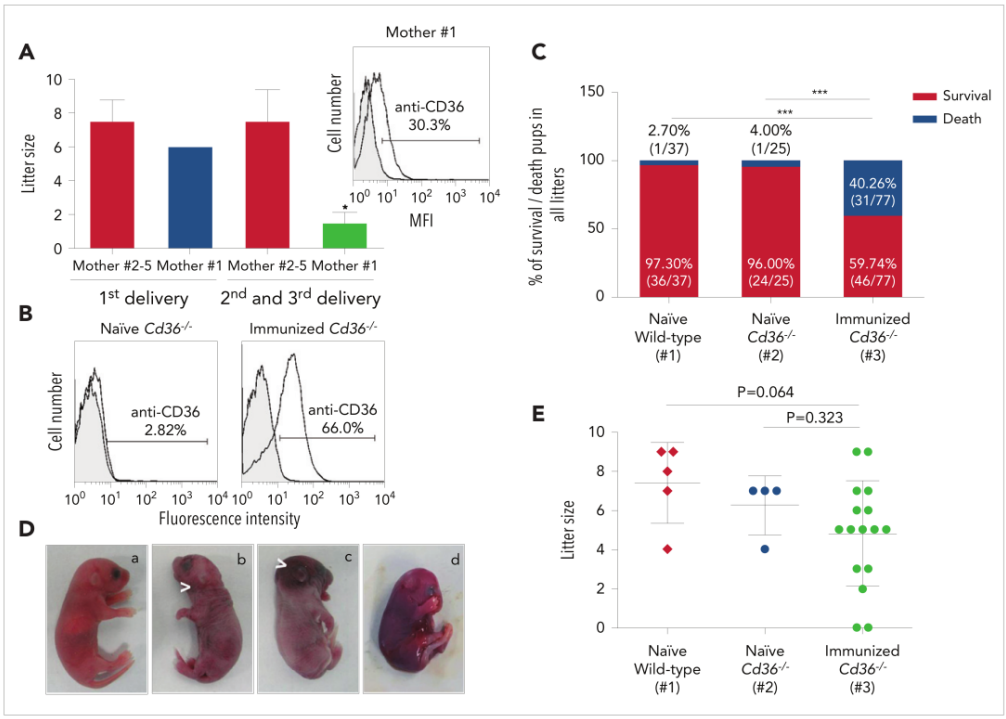

Figure 1. The effect of maternal anti-CD36 antibodies on the fate of pups delivered by immunized *Cd36*$^{-/-}$ female mice. (A) Naive *Cd36*$^{-/-}$ female mice (n = 5) were bred with WT male mice and delivered pups 3 times. The litter size was documented. One mother (#1) delivered a smaller littermate after the second and third pregnancy (litter size, 1.50 ± 0.71) compared with the other 4 mothers (#2-5) (litter size, 7.50 ± 1.93). Weak CD36-reactive antibodies were detected in mother #1 according to flow cytometry (see inset, the third delivery) but not in mothers #2 to #5 (data not shown). (B) *Cd36*$^{-/-}$ female mice (cohort #3) were immunized 3 times with WT platelets. Representative sera were then incubated with platelets from WT (white curves) or *Cd36*$^{-/-}$ mice (gray curves). Bound antibodies were detected with fluorescence-labeled anti-mouse IgG and analyzed by using flow cytometry. The percentages of positive cells (anti-CD36) are given. Sera from naive *Cd36*$^{-/-}$ were used as controls. (C) After immunization, *Cd36*$^{-/-}$ female mice (cohort #3; n = 16) were bred with WT male mice. Naive WT (cohort #1; n = 5) and naive *Cd36*$^{-/-}$ (cohort #2; n = 4) were conducted as controls. Dead pups were determined in utero or within 24 hours after delivery. The mortality of the pups in cohort #3 (31 of 77 [40.26%]) was compared with that of cohort #1 (χ^2 = 17.46; ***P < .0001) and cohort #2 (χ^2 = 11.52; ***P = .0007). (D) Severe bleeding in *Cd36*$^{+/-}$ pups delivered by immunized *Cd36*$^{-/-}$ mothers was found. (a) Healthy pup; (b) dead pup with bleeding (arrow); (c) dead pup with ICH (arrow); (d) dead pup with hydrops. (E) The litter size (including the dead pups) in naive WT, *Cd36*$^{-/-}$ mothers (cohorts #1 and #2), and immunized *Cd36*$^{-/-}$ mothers (cohort #3) is presented. The litter size in immunized *Cd36*$^{-/-}$ (cohort #3) was small but not significantly different compared with the naive WT (cohort #1) (4.81 ± 2.69 vs 7.40 ± 2.07; P = .064) and *Cd36*$^{-/-}$ (cohort #2) mothers (4.81 ± 2.69 vs 6.25 ± 1.50; P = .323) using a two-tailed unpaired Student t test.

Induction of FNAIT and treatment with IVIG or deg-anti-CD36 antibodies

Naive or immunized *Cd36*$^{-/-}$ female mice were bred with WT male mice. Anti-CD36 developed in *Cd36*$^{-/-}$ mothers was monitored by flow cytometry. During pregnancy, mothers were untreated or treated with human IVIG (1 g/kg), deg-anti-CD36 polyclonal IgG (5 mg/kg), or deg-anti-CD36 mAb (5 mg/kg) intravenously 3 times.[3,29] Dead pups were determined in utero or within 24 hours after delivery. Miscarriage during pregnancy (a drop of body weight >1 g), platelet counts (within 24 hours), and bleeding in the pups after delivery were analyzed, as previously described.[31]

Histologic analysis of murine placenta

Murine placentas were collected at 16.5 days' postcoitum and embedded in paraffin. Placental sections (3 μm) were stained with hematoxylin/eosin to determine the labyrinth zone ratio of the whole placenta. For immunohistochemistry, placental sections were incubated with anti-CD31 at 4°C overnight and stained with enzyme-labeled secondary antibody (Servicebio, Wuhan, China). Two fields per placenta labyrinth zone were selected, and the CD31$^+$ area was quantified by using Image J (National Institutes of Health, Bethesda, MD).

Tube formation assay

Human placental microvascular endothelial cells (HPVEC; 4 × 10^5/mL; FuDan, IBS Cell Center, Shanghai, China) in 50 μL Dulbecco's modified Eagle medium (serum free) were seeded onto the Matrigel (Corning, Bedford) and incubated for 30 minutes at 37°C. Thrombin (1 U; MilliporeSigma, Burlington, MA), 4 μg mouse IgG, mAb 32-106, or deg-32-106 in 50 μL Dulbecco's modified Eagle medium containing 5% mouse serum was added. For

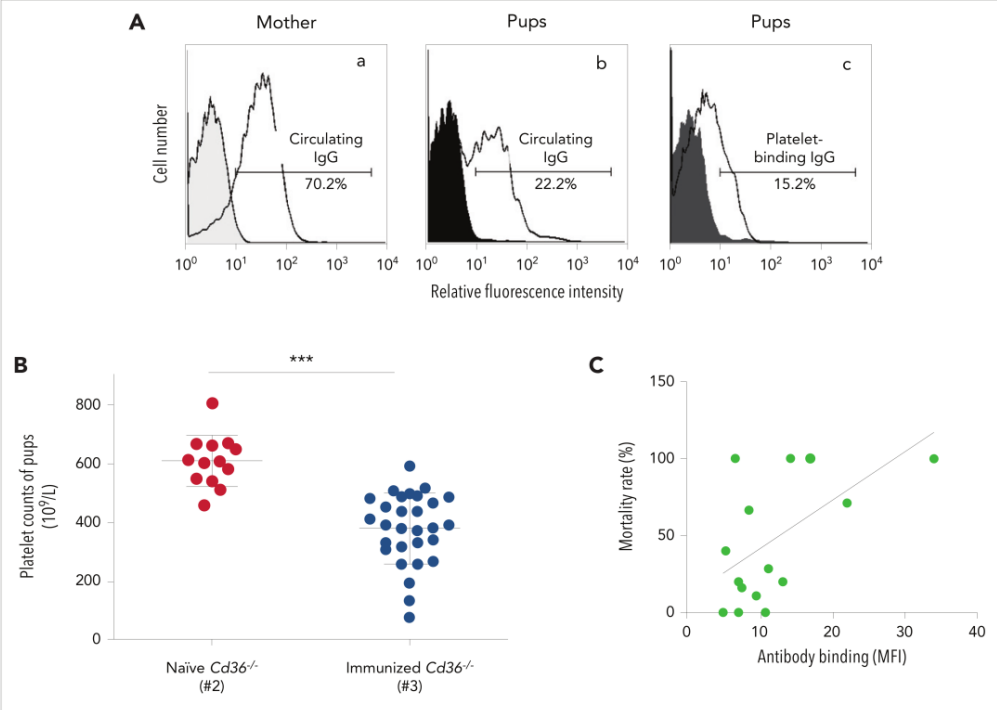

Figure 2. Maternal anti-CD36 antibodies crossed the placenta and caused thrombocytopenia and fetus death. (A) Flow cytometry analysis: (a) circulating anti-CD36 IgG from an immunized *Cd36*$^{-/-}$ mother (white curve) was analyzed by using normal mice sera as control (gray curve). (b) Circulating anti-CD36 in the pups' serum was measured by using pups' serum from the naive mother as control (black curve). (c) Anti-CD36 IgG bound to pups' platelets (platelet-binding IgG) was analyzed by using normal pups' platelets from the naive mother as control (dark gray curve). (B) Platelet counts of pups from naive and immunized *Cd36*$^{-/-}$ mothers were counted by flow cytometry using counting beads as standard. The pups' platelets (29 among 46 survival pups) from *Cd36*$^{-/-}$ immunized mothers (cohort #3; n = 7) is significantly decreased compared with pups' platelets (13 among 24 survival pups) from naive mothers (cohort #2; n = 3). Data are expressed as mean ± standard deviation. Significance (***$P <$ .0001) was analyzed by using a two-tailed unpaired Student *t* test. (C) Sera from immunized *Cd36*$^{-/-}$ mothers (n = 16) were analyzed by using flow cytometry. The reactivity of anti-CD36 antibodies in each maternal serum as relative fluorescence intensity (geometric mean) related to the percentage of mortality (number of dead pups/total pups) in the respective mothers is presented. Significance was analyzed by Pearson analysis (P = .0189; 95% confidence interval, 0.1161-0.8349). MFI, median fluorescence intensity.

the blocking experiment, 1 μg anti-FcγRIIa (clone IV.3; Stemcell Technologies, Vancouver, BC, Canada) was added with mAb 32-106. Furthermore, 50 μL anti-CD36 sera (1:10, 1:40) was transferred into the gel and incubated with HPVEC at 37°C for 6 hours in the absence or presence of deg-32-106. Images were taken from 2 to 3 selected areas per well. The tube formation assay was repeated 3 times. The total tube length was quantified by using Wimasis Image Analysis (Onimagin Technologies, Córdoba, Spain).

