Thrombosis Research 176 (2019) 108–114

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Review Article

Molecular mechanisms of immunoreceptors in platelets

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ARTICLE INFO

Keywords:
Platelets
ITIM
ITAM
Haemostasis
Integrin αIIbβ3

ABSTRACT

Background: The main role of platelets is to control haemostasis when there is a blood vessel injury in order to minimise blood loss at the injury site. Under normal circumstances, platelets flow freely within blood vessels as the endothelial cells provide a non-adhesion surface. Naturally, bioactive mediators are released from endothelial cells to prevent and control platelet activation. However, when the vascular endothelium is ruptured, the local concentration of nitric oxide and prostaglandin is diminished and receptors containing a sequence of amino acids known as, immunoreceptor tyrosine-based inhibition motifs (ITIMs), serve as natural inhibitors within platelets. The main role of ITIMs is to decrease immunoreceptor tyrosine-based activation motif (ITAM) signalling in platelets; however, some studies have revealed their novel role in integrin αIIbβ3 activation. This review highlights the main structural and functional features of immunoreceptors in platelets.

1. Introduction

Platelets are the smallest fragments in blood which are derived from megakaryocytes (MKs). The average lifespan of circulating platelets is generally accepted to be less than 10 days. During their lifespan, platelets decrease in size such that old platelets are smaller than the young platelets [1]. At the end of their lifespan platelets are destroyed by neutrophils and macrophages in the spleen and liver.

Platelets play an important role in physiological and pathological thrombosis. In addition, they play an important role in angiogenesis during adulthood and childhood [2,3]. Recently, platelets' contribution to the immune system has been widely investigated, and they are considered to contribute to innate immune responses, the inflammation response as well as to wound healing [4,5].

In the flowing blood, platelets are marginated near the vessel wall to monitor blood vessel integrity, while red blood cells (RBCs) are concentrated in the centre of the blood stream. Under normal conditions, circulating platelets do not interact with endothelial cells, which provides a non-adhesive surface to platelets [1]. When endothelium is removed, platelets slow down at the site of injury and come in contact with the subendothelial matrix that contains both collagen and von Willebrand factor (vWF). Platelets then bind to vWF via the platelet receptor GPIb/IX/V complex (platelet tethering) [6]. Thereafter, platelets exposed to collagen will bind directly or indirectly to collagen receptors. Integrin $\alpha_2\beta_1$ binds to collagen and allows for further binding to collagen via GPVI which initiates transmembrane and intracellular signalling leading to platelet activation [7].

The signalling pathway by GPVI/FcRγ chain initiates with cross-linking of the glycine-proline-hydroxyproline (GPO) repeat motif in collagen with the immunoglobulin (Ig) domains of GPVI dimers. The intracytoplasmic FcRγ chain of the immunoreceptor tyrosine-based activation motif (ITAM) on the cytoplasmic portion of the FcRγ chain is then phosphorylated by the sarcoma (Src) family kinases (SFK) Fyn/Lyn [8]. Downstream signalling cascades lead to activation of phospholipase Cγ2 (PLCγ2) and phosphoinositide-3 kinase (PI3K) which, in turn activate the fibrinogen receptor (integrin αIIbβ3) (inside-out signalling), and ultimately cause Ca^{2+} mobilisation, granule secretion and platelet aggregation [9].

Activated platelets undergo protein cytoskeleton reorganisation, resulting in extensive formation of spiny spheres with long filopodia extending from the plasma membrane [10]. This increases the surface area of the platelet, allowing more interaction with adjacent platelets and also provides greater surface for binding of the coagulation proteins of the secondary haemostatic mechanism. Platelets then release their granule contents via platelet exocytosis. Some released contents such as ADP and serotonin attract more platelets to the site of injured vessel wall and active them, whiles thromboxane A2 initiates vasoconstriction to minimise blood loss and activates surrounding platelets. ADP binding to its receptors such as $P2Y_1$ and $P2Y_{12}$ (ADP receptors) triggers signalling pathways that eventually converts the fibrinogen receptor (αIIbβ3) from the resting to an activated state [11–13]. Activation of integrin αIIbβ3 in the plasma but not on surface is essential for fibrinogen to bind and bridge adjacent platelets (aggregation). The two loops of plasma fibrinogen then binds to the extracellular domain of the

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https://doi.org/10.1016/j.thromres.2019.01.019
Received 8 November 2018; Received in revised form 20 January 2019; Accepted 28 January 2019

activated integrin αIIbβ3 to form a huge meshwork that forms a platelet plug and arrests blood loss from the injured vessel.