Statistical analysis

Data are presented as mean ± standard deviation and were analyzed by using Prism version 5.0 (GraphPad Software, La Jolla, CA). Comparisons of the 2 groups were assessed by using the two-tailed unpaired Student *t* test and χ^2 test. One-way analysis of variance was used for the multiple comparisons, and the Fisher exact test was used for small sample sizes. Correlations between anti-CD36 titers and mortality rates were analyzed by using the Pearson correlation method. P values $<.05$ were considered significant.

Results

Antibodies developed in immunized *Cd36*$^{-/-}$ female mice caused severe FNAIT in the animal model

We initially investigated whether *Cd36*$^{-/-}$ female mice could develop natural anti-CD36 antibodies during the first pregnancy when crossed with WT male mice. An analysis of maternal sera by flow cytometry showed that immunization rarely occurred. Only one-fifth of mothers developed anti-CD36 at low titers and only after the second and third pregnancy. Interestingly, one mother (#1) delivered a smaller littermate (litter size, 1.50 ± 0.71) compared with mothers (#2-5) that lacked anti-CD36 antibodies (litter size, 7.50 ± 1.93) (Figure 1A), indicating that miscarriages and fetal death occurred during pregnancy.

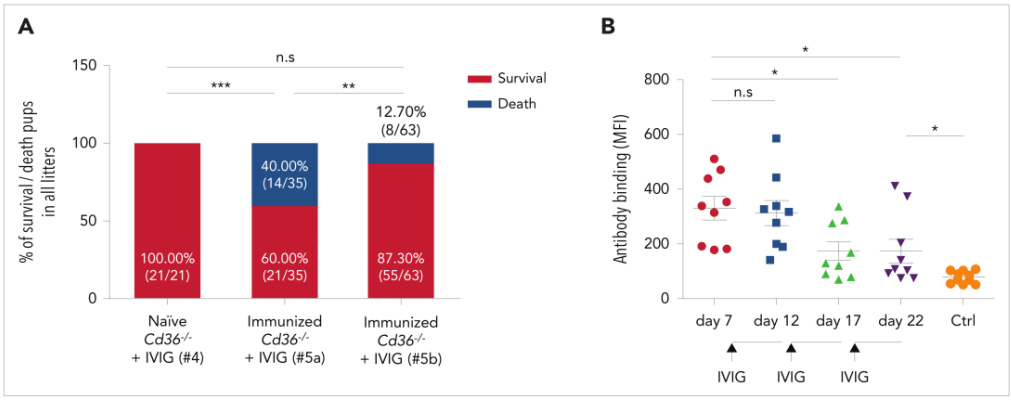

Figure 3. Early treatment with IVIG prevents FNAIT caused by maternal anti-CD36 antibodies. (A) Naive and immunized *Cd36*$^{-/-}$ female mice were bred with WT mice. During pregnancy, naive (cohort #4; n = 3) or immunized *Cd36*$^{-/-}$ mothers were treated with IVIG (1 g/kg) on days 10, 15, and 20 (cohort #5a; n = 7), or on days 7, 12, and 17 (cohort #5b; n = 9). High mortality of pups was observed in immunized mothers (cohort #5a) receiving IVIG on days 10, 15, and 20 compared with naive mothers (cohort #4) ($P < .001$). The number of dead pups was significantly decreased in immunized *Cd36*$^{-/-}$ mothers treated with IVIG 3 days earlier (cohort #5b), on days 7, 12, and 17 ($P < .01$). Significance was analyzed by using the χ^2 test (**$P < .01$; ***$P < .001$). (B) Anti-CD36 antibodies in *Cd36*$^{-/-}$ immunized (cohort #5b; n = 9) were measured before (on day 7) and after (on days 12, 17, and 22) IVIG administrations by flow cytometry and are represented as median fluorescence intensity (MFI). Sera from naive mothers (Ctrl) were run as controls (n = 10). Data are expressed as mean ± standard deviation. Significance (*$P < .05$) was analyzed by using a two-tailed unpaired Student *t* test. n.s, not significant.

To establish a murine model of anti-CD36–mediated FNAIT at the first pregnancy, we immunized *Cd36*$^{-/-}$ female mice 3 times with 10^8 WT platelets before breeding with WT male mice. This study designed 8 cohorts (#1-8) (supplemental Table 1 [available on the *Blood* Web site]; Figure 1). Naive WT (#1) and *Cd36*$^{-/-}$ female mice (#2) were conducted as control cohorts. Flow cytometry analysis showed that *Cd36*$^{-/-}$ mice (#3) developed anti-CD36 antibodies after immunization (Figure 1B). Interestingly, these antibodies also reacted with human *Cd36*$^{+/+}$ platelets (supplemental Figure 2). These results also pointed out that besides anti-CD36, no other platelet-reactive antibodies are involved in our animal model. In contrast, anti-CD36 was undetectable in naive *Cd36*$^{-/-}$ and naive *Cd36*$^{+/+}$ mice (cohorts #1-2). Notably, 31 (40.26%) of 77 pups that were delivered from 16 immunized *Cd36*$^{-/-}$ mothers were found dead: 3 in utero and 28 pups after deliveries (bleeding, ICH, and hydrops) (Figure 1C-D). The mortality of pups in this cohort was significantly higher than in cohort #1 ($\chi^2 = 17.46$; $P < .0001$) and cohort #2 ($\chi^2 = 11.52$; $P = .0007$). In total, only 2.70% to 4.00% of dead pups were documented in the control cohorts (#1-2). Furthermore, litter size numbers from immunized *Cd36*$^{-/-}$ mothers with detectable anti-CD36 antibodies (cohort #3) showed a trend to be smaller than control cohorts; however, it was not statistically significant compared with naive WT ($P = .064$) and *Cd36*$^{-/-}$ ($P = .323$) mothers (Figure 1E). This smaller litter size was probably related to 2 miscarriages found in cohort #3, which were not found in the control cohorts.

Analysis of anti-CD36 antibodies in immunized *Cd36*$^{-/-}$ mothers and their corresponding pups showed that maternal anti-CD36 antibodies could not only be detected in fetal sera (circulating IgG) but also on fetal platelets (platelet-binding IgG) (Figure 2A). In these mothers, the platelet counts in the surviving pups were significantly lower than in pups from naive *Cd36*$^{-/-}$ mothers ($377.12 \pm 121.14 \times 10^9$/L vs $607.76 \pm 87.80 \times 10^9$/L; $P < .0001$) (Figure 2B). A significant correlation (95% CI, 0.1161-0.8349; $P = .0189$) between anti-CD36 antibody titer and pups' mortality rates was observed (Figure 2C).

These findings showed that maternal anti-CD36 antibodies could cross the placenta, bind to fetal platelets, and induce severe FNAIT with a high frequency of fetal death.

Early treatment with IVIG prevents FNAIT caused by maternal anti-CD36 antibodies

Subsequently, we queried whether IVIG could prevent the severe FNAIT found in our animal model. IVIG (1 g/kg) was intravenously administered to immunized *Cd36*$^{-/-}$ mothers 3 times on days 10, 15, and 20 after breeding. In the control experiment, no fetal death was detected in naive *Cd36*$^{-/-}$ mothers treated with IVIG (cohort #4) (Figure 3A). Surprisingly, this treatment did not prevent the severe clinical outcome of FNAIT. A high frequency of fetal death was still documented (40.00% vs 0.00%; $P < .001$) (cohort #5a). However, we found a significant reduction in fetal death (12.70% vs 40.00%; $P < .01$) when IVIG was administered earlier, on days 7, 12, and 17 (cohort #5b), although similar titers of maternal anti-CD36 antibodies before IVIG administration were observed in both cohorts (data not shown). In accordance, a reduced antibody titer was first observed on day 17 after the second antenatal administration of IVIG ($P < .05$) (Figure 3B).

These results show that IVIG is suitable for the antenatal treatment of anti-CD36–mediated FNAIT, but particular care is required due to the delayed response of this therapy management.

deg-anti-CD36 antibodies prevent FNAIT caused by maternal anti-CD36 antibodies

Based on our previous studies, we sought to investigate whether deg-anti-CD36 antibodies could ease thrombocytopenia and

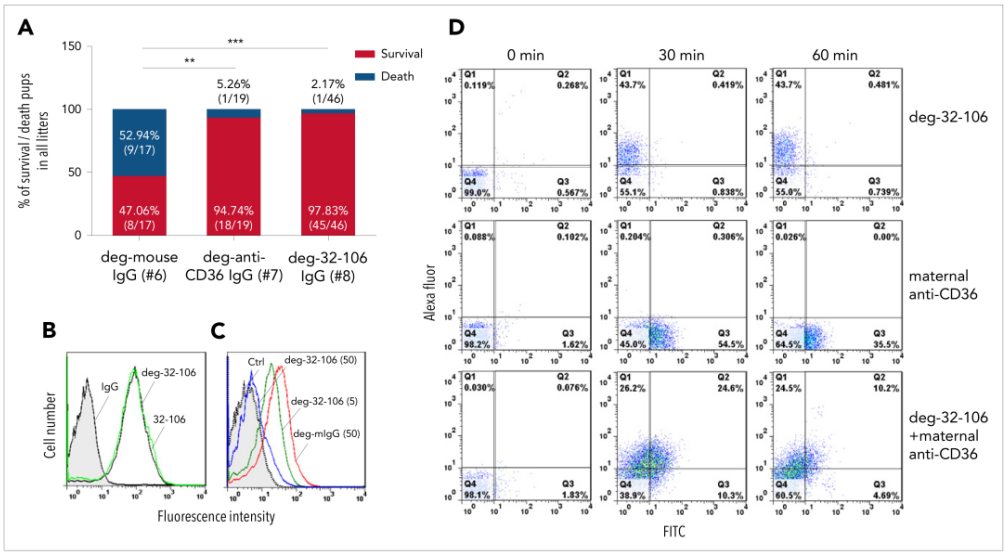

Figure 4. deg-anti-CD36 antibodies and deg-32-106 prevent FNAIT caused by maternal anti-CD36 antibodies. (A) Immunized *Cd36*$^{-/-}$ female mice were bred with WT mice. During pregnancy, *Cd36*$^{-/-}$ mothers were treated with deg-normal mouse IgG (cohort #6; n = 4), deg-anti-CD36 polyclonal antibodies (cohort #7; n = 3), or deg-32-106 (cohort #8; n = 6) in a dose of 5 mg/kg body weight on days 10, 15, and 20. The mortality of the pups treated with deg-anti-CD36 IgG (cohort #7; $P < .01$) or deg-32-106 (cohort #8; $P < .0001$) was significantly lower than that of mothers treated with deg-mouse IgG (cohort #6). Significance was analyzed by using the χ^2 test (**$P < .01$; ***$P < .001$). (B) WT mice platelets were incubated with mAb 32-106 (black) and deg-32-106 (green) using isotype IgG as controls (gray). The binding of both antibodies was compared by using flow cytometry. (C) As indicated, 50 ng deg-normal mouse IgG (red), 50 ng deg-32-106 (blue), or 5 ng deg-32-106 (green) was incubated with WT mice platelets. After washings, platelets were incubated with fluorescence-labeled (fluorescein isothiocyanate [FITC]) maternal IgG containing anti-CD36 antibodies (13.8 μg IgG) and analyzed by flow cytometry. Fluorescence-labeled (FITC) mouse IgG (dotted line) was used as a negative control. Note the significant left shift of fluorescence intensity in the presence of deg-32-106 compared with normal mouse IgG. (D) Alexa Fluor–labeled deg-32-106 (1 mg/kg) was injected into WT female mice via the tail vein. After 10 minutes, FITC-conjugated maternal IgG containing anti-CD36 antibodies was administered. Subsequently, the binding of anti-CD36 and deg-32-106 antibodies was evaluated at 30 and 60 minutes. Note the decreasing frequency of FITC-labeled platelets (Q2 + Q3) when deg-32-106 was injected (54.8% vs 34.9%; 35.5% vs 14.9%). A single administration of labeled deg-32-106 or maternal IgG containing anti-CD36 was run as a control.

prevent the severe effects of maternal anti-CD36 antibodies on the fetus during pregnancy.[28] For this purpose, a pool of IgG purified from sera of immunized *Cd36*$^{-/-}$ mice (n = 10) and nonimmunized (n = 10; as control) mice were deglycosylated with PNGase F enzyme. Because undigested anti-CD36 IgG antibodies can worsen the pups' fate, only pure deg-anti-CD36 was used for this in vivo study (supplemental Figure 3). When deg-anti-CD36 (5 mg/kg) was injected into immunized *Cd36*$^{-/-}$ mothers 3 times on days 10, 15, and 20 after breeding, the numbers of dead pups (cohort #7) were significantly lower than those of mothers treated with deg-mouse IgG (cohort #6) (5.26% vs 52.94%; $P < .01$) (Figure 4A).

Given the marked advantages of deg-anti-CD36 for the antenatal treatment of FNAIT, mouse mAbs against mouse CD36 were generated. One clone producing a high-affinity IgG2a antibody against mouse and human CD36, termed 32-106, was selected and deglycosylated. Flow cytometry analysis showed that both native and deg-32-106 IgG bound with similar affinity to WT platelets (Figure 4B). Furthermore, the competitive inhibition study in vitro (Figure 4C) showed that deg-32-106 could completely block the binding of maternal anti-CD36 antibodies into WT platelets. In vivo, administration of deg-32-106 significantly reduced the binding of maternal IgG containing anti-CD36 antibodies after 30 and 60 minutes (54.8% vs 34.9%; 35.5% vs 14.9%) (Figure 4D). However, the frequency of fluorescein isothiocyanate–labeled platelets (sensitized platelets) decreased after 60 minutes, most probably due to platelet clearance. Finally, we administered deg-32-106 (5 mg/kg) into immunized *Cd36*$^{-/-}$ mothers by the same therapy protocol (cohort #8). Only 1 dead pup (1 of 46 [2.17%]) was found in this cohort ($P < .0001$) (Figure 4A).

In addition, treatment with deg-32-106 led to increased pup platelet counts compared with the nontreated cohort (468.24 ± 85.20 $\times 10^9$/L vs 377.12 ± 121.14 $\times 10^9$/L; $P < .05$) (Figure 5A). Increased platelet counts (463.30 ± 30.88 $\times 10^9$/L vs 377.12 ± 121.14 $\times 10^9$/L; $P < .05$) were also observed with IVIG therapy; however, this was noted only when IVIG was administered early. These results showed that deg-32-106 could prevent the fatal severe effect of maternal anti-CD36 antibodies and improve the thrombocytopenic status of the fetus. More importantly, this treatment's positive effect occurred more rapidly and was more effective compared with IVIG therapy. A similar phenomenon was observed with the litter size (Figure 5B).

Placental dysfunction in FNAIT mediated by maternal anti-CD36 antibodies

Analysis of the placenta of immunized *Cd36*$^{-/-}$ mothers revealed a significant reduction in the placental labyrinth area compared

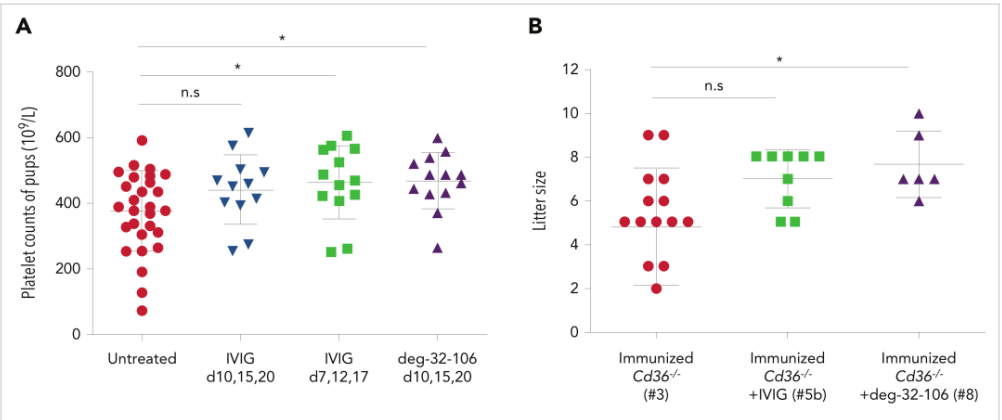

Figure 5. The effect of anti-CD36 on pups' platelet counts and litter size from *Cd36*$^{-/-}$ immunized mothers treated with IVIG or deg-32-106 antibodies. (A) Platelets from survivor pups derived from untreated, IVIG-treated (1 g/kg), or deg-32-106–treated (5 mg/kg) mothers were collected and quantified by flow cytometry using counting beads as standard. The pups' platelets from the untreated group were compared with those from 3 treated groups, including treatment with IVIG after breeding on days 10, 15, and 20; treatment with IVIG on days 7, 12, and 17; and treatment with deg-32-106 after breeding on days 10, 15, and 20. For each group, the platelets of 12 to 29 pups were counted. Data are expressed as mean ± standard deviation. Significance (*$P < .05$) was analyzed by using a two-tailed unpaired Student *t* test. (B) The litter size numbers derived from immunized *Cd36*$^{-/-}$ mothers treated with IVIG (cohort #5b; n = 9) or deg-32-106 (cohort #8; n = 6) were compared with those of untreated *Cd36*$^{-/-}$ mothers (cohort #3; n = 16). The data were analyzed by one-way analysis of variance, followed by the Bonferroni post hoc test (*$P < .05$). n.s, not significant.

with that of the naive cohort (55.75 ± 3.28% vs 62.88 ± 2.58%; $P < .005$) (Figure 6A-B). Furthermore, the quantification of vascular density in the placenta labyrinth by staining of endothelial cells with anti-CD31 antibodies showed lower fetal capillary numbers in the placenta labyrinth from immunized *Cd36*$^{-/-}$ mothers compared with naive cohort (9.86 ± 1.40 vs 13.98 ± 1.48; $P < .0005$) (Figure 6C-D).

Subsequently, tube formation assay was performed by using the placenta microvascular endothelial cells (HPVEC) expressing human CD36 to study the influence of mouse sera containing anti-CD36 on the angiogenesis process. Our flow cytometry analysis showed that human CD36 could react with mAb 32-106 against mouse CD36 (supplemental Figure 4). As shown in Figure 7A to 7B, sera containing anti-CD36 antibodies significantly reduced the tube length compared with the control sera ($P < .0005$). A similar phenomenon was observed with 32-106. In contrast, deg-32-106 did not alter angiogenesis (Figure 7C-D), indicating the important role of FcγR. Indeed, blocking endothelial FcγRIIa with mAb IV.3 abolished the antiangiogenic effect of mAb 32-106 (supplemental Figure 5). Finally, preincubation of HPVEC with deg-32-106 restored the antiangiogenic effect caused by the anti-CD36 sera, both at 1:40 and 1:10 serum dilutions ($P < .005$). These results suggest that impaired angiogenesis of fetal placenta endothelial cells caused by maternal anti-CD36 antibodies could lead to the fetal death observed in our mouse model of FNAIT.

Discussion

This study established the first animal model of anti-CD36–mediated FNAIT and compared the capability of IVIG and deg-anti-CD36 antibodies to lessen FNAIT. After immunization with WT platelets, *Cd36*$^{-/-}$ female mice were bred with WT male mice. A high mortality rate of pups (40.26%) was detected. Furthermore, massive skin bleeding, hydrops fetalis, and ICH was identified. The surviving pups' platelet counts (377.12 ± 121.14 ×10^9/L) were lower than those of the control cohorts. However, more severe thrombocytopenia (platelet counts, 132.2 ± 10.5 ×10^9/L) in pups caused by maternal anti-β3 antibodies was observed in a similar FNAIT model.[29] The reason for this mild thrombocytopenia in our cohorts may be the low level of CD36 surface expression on mouse platelets (<25,000 copies) and the broad cellular distribution of the CD36 antigen (platelets, monocytes, macrophages, and endothelial cells).[32] In contrast, high copy numbers of the αIIbβ3 integrin (110,000 to 130,000 copies/platelets) and the more restricted cellular distribution of mouse β3 integrin (platelets and endothelial cells) have been documented.[33]

In humans, variable clinical pictures of FNAIT caused by anti-CD36 antibodies have been observed, including widespread petechial hemorrhages, severe thrombocytopenia, ICH, and hydrops fetalis.[10,15,16,20,34] All these clinical pictures were found in our FNAIT animal model, indicating that this model is suitable for studying the mechanism of anti-CD36–mediated FNAIT and proving certain therapy strategies.