The platelet plug will arrest blood flow but requires stablilisation by the secondary haemostatic process which results in a fibrin network throughout the platelet plug.

In the subendothelial space, the tissue factor bearing cells from a complex with factor VIIa which then activates factor X and factor IX [14]. A small amount of thrombin is then generated which can activate platelets and release factor VIII from vWF (initiation phase) [15]. Amplification of secondary haemostasis occurs upon platelet granule secretion and exposure of phosphatidyl serine on the platelet surface. The propagation phase of haemostasis results in massive thrombin generation which cleaves fibrinogen to fibrin and stabilises the platelet plug [16].

Critical to the platelet thrombosis and haemostasis processes is signalling provided by platelet immunoreceptors. With the increase in the number of immunoreceptors identified in platelets and their diverse functional roles in platelet activation or inhibition, we will review the recent literate that highlights and summarises structural and functional features of the following immunoreceptors: GPVI, FcγRIIa, CLEC-2, PECAM-1, CEACAM-1, CEACAM-2, G6b and TLT-1.

2. ITAM-containing receptors in platelets

The immunoreceptor tyrosine-based activation motifs (ITAMs) are important signalling motifs in immune cells and platelets. ITAMs have a unique structure which possess two identical signatures Yxx (I/L) interspersed by 6–12 amino acids [17]. There are three ITAM containing receptors: GPVI/FcRγ chain, FcγRIIa and the C-type lectin CLEC-2 expressed in human platelets, while FcγRIIa is not expressed in murine platelets.

During platelet activation, the integrin αIIbβ3 clusters and triggers the integrin associated Src family kinases (SFKs), to phosphorylate the ITAMs in the cytoplasmic domain of the receptor. Following the ITAM phosphorylation, ITAMs serve as a docking site for SH2-domain containing proteins, thus further downstream signalling pathways are propagated.

Platelet activation is regulated via ITAM and ITIM immunoreceptor families which have positive and negative regulation contribution toward integrin αIIbβ3 outside-in signalling [18].

2.1. GPVI

Glycoprotein (GP) VI is only expressed on MKs and platelets and it is the main collagen receptor [9]. It is a 55–63 kDa type 1 transmembrane glycoprotein that belongs to the immunoreceptor superfamily (IgSF). The extracellular N-terminal domain of GPVI has two immunoglobulin (Ig) like domains, followed by a highly O-glycosylated mucin-like region (Fig. 1). The 19 amino acid transmembrane domain is important for forming a salt bridge between GPVI Arg272 and Asp11 of FcRγ chain which acts as co-receptor [19,20]. In addition, the cytoplasmic tail which is important for activating intracellular signalling pathway contains 51 amino acids including ITAM motifs.

GPVI and FcRγ chain receptor form a synergistic signalling complex that mediates platelet activation by tyrosine kinase phosphorylation of the two FcRγ cytoplasmic domains (ITAM). Moreover, murine knockout studies revealed the important role of FcRγ for GPVI expression and signalling pathways. FcRγ$^{-/-}$-derived platelets lack GPVI expression on their surface and have attenuated collagen-stimulated platelet activation and thrombus formation under flow conditions [21]. The signalling pathway by GPVI initiates with crosslinking the glycine-proline-hydroxyproline (GPO) repeat motif in the collagen with Ig domains of GPVI dimers [22]. Thereafter, the proline-rich domain in the cytoplasmic tail of GPVI provides a docking site for SFK members and Lyn, which are important in tyrosine phosphorylation of the two FcRγ ITAMs. This initiates the downstream signalling events via FcRγ chain [23,24], which to ultimately activate phospholipase Cγ2 (PLCγ2) and phosphoinositide-3 kinase (PI3K) that in turn induce integrin αIIbβ3 activation, Ca^{2+} mobilisation, granule secretion, and aggregation (Fig. 2) [25].