Several treatment options have been conducted to prevent severe FNAIT caused by anti—HPA-1a antibodies, including serial fetal blood sampling, intrauterine platelet transfusions, and infusions of IVIG. A systematic review suggested that IVIG administration represents the first-line antenatal treatment, whereas fetal blood sampling and intrauterine platelet transfusion resulted in a high complication rate.[1] Nevertheless, refractory states under IVIG treatment were still observed in mothers with ICH, although the success rate is 98.7%.[35,36]

Despite the increasing use of IVIG for treating FNAIT, the precise mechanism of IVIG action is still unclear.[37] Several mechanisms

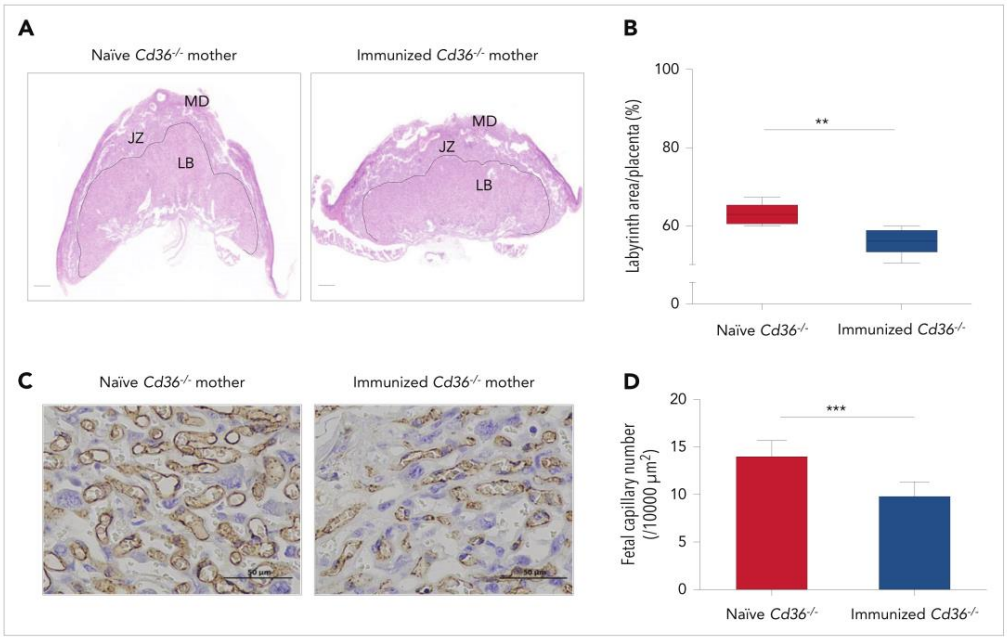

Figure 6. Anti-CD36 antibodies impaired vascularization in the immunized *Cd36*$^{-/-}$ mice placenta labyrinth zone. Additional 3 naive or *Cd36*$^{-/-}$ immunized female mice (n = 3) were crossed with WT male mice. The labyrinth zone was quantitatively analyzed at 16.5 days' postcoitum. (A) The solid line shows the labyrinth area (scale bars, 500 µm). (B) The labyrinth ratio to the whole placenta is shown as a box-and-whisker plot. (C) Representative photomicrographs of the naive and immunized *Cd36*$^{-/-}$ placenta labyrinth areas stained with anti-CD31 antibodies. Scale bars, 50 µm. (D) Numbers of fetal capillaries in the labyrinth areas. Data are expressed as mean ± standard deviation . Significance (**$P < .005$; ***$P < .0005$) were analyzed by using a two-tailed unpaired Student *t* test. JZ, junctional zone; LB, labyrinthine layer; MD, maternal decidua.

have been proposed, including decreasing maternal antibody production by inducing immune tolerance[38] and increasing pathogenic antibody clearance[39] or decreasing antibody transport by saturated FcRn.[40] In the β3$^{-/-}$ mice model of FNAIT, administration of IVIG could downregulate anti-β3 antibodies in both maternal and fetal circulations through FcRn-dependent and FcRn-independent pathways.[3,29] However, such strategies may inhibit the transfer of immune-protective maternal IgG, which might cause an increased risk of infections by the local dominant micropathogens during pregnancy and in the first weeks following birth. Therefore, more specific and effective antenatal therapies are desirable.

Our previous studies have shown that deg-mAb SZ21 against HPA-1a could pass through the placenta, inhibit the binding of maternal anti–HPA-1a antibodies, and prevent the clearance of fetal platelets by macrophages.[28] Furthermore, we found that some anti–HPA-1a antibodies explicitly bound to αvβ3 expressed by endothelial cells could induce endothelial dysfunction responsible for developing ICH in the fetus with severe FNAIT.[41,42] More recently, we observed that deg-SZ21 could prevent not only thrombocytopenia but also inhibit endothelial dysfunction caused by anti-HPA-1a antibodies (S.S. manuscript in preparation). Based on this knowledge, an antenatal therapy for severe FNAIT based on epitope-specific competitive antibodies should be feasible.

Earlier studies have shown that most mAbs against CD36 and anti-CD36 sera recognized epitopes within amino acids 155-183, indicating the important role of this domain as an immune-dominant target for anti-CD36 antibodies.[43,44] In the current study, we selected one mAb, 32-106, from our panel. Our in vitro and in vivo data showed that deg-mAb 32-106 could inhibit the binding of maternal anti-CD36 antibodies, indicating that both antibodies also react with the immune-dominant region. Accordingly, we found that the administration of deg-32-106 (5 mg/kg body weight) to the immunized *Cd36*$^{-/-}$ mothers 3 times (on days 10, 15, and 20) after breeding significantly increased not only fetal platelet counts (377.12 ± 121.14 $\times 10^9$/L to 468.24 ± 85.20 $\times 10^9$/L; $P < .05$) but also significantly reduced fetal death (40.26% to 2.17%; $P < .005$).

Surprisingly, similar antenatal treatment with IVIG administered in a dose of 1 g/kg body weight on days 10, 15, and 20 after breeding did not result in increased platelet counts and did not restore fetal death (40.00%). Based on risk stratification, weekly doses of IVIG (0.5 or 1 g/kg) are recommended to prevent bleeding complications in pregnancies complicated by FNAIT.[37] At the start of treatment, the gestational age is mainly based on the estimated onset of ICH, ranging from 20 until 28 weeks of gestation. In women with a previous child with ICH, IVIG is commonly introduced earlier, at 12 weeks of gestation. This management indicates that early antennal treatment with IVIG prevents severe

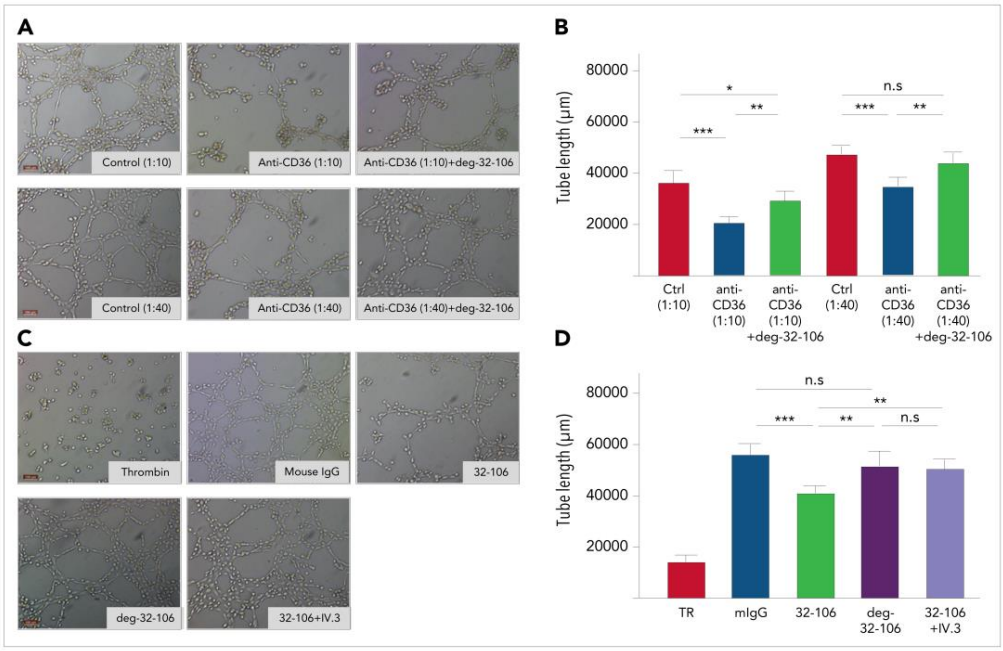

Figure 7. deg-32-106 prevented angiogenesis disturbance caused by the anti-CD36 antibodies. (A) HPVEC in Matrigel-coated wells were incubated with anti-CD36 sera (1:10, 1:40 dilution) in the absence or presence of deg-32-106. Diluted sera from naive mice were run as controls (control). (C) Thrombin (TR), 32-106, deg-32-106, or 32-106 together with mAb IV.3 against FcγRIIa was added to HPVEC as indicated. Mouse IgG was used as control. Scale bars, 100 µm. (B, D) Results from 3 experiments are presented. Data are expressed as mean ± standard deviation. Significance (*$P < .05$; **$P < .005$; and ***$P < .0005$) were analyzed by using a two-tailed unpaired Student *t* test.

FNAIT. Following this view, IVIG administration 3 days earlier to the immunized *Cd36*$^{-/-}$ mothers (ie, days 7, 12, and 17) decreased fetal death (40.00% to 12.70%). However, fetal death frequency is still significantly higher than that of antenatal treatment with deg-32-106 (2.17%). The late response of IVIG may be attributed to the slow downregulation of maternal antibodies. In our animal model, downregulation of maternal anti-CD36 antibodies was observed only after the second administration of IVIG, which agrees with the current view mechanism that IVIG can prevent serious FNAIT by downregulation of maternal anti-CD36 titer due to clearance of IgG via FcRn.[39] However, other mechanisms, such as direct inhibition of placenta transport by IVIG, should be considered.[40] Nevertheless, the effect of IVIG seems to be delayed.

Furthermore, Leontyev et al[45] reported that C57BL/6 mice are much less sensitive than BALB/c mice to IVIG-mediated attenuation of autoimmune thrombocytopenia (ITP), requiring ~2.5-fold more IVIG (2.5 g/kg) than BALB/c mice. In the previous FNAIT model, mice on the BALB/c background were used to study the effect of IVIG on anti-β3 antibody-mediated FNAIT.[3] Here, our results were based on the experiments with C57BL/6 mice, which may explain the low sensitivity of the IVIG treatment in our FNAIT model.