Most recent work have revealed a new important role of GPVI in thrombus growth and stability. In vivo thrombus studies showed that GPVI-deficient mice had a marked delay in occlusion or with constant embolisation [26,27]. Moreover, in vitro thrombus formation studies have showed that GPVI can bind to D-dimer and the D-domain site on fibrin but not fibrinogen to induce platelet spreading via GPVI activation [22,28]. Therefore, these findings suggest the novel role of fibrin-GPVI crosslinking in thrombus propagation and stability.

2.2. FcγRIIa

FcγRIIa is an important member of the immunoglobulin gene superfamily that is expressed on human platelets [29]. It is a 40 kDa transmembrane glycoprotein with two Ig-like domains and a cytoplasmic tail that contains two intrinsic ITAMs with a typical sequence Yxx(I/L)x(6–12)Yxx(I/L) (Fig. 1) [30].

FcγRIIa is an activating form of Fc receptor (FcR) which has low affinity to the Fc of IgG-containing immune complexes. FcγRIIa has been demonstrated to be a SFK target. Once cross-linked, the ITAMs become phosphorylated and create a docking site for the SH2 domain of tyrosine kinase Syk [31]. As a result of Syk activation, which is necessary for integrin-mediated outside-in signalling pathway this leads to activation of phospholipase Cγ2. Furthermore, intracellular Ca^{2+} concentration increases followed by granule secretion, integrin activation, platelet aggregation and then thrombus formation (Fig. 2) [32].

2.3. The C-type lectin CLEC-2 in platelets

C-type lectin-like receptor 2 (CLEC-2) is a 32 kDa type 2 transmembrane glycoprotein with an extracellular carbohydrate-like recognition domain (CRD-like) [33]. Unlike other ITAM containing receptors, CLEC-2 has only a single YxxL motif in its cytoplasmic domain that is known as atypical or hemITAM motif (Fig. 1) [34]. Historically, CLEC-2 was first discovered as the target receptor for the snake venom rhodocytin. Another ligand for CLEC-2 is the endogenous cellular podoplanin which presents on cancer cells and lymphatic endothelial cells [34].

CLEC-2 and GPVI roles in vascular processes have been studied and been found that they contribute in maintenance of vascular integrity, angiogenesis and lymphogenesis. CLEC-2 role also in haemostasis was unclear and has been reported as having no contribution or a slight contribution [35–37]. More recently, combined deletion of GPVI and CLEC-2 in mice demonstrated severely impaired haemostasis and a marked thrombus growth defect compared to single knockout mice [38]. Which is by far more than that in a single GPVI or CLEC-2 depleted mice.

CLEC-2 activation signalling pathway is similar to the GPVI or FcγRIIa signalling pathways [39,40]. Cross-linking of CLEC-2 with its ligand podoplanin or the snake venom rhodocytin initiates the single ITAM phosphorylation which becomes a docking site for Syk. Afterwards, Syk signalling pathway leads to the activation of PLCγ2 which ultimately activates the fibrinogen receptor (αIIbβ3), Ca^{2+} mobilisation, granule secretion, and platelet aggregation (Fig. 2) [41].

3. ITIM-containing receptors in platelets

3.1. PECAM-1

Platelet endothelial cell adhesion molecule-1 (PECAM-1;CD31) is a 130-kDa member of the immunoglobulin superfamily (IgSF) that is expressed on the most of nonerythroid haematopoietic cells, including platelets and leukocytes [42,43]. PECAM-1 is also abundantly