Disturbance of placenta vascular development and function could dramatically alter fetal growth development and thereby neonatal survival. In this process, placental vascularization and angiogenesis play critical roles.[46,47] During the third trimester of pregnancy, placenta preferential transport of maternal plasma fatty acids is critical for fetal growth and development.[48] CD36 is found on placental membranes, microvillus, and basal membrane.[49] The central role of CD36 (also known as fatty acid translocase) as a high-affinity receptor for fatty acid uptake and lipid metabolism has been well documented.[50,51] It is conceivable that the inhibition of fatty acid uptake via CD36 receptor by anti-CD36 antibodies could lead to inadequate placental angiogenesis. Indeed, the placenta analysis from immunized *Cd36*$^{-/-}$ mothers revealed a significant reduction of placental labyrinth area and decreased fetal capillary numbers compared with the naive cohort.

Previous studies reported on FcγRIIa (CD32) expression in the placenta microvascular endothelial cells.[52] Accordingly, preincubation of HPVEC with mAb against FcγRIIa restored the antiangiogenic effect of anti-CD36 antibodies. Consequently, the decisive effect of anti-CD36 antibodies could be restored by the addition of deg-mAb 32-106. One study showed that antibodies bound to endothelial cells significantly increased polymorphonuclear leukocyte adhesion in an FcγRIIa-dependent manner in cooperation with CXCR1/2, a mechanism of tissue injury during the inflammatory response.[53] The use of deg-32-106 may prevent antibody-mediated endothelial activation and recruitment of other blood cells such as monocytes and platelets. Taken together, our results showed that despite thrombocytopenia, maternal anti-CD36 antibodies contribute to fetal death by affecting placental angiogenesis.

In summary, we established a mouse model of FNAIT that reproduced the symptoms of human FNAIT induced by anti-CD36 antibodies. Although only mild thrombocytopenia was observed in the fetus, maternal anti-CD36 antibodies can cause severe bleeding, miscarriage, and fetal death. These severe clinical symptoms could be prevented by antenatal treatment with IVIG and deg-mAb 32-106 against CD36. Notably, treatment with deg-mAb against CD36 seems more beneficial than IVIG for various reasons, including the use of a lower dose (at least 200-fold less), the later start of treatment, and therapy success, despite other general IVIG disadvantages. Because our mouse hybridoma, 32-106, also recognizes human CD36 and can inhibit the binding of maternal anti-CD36 from FNAIT cases (supplemental Figure 6), humanized deg-mAb 32-106 should be feasible for immunotherapy in the near future.

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Authorship

Contribution: X.X., X.Y., Y.F., and S.S. conceived and designed the research; X.X., D.C., Y.X., Y.C., Y.S., J.D., H.D., J.L., and J.W performed experiments; X.X., S.S., D.C., X.Y., and W.X. analyzed data; X.X., Y.F., and S.S. wrote the manuscript; and H.N. analyzed and revised the manuscript.

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Footnotes

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All data-sharing requests may be submitted to the corresponding authors (Yongshui Fu [e-mail: fuyongshui@sina.com] or Sentot Santoso [e-mail: sentot.santoso@immunologie.med.uni-giessen.de]).

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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4. Discussion

Platelet reactive antibodies against polymorphic structures residing on platelet glycoproteins are a common cause of immune-mediated thrombocytopenias such as platelet transfusion refractoriness (PTR), post-transfusion purpura (PTP), or foetal and neonatal alloimmune thrombocytopenia (FNAIT). In contrast to Caucasians, alloantibodies against HPA-1a do not seem to play an important role in Asian populations. Until recently, the role of anti-CD36 antibodies in the pathomechanism of immune-mediated thrombocytopenia has been underappreciated. Recent data has indicated that CD36 isoantibodies produced by CD36 type I-deficient individuals seem more common in Asian and African populations.

4.1. The Molecular Basis of CD36 Deficiency in South China

Three decades ago, the first case of PTR caused by anti-Naka antibodies was reported in Japan (Ikeda H et al., 1989). Further study showed that Naka negative platelets failed to express CD36 (CD36null) on the cell surface, suggesting that Naka is an isoantigen rather than an alloantigen (Tomiyama Y et al., 1990). Two types of CD36 deficiency are known: type I lacking CD36 surface expression both on platelets and monocytes, and type II lacking CD36 on platelets only (Yamamoto N et al., 1994). The frequency of CD36 deficient in Japanese, African and Caucasian had been described. However, little is known about the relevance of CD36 in immune-mediated platelet disorders among Chinese populations.

In the first paper, we analyzed the frequency of type I and type II CD36 deficiencies among healthy blood donors in South China and found that the frequency of type I CD36 deficiency (> 0.5%) was similar between Japanese populations and our cohort. Nowadays, more than 30 mutations in the coding region of the CD36 gene responsible for type I CD36 deficiency have been described (Li R, et al., 2015). Homozygous or compound heterozygous mutations in the CD36 gene associated with the absence of CD36 surface expression both on platelets and monocytes have been discovered. Four major CD36 gene mutations, including C268T, 329-330*del*AC, 949*ins*A, and 1228-1239*del*ATTGTGCCTATT, cover almost 90% of CD36 genetic defects among Japanese, of which the C268T (Pro90Ser) mutation is the most frequent mutation (>50%) (Kashiwagi H et al., 2001). However, the Pro90Ser mutation did not represent the most

common cause of type I CD36 deficiency in South Chinese populations. No individual carrying Pro90Ser was detected in this study (manuscripts 1). In contrast, the most common mutations found in this Chinese population were 329-330*del*AC and 1228-1239*del*ATTGTGCCTATT, which was similar to an analysis of CD36 deficiency among Southeast Asian populations (Xia et al., 2016). In addition, three new CD36 mutations, C220T, 429+4*ins*g and 1200-5*inv*49bp associated with type I CD36 deficient individuals were identified. By CD36 transcript analysis we found that the mutations C220T, 429+4*ins*g and 1200-5*inv*49bp leads to premature stop codon 220 TAG (Gln74Term), and skipping of exon 6 and exon 13, respectively. To date, the molecular mechanism of CD36 deficiency is unknown, and unfortunately, no mutations have been found in some of these CD36 type II-deficient individuals. Thus, further study is necessary to unravel the mechanism(s) of CD36 deficiency on platelets, especially in type II deficiency.

4.2. PTR and FNAIT caused by anti-CD36 Antibodies

Recently, several cases of PTR associated with anti-CD36 antibodies, primarily in Asian and African populations were reported (Curtis BR et al., 1996; Lee K et al., 1999; Fujino H et al., 2001; Saw CL et al., 2010; Yin XL et al., 2011). In manuscripts 2, two cases of PTR associated with strong anti-Nak^{a} antibodies in patients receiving multiple transfusions of random platelets were reported. Both patients did not respond to apheresis random donor and subsequent HLA-compatible platelet transfusions. Antibody detection using commercial ELISA kit (Pakplus, GTI) showed positive reaction against CD36. Flow cytometry analysis of platelets and monocytes from patients using FITC-labelled mAb FA6-152 against CD36 showed deficient of CD36 surface expression. In comparison to transfusions with non-compatible apheresis platelets (CD36 positive), significant improvement of corrected count increment (CCI) was obtained after transfusions with compatible platelets (CD36 negative). Transfusion with CD36-deficient platelets is the method of choice for preventing bleeding in patients with PTR caused by anti-CD36 antibodies (Fujino H et al., 2001).

After the discovery of anti-CD36 antibodies as a cause of PTR in Japan, the first case of FNAIT caused by anti-CD36 antibodies was reported in Thailand (Kankirawatana S et al., 2001). Recently we described several cases of anti-CD36-mediated FNAIT with severe intracranial hemorrhaging or abortions of the fetus of type I CD36 deficient

multi gravida mothers (manuscripts 1, 2, 3 and 4). In addition, a clinical case of FNAIT in Taiwan caused by anti-CD36 antibodies was firstly reported (manuscript 6). Interestingly, the clinical presentation of FNAIT mediated by anti-CD36 antibodies is heterogeneous and ranges from widespread petechial hemorrhages to thrombocytopenia, gastrointestinal bleeding, severe anemia, ICH, and hydrops fetalis (Kankirawatana et al., 2001; Curtis et al., 2002; Taketani et al., 2008; Okajima et al., 2006).

Based on these observations, screening for anti-Nak^{a} antibodies should be considered for suspected immune mediated thrombocytopenia in Asian populations. A donor registry of CD36 deficient individuals should be established to supply for patients with these disorders.

4.3. Successful Management of FNAIT Cases caused by anti-CD36 Antibodies

In Caucasians, more than 75% of FNAIT cases are induced by *allo*antibodies against human platelet antigen (HPA)-1a (Mueller-Eckhardt C et al., 1989), whereas the most common antibodies related to FNAIT for Japanese are anti-HPA-4b antibodies (Ohto H et al., 2004). However, recent data showed that anti-CD36 *iso*antibodies developed in type I CD36 deficient mothers are frequently reported as the cause of FNAIT in China (Wu G et al., 2017). Little is known about the management of FNAIT induced by anti-CD36 *iso*antibodies.

Recently, we successfully managed three cases of FNAIT caused by anti-CD36 *iso*antibodies, of which two were life-threatening cases of FNAIT with hydrops fetalis, serious anaemia, and thrombocytopenia (patient 1 and patient 2; manuscript 3 and 4), while the third one only suffered from thrombocytopenia with a platelet count of 36.000/µL (Patient 3; manuscript 6). Here, two approaches, including intrauterine transfusions and IVIG, were used to restore the platelets of the fetus or in neonatal patients. For patient 3, the administration of IVIG seemed to be effective to increase the platelet count, whereas in patients 1 and 2, intrauterine transfusions with compatible RBC and CD36*null* platelets were necessary to manage severe fetal anemia and the development of hydrops fetalis. In paper of Curtis et al, five FNAIT cases caused by anti-CD36 antibodies were described, but neither prenatal nor postnatal anaemia was observed (Curtis BR et al., 2002). In two of these infants, the platelet counts rose to the normal range within 2 weeks without any specific treatment. However, Okajima et al.

described a case of two siblings with hydrops fetalis due to anti-CD36. Foetal anaemia was observed in the elder sister. She was managed by intraperitoneal transfusion with RBC and delivered at 30 weeks of gestation. The younger sister was delivered at 29 weeks of gestation without intrauterine intervention (Okajima et al., 2006). In the third and fourth papers, we presented a careful elaboration of these cases caused by anti-CD36 antibodies. This included laboratory diagnosis as well as successful management. Since hydrops fetalis and anemia rather than thrombocytopenia seem to threaten a fetus, immediate RBC transfusion should be recommended as the first priority upon diagnosis.

4.4. Possible underlying Mechanism for Hydrops Fetalis associated with anti-CD36 Antibodies in FNAIT

In FNAIT, anti-CD36 antibodies cause not only thrombocytopenia and ICH, but they also cause recurrent early fetal loss and hydrops. Two cases of FNAIT caused by anti-CD36 antibodies associated with hydrops fetalis (HF) were reported by Okajima et al. However, little is known regarding the pathological mechanism underlying the induction of hydrops fetalis by these antibodies (Okajima et al., 2006). HF is a clinical condition in which excessive fluid accumulation in the extravascular compartment of the fetus leads to widespread oedema. The pathophysiology of HF is decidedly more complex. In contrast to anti-HPA-1a, anti-CD36 antibodies react with platelets and endothelial cells, and also erythrocytes and monocytes of the fetus. The CD36 expression was related to the differentiation stage of the erythroid progenitors. It is expressed on mature BFU-E with an antigenic density increasing until day five CFU-E (Edelman et al., 1986). In the fourth paper, we found that maternal anti-CD36 antibodies caused a significant reduction of burst-forming unit-erythroid and colony-forming unit-erythroid (BFU-E/CFU-E) formation associated with an increased number of apoptotic CD34+ erythroid/myeloid precursor cells. This mechanism may be responsible for fetal anemia and consecutive anemic hydrops fetalis (AHF). However, other mechanisms, including hemolysis due to complement activation by anti-CD36, may also contribute to the pathogenesis of AHF.

4.5. The Detection of anti-CD36 Antibodies by Different Assays

It has been suggested that Type I CD36-deficient individuals, not type II individuals are at risk of producing anti-CD36 *iso*antibodies through blood transfusion and pregnancy.

In China around 0.5% of CD36 type I deficient individuals are at risk to be immunized through blood transfusion or pregnancy. Similarly, 0.6% of Taiwanese population failed to express CD36 on platelets and monocytes (Lo et al., 2016). Until today, several cases of PTR and FNAIT caused by anti-CD36 antibodies have been documented.Therefore, the detection of anti-CD36 antibodies should be considered for Asian and African patients suffering from immune thrombocytopenia.

In the sixth paper, stably transfected HEK293T cells expressing human CD36 were established to characterize the antibody specificity. The presence of anti-CD36 antibodies in the maternal serum was identified by flow cytometry as well as by immunoprecipitation analysis using this transfected cell line. However, a negative result was obtained when an antigen capture assay (MAIPA) was performed with platelets using mAb FA6-152 as the capture antibody. This was probably due to competitive inhibition between the monoclonal antibody (mAb) used for MAIPA and human anti-CD36 antibodies (Morel-Kopp et al., 1996). To avoid this problem, recombinant CD36 carrying the V5-peptide (GKPIPNPLLGLDST) was used to react with human anti-CD36 antibodies. Since these antibodies do not bind to the extracellular domain of CD36 directly, competitive inhibition by any human anti-CD36 antibodies would be prevented.

Currently, the detection of anti-CD36 antibodies by standard MAIPA assay can be hampered by the high frequency of false negative results. Previous studies have demonstrated that most mAbs against CD36, including FA6-152, OKM5, and 10/5, bind epitopes within the domain comprising amino acids 155–183, and the binding of these mAbs prevents the binding of anti-Nak^{a} antibodies (Daviet et al., 1995a &1995b). Due to this problem, the choice of mAbs is critically important for the reliable diagnosis of anti-CD36 antibodies in MAIPA. In our fifth paper, we generated a panel of mouse mAbs against CD36 and selected two mAbs GZ-70 and GZ-608 for MAIPA assay. In comparison to reference mAb FA6-152 in this MAIPA assay, mAbs GZ-70 and GZ-608 could detect a majority of anti-CD36 antibodies in 14 samples containing anti-CD36 (92.86% vs. 42.86%). MAIPA may be reliably used for the detection of anti-CD36 antibodies when certain capture mAbs are used. The use of MAIPA will facilitate the identification of new platelet antigens and antibodies against CD36 that are involved in immune mediated thrombocytopenia. However, further study with other cohorts is

necessary to evaluate the applicability of this MAIPA approach for the detection of anti-CD36 antibodies.

In addition to the MAIPA method, other approaches can also be used to detect anti-CD36 *iso*antibodies, including platelet suspension immunofluorescence testing (PSIFT) and commercial assays such as solid-phase assay (PakPlus) and Luminex bead-based platelet antibody detection assay (Pak Lx), but they still have some drawbacks. PSIFT is a simple and fast flow cytometry based method. However, this method needs fresh CD36 deficient platelets as a negative control to exclude the presence of other platelet reactive antibodies in serum samples (such as anti-HPA or anti-HLA antibodies). In addition, mAb-independent ELISA using purified CD36 protein is commercially available. However, the fact that purified antigens immobilized on a solid platform may undergo epitope changes, leading to false negative or positive results, cannot completely be excluded. In the near future, comparable studies should be performed to find a suitable method for the routine laboratory diagnosis of anti-CD36 antibodies. Moreover, the analysis of anti-CD36 containing sera using mAb-dependent (e.g. with anti-CD36 mAb) and mAb-independent assays (e.g., with anti-V5 antibody) may allow better characterization of the different epitopes recognized by human anti-CD36 antibodies. This knowledge may help us to understand the variable clinical pictures of FNAIT caused by anti-CD36 antibodies.

4.6. The Patho-mechanism and Antenatal Treatment of anti-CD36-mediated FNAIT in a Mouse Model

Although anti-CD36 *iso*antibodies turn out to be the most frequent platelet antibodies found in Asia and could cause varied clinical outcomes of FNAIT, little is known about the patho-mechanism and antenatal treatment of anti-CD36-mediated FNAIT. Compared to anti-HPA-1a antibodies, anti-CD36 antibodies cause more common hydrops and recurrent miscarriages (Okajima et al., 2006; Xu et al., 2018; Wu et al., 2020). The reason for this phenomenon is currently unknown. Therefore, the current approach for treating FNAIT caused by anti-HPA-1a may not be adaptable for the disease caused by anti-CD36 antibodies. In the last paper, we established a novel animal model to examine the clinical features of pups from immunized *Cd36*-/- female mice after breeding with wild type male mice. Mild thrombocytopenia was observed, but high pup mortality was also documented (40.26%). The reason for this mild

thrombocytopenia in our cohorts may have been the low level of CD36 surface expression on mouse platelets (<25,000 copies) and the broad cellular distribution of the CD36 antigen (platelets, monocytes, macrophages, and endothelial cells) (Kuijpers et al., 2014).

The current clinical challenge for managing FNAIT relies on the prevention of fetal bleeding complications through antenatal therapy since the prophylaxis is currently not available. However, the antenatal management of FNAIT is still a matter of debate (Winkelhorst et al., 2017). Currently, several treatment options have been conducted to prevent severe FNAIT caused by anti-HPA-1a antibodies, including serial fetal blood sampling (FBS), IUPTs, and infusions of IVIG (Winkelhorst et al., 2017; Bertrand G et al., 2011). A recent systematic review suggested that the non-invasive maternal treatment with IVIG represents antenatal therapy's first line of defense (Winkelhorst et al., 2017). However, such strategies may inhibit the transfer of immune-protective maternal IgG, which may cause an increased risk of infections via local dominant micropathogens during pregnancy and in the first weeks after birth (Mathiesen et al., 2013). Therefore, more specific and effective antenatal therapies are desirable.

Ghevaert and colleagues introduced such a strategy using human recombinant scFv (single-chain fragment variable) anti-HPA-1a antibodies with a modified Fc part. These modified recombinant anti-HPA-1a antibodies could pass the placenta and ease FNAIT in immunized mothers (Ghevaert et al., 2013 & 2008).

In recent years, the therapeutic potential of deglycosylated IgG (deg-IgG) antibodies for autoimmunity treatment have been widely recognized (Crispin, 2013). Removal of the N-glycan (linked to asparagine 297), located on the Fc part, leads to a significant reduction of IgG binding to the macrophages' cellular receptors (FcγRs) and its ability to activate complement factor C1q. However, these deg-IgG antibodies can still be transported from maternal circulation to the fetus via the neonatal Fc receptor (FcRn) (Nimmerjahn and Ravetch, 2008).

Accordingly, our previous studies demonstrated that deglycosylated mAb SZ21 against HPA-1a could pass through the placenta, inhibiting the binding of maternal anti-HPA-1a antibodies, and prevent the clearance of fetal platelets by macrophages (Bakchoul et al., 2013). Furthermore, we demonstrated that some anti-HPA-1a antibodies explicitly

bound to αvβ3 expressed by endothelial cells could induce the endothelial dysfunction responsible for developing ICH in a fetus with severe FNAIT (Yougbaré et al., 2015; Santoso et al., 2016). Based on this knowledge, an antenatal therapy of severe FNAIT based on epitope-specific antibodies should be feasible.

In the last paper, we generated mouse mAbs against mouse CD36, and one clone producing high-affinity anti-CD36 (termed 32-106) effectively inhibited maternal antibody binding and was therefore selected. Accordingly, we found that the administration of deg-32-106 (5 mg/kg body weight) to immunized *Cd36*-/- mothers three times (on days 10, 15, and 20 after breeding) significantly increased not only the fetal platelet counts (377.12 ± 121.14 $x10^9$ / L to 468.24 ± 85.20, $P < 0.05$) but also completely prevented fetal death (40.26% to 2.17%; $P < 0.005$) caused by maternal anti-CD36 antibodies. Surprisingly, similar antenatal treatments with IVIG administered at a dose of 1 g/kg body weight on days 10, 15, and 20 after breeding did not result in increased platelet counts and did not rescue fetal death rates (40.00%). Interestingly, IVIG administration three days earlier to immunized *Cd36*-/- mothers (on days 7, 12, and 17 instead on days 10, 15 and 20) decreased fetal death rates (40.00% to 12.70%). However, the number of fetal deaths was still significantly higher than that with antenatal treatment using deg-32-106 (2.17%). The late response to IVIG could be attributed to the slow down-regulation of maternal antibodies.