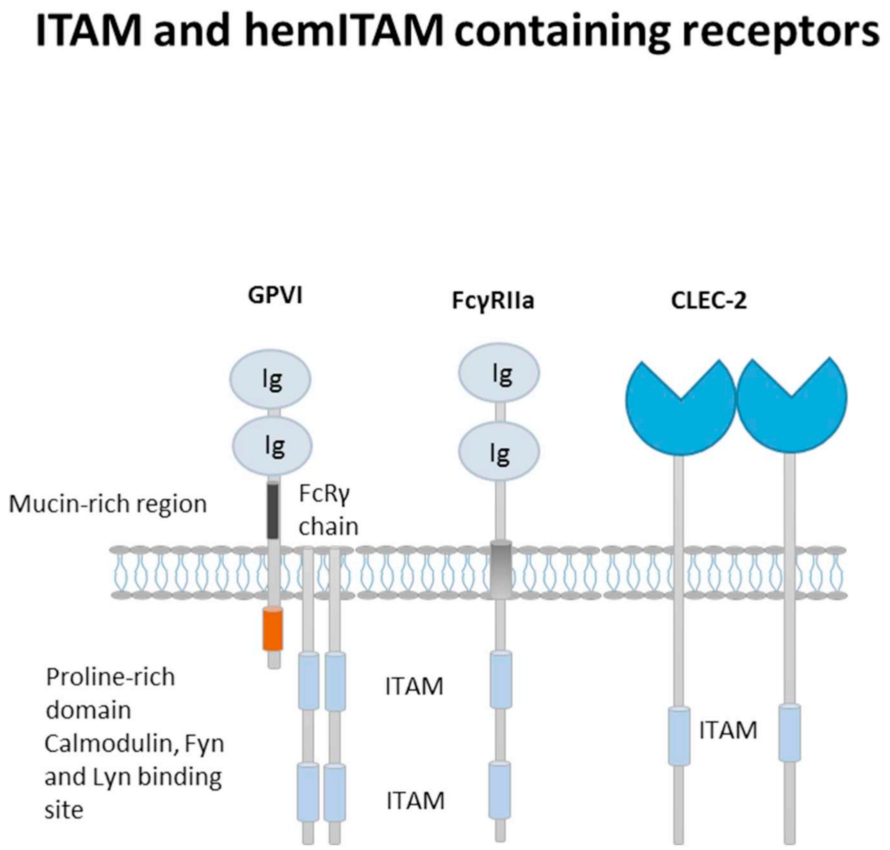

Fig. 1. Schematic diagram of ITAM containing receptors GPVI, FcγRIIa and CLEC-2. The main structural features including the extracellular Ig like domains or a C terminal domain. The intracellular tail contains dual ITAMs in GPVI and FcγRIIa but only a single ITAM in CLEC-2.

expressed at the intercellular junctions of endothelial cells [44]. PECAM-1 has a highly glycosylated extracellular domain that is composed of 6 immunoglobulin homology domains, a transmembrane domain, and a 118 amino acid cytoplasmic tail that mediates cell-cell interaction and cell signalling (Fig. 3) [45]. In humans, the localisation of the PECAM-1 gene has been determined as the long arm of chromosome 17q23, while it is on chromosome 6 in mice [46,47].

The function of PECAM-1 in intracellular junctions between endothelial cells is to mediate transmigration of leukocytes between the vasculature and subendothelial space [48]. In addition, it functions to maintain the cellular adhesive interactions to preserve the integrity of vascular endothelial cell junctions under inflammation and thrombotic stress conditions [49]. Moreover, PECAM-1 has been identified to mediate leukocyte migration through endothelial cells during diapedesis [50].

Previous studies have shown that, the cytoplasmic tail of PECAM-1 that conveys signalling functionality, contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based motif (ITSM) [45,51–53]. Once platelets are activated, both ITIM and ITSM become tyrosine phosphorylated, thereby PECAM-1 becomes a docking site for the protein tyrosine phosphatases SHP-2 predominantly, but also SHP-1 [54]. These two protein tyrosine phosphatases initiate inhibitory effects by removing the phosphate group from the ITAM and thus dampening its signalling pathway (Fig. 2) [51,54].

The role of PECAM-1 in thrombus formation in vitro has been studied using type 1 collagen, and PECAM-1 deficient platelets display hyper-responsive platelet adhesion with larger and more stable thrombus [55]. This finding supports the concept that PECAM-1 acts as an inhibitory co-receptor in platelets to negatively regulate collagen-GPVI-mediated platelet responses. However, platelets isolated from PECAM-1 deficient mice show weak adhesion and restricted cytoskeleton recognition on a fibrinogen-coated surface compared with wild type-derived platelets [56]. These findings raises the potential that PECAM-1 is also a positive regulator of the integrin αIIbβ3 mediated pathway [56]. However, the underlying mechanism of PECAM-1 into maintaining the normal integrin αIIbβ3-mediated platelet function is still not well understood.