Furthermore, immunized *Cd36*-/- mothers showed placenta deficiencies. Accordingly, maternal anti-CD36 antibodies inhibited the angiogenesis of placenta endothelial cells, which could be restored by treatment with deg-32-106. In summary, maternal anti-CD36 antibodies caused a high frequency of fetal death in our animal model, associated with placental dysfunction. These severe clinical symptoms could be prevented by antenatal administration of IVIG and deg-mAb 32-106 against CD36. Notably, therapy with deg-mAb against CD36 showed more beneficial than IVIG for different factors, including the use of a lower dose (at least 200 fold less), the later start of treatment, and therapy success, despite other general IVIG disadvantages. Since deg-32-106 could react with human CD36, humanized deg-mAb 32-106 could be feasible for immunotherapy in the near future.

4.7. Summary Discussion

In summary, in this study, we highlighted the clinical relevance of anti-CD36 in the development of FNAIT, developed new laboratory diagnostics for the identification of anti-CD36 antibodies, and established an animal model to understand the pathomechanism of anti-CD36-mediated thrombocytopenia and used this to test different treatment approaches.

5. Summary

Although the role *allo*antibodies against HPA-1a on the pathomechanism of FNAIT in Caucasian populations is well established, these *allo*antibodies do not seem to play significant role in Asian and African populations. In contrast, anti-CD36 *iso*antibodies developed by immunized CD36 negative individuals turn out to be important as causes of immune mediated thrombocytopenia (PTR and FNAIT) in Chinese populations. In this study, we determined the frequency of CD36 defective individuals in China, underlined the clinical relevance of anti-CD36 antibodies on FNAIT, developed new laboratory diagnostics for the identification of anti-CD36 antibodies, and established an animal model to understand the pathomechanism of anti-CD36-mediated thrombocytopenia and to test different treatment approaches. Overall, this study illustrates a new scientific platform to improve our understanding of the relevance antibody-mediated cell destruction.

In our first study, we found that more than 0.5% of CD36 type I deficient individuals in a South Chinese population were at risk of developing anti-CD36 antibodies through blood transfusions or pregnancy. Based on this observation, the involvement of anti-CD36 antibodies should be considered for Chinese patients suffering from immune thrombocytopenia.

Furthermore, our second, third, and fourth studies showed that the clinical the foetus caused by anti-CD36 antibodies were heterogeneous, ranging from moderate to severe. In serious FNAIT, anti-CD36 antibodies may cause anemia and trigger hydrops through apoptosis of CD34+ erythroid/myeloid precursor cells. Intrauterine transfusions with compatible RBC and CD36*null* platelets are useful in preventing deleterious clinical effects. These studies underline the clinical significance of anti-CD36 antibodies in our population, substantiate the necessity of laboratory diagnostics for the identification of these antibodies, and emphasize the importance of generating a registry of CD36*null* donors for the treatment of these patients.

In the fifth and sixth studies, we provided several tools to improve the laboratory diagnosis of anti-CD36 antibodies, including the development of suitable capture mAbs against CD36 for antigen capture assays and generated transfected cell lines expressing recombinant CD36 for the characterization of anti-CD36 antibodies by flow cytometry,

immunoprecipitation, and antigen-capture assays. These approaches offer reliable identification of anti-CD36 antibodies.

In the last study, through an animal model we showed that maternal anti-CD36 antibodies could cause a high frequency of fetal death which associated with placental dysfunction. This serious effect could be prevented by the antenatal administration of IVIG and deg-mAb 32-106. Interestingly, therapy with deg-32-106 appeared more beneficial than IVIG considering the lower dose, later start of application and the success rate of treatment.

6. Zusammenfassung

Obwohl die Rolle von Alloantikörpern gegen HPA-1a auf den Pathomechanismus der FNAIT in der kaukasischen Bevölkerung gut etabliert ist, scheinen diese Alloantikörper in der asiatischen und afrikanischen Bevölkerung keine bedeutende Rolle zu spielen. Im Gegensatz dazu erweisen sich anti-CD36-Isoantikörper, die von immunisierten CD36-negativen Personen entwickelt werden, als wichtige Ursache für immunvermittelte Thrombozytopenie (PTR und FNAIT) in der chinesischen Bevölkerung. In dieser Studie haben wir die Häufigkeit von CD36-defizienten Individuen in China bestimmt, die klinische Relevanz von anti-CD36-Antikörpern bei FNAIT unterstrichen, neue Labordiagnostik zur Identifizierung von Anti-CD36-Antikörpern entwickelt und ein Tiermodell etabliert, um den Pathomechanismus der anti-CD36-vermittelten Thrombozytopenie zu verstehen und verschiedene Behandlungsansätze zu testen. Insgesamt eröffnet diese Studie eine neue wissenschaftliche Plattform, um unser Verständnis über die Relevanz antikörpervermittelter Zellzerstörungen zu verbessern.

In unserer ersten Studie fanden wir heraus, dass in der südchinesischen Bevölkerung mehr als 0,5 % der Personen mit CD36-Typ-I-Defiziens. Diese Personen können somit durch Bluttransfusionen oder während der Schwangerschaft anti-CD36 Antikörper entwickeln, die für Entstehung von PTR beziehungsweise FNAIT verantwortlich sein können. Aufgrund dieser Beobachtung sollte die Beteiligung von Anti-CD36-Antikörpern bei chinesischen Patienten, die an Immunthrombozytopenie leiden, in Betracht gezogen werden.

Darüber hinaus zeigten unsere zweiten, dritten und vierten Studien, dass die durch Anti-CD36-Antikörper verursachten Krankheitsbilder von FNAIT heterogen sind und zwar von moderat bis schwer reichen. Bei schwerer FNAIT können Anti-CD36-Antikörper eine Anämie verursachen und einen Hydrops durch Apoptose von CD34+ erythroiden/myeloischen Vorläuferzellen auslösen. Intrauterine Transfusionen mit kompatiblen Erythrozyten und CD36null Thrombozyten sind nützlich, um die schädlichen klinischen Auswirkungen zu verhindern. Diese Studien unterstreichen die klinische Bedeutung von Anti-CD36-Antikörpern in unserer Population, belegen die Notwendigkeit der Labordiagnostik zur Identifizierung dieser Antikörper und betonen die Wichtigkeit des Registers von CD36null-Spendern für die Behandlung dieser Patienten.

In der fünften und sechsten Studie stellten wir mehrere Werkzeuge zur Verbesserung der Labordiagnostik von Anti-CD36-Antikörpern zur Verfügung; darunter sind die Entwicklung geeigneter Fänger-mAbs gegen CD36 für den Antigen-Capture-Assay, stabilen CD36 Transfektanten für die Detektion von Anti-CD36-Antikörpern mittels Durchflusszytometrie, Immunpräzipitation und Antigen-Capture-Assay. Diese Ansätze bieten eine zuverlässige Identifizierung von Anti-CD36-Antikörpern.

In der letzten Studie konnten wir zeigen, dass mütterliche Anti-CD36-Antikörper in unserem Tiermodell eine hohe Häufigkeit des fetalen Todes verursachen können, der mit einer Dysfunktion der Plazenta einhergeht. Dieser schädliche Effekt konnte durch die vorgeburtliche Gabe von IVIG und deg-mAb 32-106 vermindert werden. Interessanterweise scheint die Behandlung mit deg-32-106 in Anbetracht der niedrigeren Dosis, des späteren Behandlungsbeginns und des Therapieerfolgs vorteilhafter zu sein als IVIG.

7. List of Abbreviations

ACA	Antigen Capture Assay
AHF	Anemic hydrops fetalis
BFU-E/CFU-E	Burst-forming unit-erythroid and colony-forming unit-erythroid
DITP	Drug-induced immune thrombocytopenia
FcγRs	Macrophages' cellular receptors
FcRn	Neonatal Fc receptor
FNAIT	Fetal and Neonatal Alloimmune Thrombocytopenia
HEK 293	Human Embryonic Kidney 293 Cells
HDFN	Hemolytic disease of the fetus and newborn
HF	Hydrops fetalis
HIT	Heparin-induced thrombocytopenia
HLA	Human Leukocyte Alloantigen
HPA	Human Platelet Alloantigen
HSCs	Hematopoietic Stem Cells
HUS	Hemolytic Uremic Syndrome
ICH	Intracranial Hemorrhage
IgG	Immunoglobulin G
ITP	Immune Thrombocytopenia
IUPT	Intrauterine Platelet Transfusion
IVIG	Intravenous Immunoglobulin G
mAb	Monoclonal antibody
MAIPA	Monoclonal Antibody Immobilization of Platelet Antigens
NHTRs	Non-hemolytic Transfusion Reactions
Ox-LDL	Oxidized low-density lipoprotein
Ox-PL	Oxidized phospholipids
PSIFT	Platelet Suspension Immunofluorescence Test
PTR	Platelet Transfusion Refractoriness
PTP	Post-transfusion Purpura
Plts	Platelets
TRALI	Transfusion-Related Acute Lung Injury
UTRs	Untranslated Regions

8. List of Figures

9. List of Tables

10. References

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11. Publikationsverzeichnis, Auflistung der Kongressbeiträge, (Poster, Vortrag)

(1). Studies on CD36 Deficiency in South China: Two Cases Demonstrating the Clinical Impact of anti-CD36 Antibodies

Xiuzhang Xu, Xin Ye, Wenjie Xia, Jing Liu, Haoqiang Ding, Jing Deng, Yangkai Chen, Yuan Shao, Jiali Wang, Yongshui Fu, Sentot Santoso

Thromb Haemost 2013;110:1199-1206. doi: 10.1160/TH13-05-0435. Epub 2013 Aug 22.

(2). The Detection of anti-Nak^a Antibodies by the use of CD36 Transfected cells: Comparison with other Methods (Oral presentation)

Xiuzhang Xu, Wenjie Xia, Xin Ye, Jing Deng, Yangkai Chen, Yongshui Fu and Sentot Santoso

The 24th Regional Congress of the ISBT, Kuala Lumpur, Malaysia, 1.- 4. December 2013.

(3). A Murine Model of Foetal/Neonatal Alloimmune Thrombocytopenia caused by CD36 Antibodies (Oral presentation)

Xiuzhang Xu, Xin Ye, Wenjie Xia, Dawei Chen, Sentot Santoso

The 26th Regional Congress of the ISBT, Bali, Indonesia, 14.- 16. November 2015.

(4). The Immunization of CD36-/- Mouse by the use of Mouse CD36 Transfected Cells: Generation of Novel anti-CD36 Antibodies (Oral presentation)

Xiuzhang Xu, Dawei Chen, Sentot Santoso

The 34th International Congress of the ISBT, Dubai, 3.- 8. September 2016.

(5). Deglycosylated anti-CD36 Antibody for Treatment of FNAIT caused by Maternal CD36 Antibodies in a Murine Model (Oral presentation)

Xiuzhang Xu, Xin Ye, Wenjie Xia, Dawei Chen, Sentot Santoso

The 28th Regional Congress of the ISBT, Guangzhou, China, 25.- 28. November 2017.

(6). Role of CD36 in Immune Thrombocytopenia among Asian Populations (Oral presentation)

Xiuzhang Xu, Sentot Santoso

The 28th Regional Congress of the ISBT, Guangzhou, China, 25.- 28. November 2017.

(7). Successful Management of a Hydropic Fetus with Severe Anemia and Thrombocytopenia caused by anti-CD36 Antibody

Xiuzhang Xu, Lin Li, Wenjie Xia, Haoqiang Ding, Dawei Chen, Jing Liu, Jing Deng, Yangkai Chen, Zhiming He, Jiali Wang, Yuan Shao, Sentot Santoso, Xin Ye, Qun Fang

Int J Hematol 2018;107(2):251-256. doi: 10.1007/s12185-017-2310-5. Epub 2017 Aug 16.

(8). Role of CD36 in Immune-mediated Thrombocytopenia in Asian Populations

Xiuzhang Xu, Sentot Santoso

ISBT Science Series 2018; 13, 317-322. First published: 14 February 2018. https://doi.org/10.1111/voxs.12414.

(9). Various CD36 Gene Mutations and Their Correlations with CD36 Expression Levels on Monocytes among Type II CD36 Individuals in Guangdong Province (Poster)

Xiuzhang Xu, Yaori Xu, Xin ye, Wenjie Xia, Jing Liu, Sentot Santoso

The 30th Regional Congress of the ISBT, Bangkok, Thailand, 16.-19. November 2019.

(10). Acute Hypothermia and Thrombocytopenia Induced by an anti-CD36 Monoclonal Antibody in Mice (Oral presentation, On line)

Xiuzhang Xu, Yaori Xu, Xin ye, Wenjie Xia, Yongshui Fu, Sentot Santoso

The 36th International Congress of the ISBT, Barcelona, Spain, 12.-16. December 2020.

(11). Successful prenatal therapy of anti-CD36-mediated severe FNAIT by deglycosylated antibodies in a novel murine model

Xiuzhang Xu, Dawei Chen, Xin Ye, Wenjie Xia, Yaori Xu, Yangkai Chen, Yuan Shao, Jing Deng, Haoqiang Ding, Jing Liu, Jiali Wang, Heyu Ni, Yongshui Fu, Sentot Santoso

Blood 2021;138(18):1757-1767. doi: 10.1182/blood.2021011131.

12. Ehrenwörtliche Erklärung

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Guangzhou,16.10.2023 Xu Xiuzhang

Ort/Datum Unterschrift

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14. Anhang

14.1. Affidavit of Approval of Animal Ethical Welfare

中山大學 SUN YAT-SEN UNIVERSITY

Institutional Animal Care and Use Committee
(IACUC) Sun Yat-Sen University

动物实验伦理审查同意书
Affidavit of Approval of Animal Ethical and Welfare

申请编号(东)	20143000217	批准编号 Approval No.	IACUC- IACUC-2014-0303

本《动物实验方案》经过实验动物伦理委员会审核，符合动物保护、动物福利和伦理原则，符合国家实验动物福利伦理的相关规定。方案的相关信息如下：

The animal use protocol listed below has been reviewed and approved by the Animal Ethical and Welfare Committee（AEWC）.

实验名称 Protocol Title	CD36基因敲除小鼠用于新生儿/胎儿血小板减少性紫癜动物模型的建立 A novel murine model（CD36 KO mouse） of fetal and neonatal alloimmune thrombocytopenia				
实验申请人 Applicant	徐秀章 Xiuzhang Xu	职称/学位 Title/Degree	硕士研究生 Master	邮箱 Email	497997452@qq.com
实验负责人 Principle Investigator (PI)	付涌水 Yongshui Fu	职称/学位 Title/Degree	其他系列正高级 chief physician	邮箱 Email	yongshuifu1969@yahoo.com
院系(部门) Department	广州血液中心 Guangzhou Blood Center			申请日期 Application date	2014/3/24
动物种系 Species or Strains	C57BL/6J 小鼠和 CD36KO 小鼠 C57BL/6J and CD36 KO mouse			动物数量 Quantity	70
计划执行时间 Period of Protocol	2014/7/24 ~ 2015/3/24	实验动物使用许可证 Number of Animal use permit		SYXK（粤）：2011-0112	
审查意见 Results of inspection	☑符合动物福利伦理要求，可以进行实验 Agree ☐调整方案后，可以进行实验 Agree after modify				
兽医师 Chief Veterinary Officer		日期 Date	2020.6.10		

中山大学实验动物伦理委员会
Animal Ethical and Welfare Committee of SYSU
主席(Chairman)：
日期(Date)：2020.6.10

补发

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14.2. Author's Contributions in Manuscript #1

Studies on CD36 deficiency in South China: Two cases demonstrating the clinical impact of anti-CD36 antibodies

Xiuzhang Xu, Xin Ye, Wenjie Xia, Jing Liu, Haoqiang Ding, Jing Deng, Yangkai Chen, Yuan Shao, Jiali Wang, Yongshui Fu and Sentot Santoso

Thromb Haemost, 2013; volume 110, pages 1199-206.

Contribution:

- Designed the study and reviewed the data: X.X, X.Y, Y.F, S.S
- Performed experiments: X.X, X.Y, W.X, J.L, H.D, J.D, Y.C, J.W
- Analyzed data: X.X, X.Y, S.S
- Wrote the manuscript: X.X, Y.F, S.S

14.3. Author's Contributions in Manuscript #2

Two cases of platelet transfusion refractoriness and one case of possible FNAIT caused by antibodies against CD36 in China

Wenjie Xia, Xin Ye, ***Xiuzhang Xu***, Haoqiang Ding, Jing Liu, Jing Deng, Yangkai Chen, Yuan Shao, Jiali Wang, Hui Li, Yongshui Fu and Sentot Santoso

Transfus Med, 2014; volume 24, pages 254-6.

Contribution:

- Wrote the manuscript: W.X, X.Y
- Managed and analyzed the results: W.X, X.Y
- Performed the ELISA and flow cytometry, and collected information from hospital: X.X, J. L, J. D, J.W, H. L
- Reviewed the manuscript: Y.F
- Read and wrote the manuscript: S.S
- Performed the sequencing analysis: H.D, Y.S, Y.C

14.4. Author's Contributions in Manuscript #3

Successful management of a hydropic fetus with severe anemia and thrombocytopenia caused by anti-CD36 antibody

Xiuzhang Xu, Lin Li, Wenjie Xia, Haoqiang Ding, Dawei Chen, Jing Liu, Jing Deng, Yangkai Chen, Zhiming He, Jiali Wang, Yuan Shao, Sentot Santoso, Xin Ye and Qun Fang

Int J Hematol, 2018; volume 107, pages 251-6.

Contribution:

- Collected the data: X.X, L.L, X.Y, Q.F
- Performed experiments: X.X, L.L, W.X, H.D, D.C, J.L, J.D, Y.C, Z.H, J.W, Y.S
- Analyzed data: X.X, L.L, X.Y, Q.F
- Wrote the manuscript: X.X, L.L, S.S

14.5. Author's Contributions in Manuscript #4

Hydrops fetalis associated with anti-CD36 antibodies in fetal and neonatal alloimmune thrombocytopenia: Possible underlying mechanism

Yongbin Wu, Dawei Chen, ***Xiuzhang Xu***, Mingqin Mai, Xin Ye, Chengyao Li, Sentot Santoso, Wenjie Xia and Yongshui Fu

Transfus Med, 2020; volume 30, pages 361-68.

Contribution:

- Designed the study: Y.F, X.Y, C.L
- Performed the research: Y.W
- Helped perform the research: X.X, W.X
- Analyzed data: Y.W, D.C
- Wrote the manuscript: Y.W, D.C
- Helped interpret the data and reviewed the manuscript: S.S, W.X, Y.F

14.6. Author's Contributions in Manuscript #5

Improvement of anti-CD36 antibody detection via monoclonal antibody immobilization of platelet antigens assay by using selected monoclonal antibodies.

Xiuzhang Xu, Dawei Chen, Xin Ye, Wenjie Xia, Yuan Shao, Jing Deng, Yangkai Chen, Haoqiang Ding, Jing Liu, Yaori Xu, Sentot Santoso and Yongshui Fu

Ann Lab Med. 2023; volume 43, pages 86-91.

Contribution:

- Designed the study and reviewed the data: Y.F, S.S
- Contributed to data collection and analysis: X.X, D.C, X.Y, W.X, Y.S, Y.C, J.D, H.D, J.L, Y.X
- Analyzed data: X.X, S.S
- Wrote the manuscript: X.X, S.S

14.7. Author's Contributions in Manuscript #6

Fetal/neonatal alloimmune thrombocytopenia due to anti-CD36 antibodies: antibody evaluations by CD36-transfected cell lines

Marie Lin, ***Xiuzhang Xu***, Hui-Lin Lee, Der-Cheng Liang and Sentot Santoso

Transfusion, 2018; volume 58, pages 189-95.

Contribution:

- Designed the study and reviewed the data: M.L, X.X, S.S
- Performed experiments: M.L, X.X, H.L, D.L
- Analyzed data: M.L, X.X, S.S
- Wrote the manuscript: M.L, S.S

14.8. Author's Contributions in Manuscript #7

Role of CD36 in immune-mediated thrombocytopenia in Asian populations

Xiuzhang Xu and Sentot Santoso

ISBT Science Series, 2018; volume 13, pages 317-22.

Contribution:

- Analyzed data: X.X, S.S
- Wrote the manuscript: X.X, S.S

14.9. Author's Contributions in Manuscript #8

Successful prenatal therapy of anti-CD36-mediated severe FNAIT by deglycosylated antibodies in a novel murine model

Xiuzhang Xu, Dawei Chen, Xin Ye, Wenjie Xia, Yaori Xu, Yangkai Chen, Yuan Shao, Jing Deng, Haoqiang Ding, Jing Liu, Jiali Wang, Heyu Ni, Yongshui Fu and Sentot Santoso

Blood, 2021; volume 138, pages 1757-67.

Contribution:

- Conceived and designed the research: X.X, X.Y, Y.F, S.S
- Performed experiments: X.X, D.C, Y.X, Y.C, Y.S, J.D, H.D, J.L, J.W
- Analyzed data: X.X, S.S, D.C, X.Y, W.X
- Wrote the manuscript: X.X, Y.F, S.S
- Analyzed and revised the manuscript: H.N