The ITSM signalling pathway in leucocytes occurs by binding SLAM adaptor protein (SAP), SHP-2 and EWS-activated transcript-2 (EAT-2) to phosphorylated (but not required) ITSM that can then induce inhibitory signalling pathway that then binds and activates Fyn [57–59]. In platelets, CD150 and CD84 have been identified as ITSM containing receptors and have SAP and EAT-2 but they are not required to tyrosine phosphorylate CD150 and CD84 which is different compared to lymphocytes [60,61].

3.2. CEACAM-1

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1 or CD66a) is a transmembrane receptor which belongs to the CEA family of adhesion molecules [62]. Previously, CEACAM-1 was named biliary glycoprotein (BGP) as it was first isolated from human bile [63]. Moreover, CEACAM-1 is widely expressed on immune, endothelial, and blood cells. Interestingly, CEACAM-1 is the only isoform of CEACAM family that is expressed in both rodents and humans [64].

The cytoplasmic domain of CEACAM-1 has an ITIM and ITSM (Fig. 3) that mediates cell-cell interactions and cell signalling [45]. During platelet activation, ITIMs become tyrosine phosphorylated and become docking site for SH2 domain-containing protein tyrosine phosphatases (SHP-1 and SHP-2). SHP-1 and SHP-2 are recruited to inhibit cell signalling by reversing the tyrosine phosphorylation process that its brought about by the tyrosine kinases action (Fig. 2) [65]. On the other hand, the extracellular domain of CEACAM-1 contains one N-

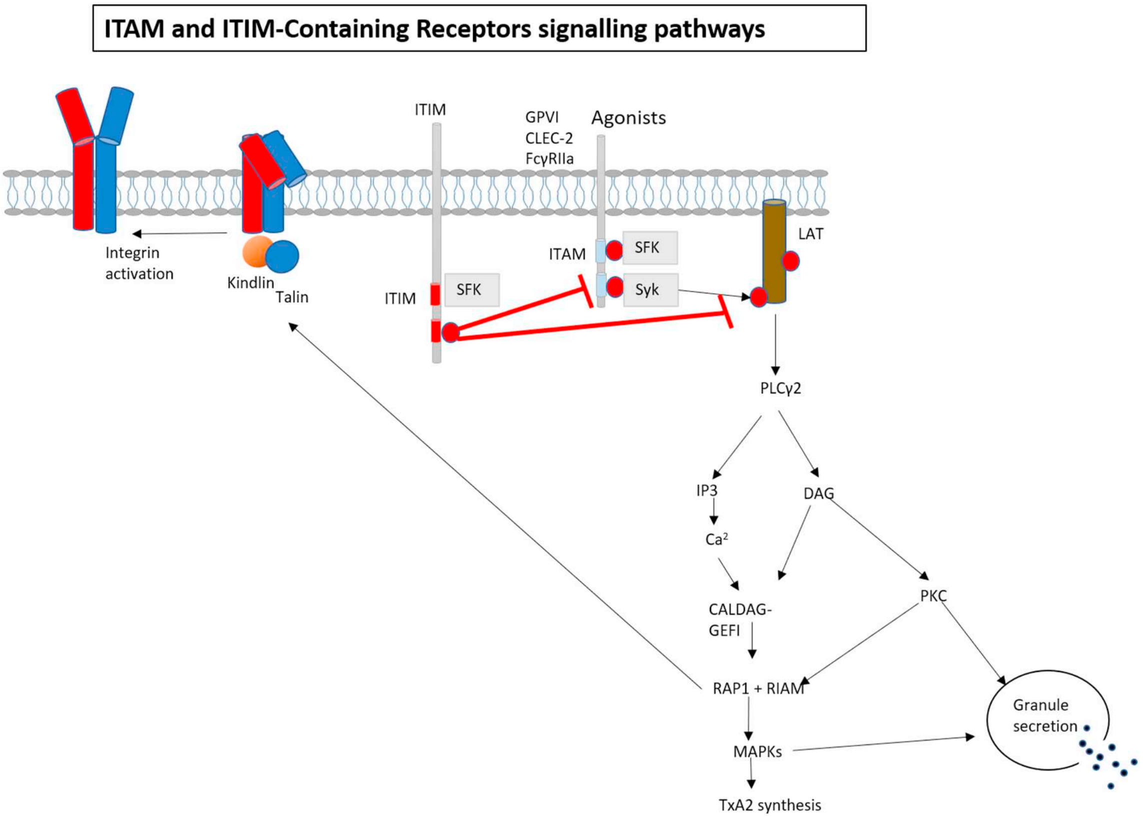

Fig. 2. Schematic diagram of ITAM containing receptors inside-out and outside-in signalling pathways. ITAM becomes phosphorylated and induces PLCγ2 signalling pathway which ultimately results in (1) intracellular calcium elevation, (2) Granule secretion, (3) TxA2 synthesis and (4) integrin αIIbβ3 activation. ITIM-bound SHP-2 dampens ITAM signalling pathway by dephosphorylating and deactivating the key components of ITAM signalling pathway.

terminal immunoglobulin variable-region-like (IgV-like) domain that mediates homophilic adhesion, followed by three immunoglobulin constant-region-type-2-like (IgC2-like) domains [66,67].

The contribution of ITAM-mediated signalling that is initiated by ITAM-bearing GPVI/FcRγ chain, and its regulation by CEACAM-1 ITIMs has been well examined in response to type 1 collagen exposure, however, much less understood about the molecular effects that are produced by platelet exposure to other agonists [64]. This raises the question whether CEACAM-1 has a similar negative regulation effect on ITAM-bearing GPVI/FcRγ chain that might be initiated by GPIb/IX/V complex signalling or G-protein coupled signalling pathways.

CEACAM-1 roles in vascular cell types have been investigated. It plays a crucial role in maintaining the strength and vascular integrity [68]. Thus, CEACAM-1 knockout mice have been investigated and increased incidence of blood vessel leakage has been reported [69]. Moreover, CEACAM-1 role in carcinogenesis have been studied and been found that CEACAM-1 has a tumour-suppression function [70]. The tissue expression of CEACAM-1 in breast cancer was significantly reduced in the cancer area compared to normal tissue. Coupled with the inhibition of cancer proliferation by ITIM mediated signalling which shows the suppressive effects of CEACAM-1 on tumour development this suggests that a strategy to restore CEACAM1 expression may be helpful for the treatment of breast cancer [70]. Furthermore, healthy lung tissues lack CEACAM-1 expression, while it has been reported to be expressed on lung tumour cells [71,72]. These findings are questioning the relation of CEACAM-1 expression and function in lung cancer and tumour progression.

3.3. CEACAM-2

CEACAM-2 is a type I transmembrane receptor that belongs to the CEACAM superfamily. Similarly, CEACAM-1,2 share some overall structure in terms of Ig domain in the extracellular part and with an ITIM and an ITSM in the cytoplasmic domain (Fig. 3). However, CEACAM-1 has four extracellular Ig-domains, while CEACAM-2 has only two Ig domains. CEACAM-2 is expressed in mouse platelets, but CEACAM-1 is predominant in human and mouse platelets [45]. Similar to CEACAM-1, the cytoplasmic tail of CEACAM-2 has a single ITIM and an ITSM that mediates cell-cell interactions and cell signalling [45].

3.4. G6b

G6b gene which codes for G6b-B is located in the major histocompatibility complex (MHC) region of chromosome 17 [73]. G6b-B is highly expressed on both mature megakaryocytes and platelets [74,75].

G6b-B consists of 242 amino acids consisting of an extracellular N-terminal domain of G6b-B that has a single extracellular variable Ig like (IgV) domain, and a single transmembrane domain. G6b-B cytoplasmic tail has an ITIM (Y194) and an ITSM (Y220) in its cytoplasmic domain (Fig. 3).

G6b-B works by inhibition of ITAM signalling pathway either from GPVI-FcRγ-chain complex, the hemITAM containing podoplanin activation receptor CLEC-2 or GPVI and ADP induced platelet aggregation after antibody-mediated crosslinking (Fig. 2) [76].

The physiological function of G6b-B was thoroughly investigated by using G6b-B knockout mouse models [77]. Interestingly, G6b-B knockout mice had a bleeding disorder due to impairment in platelet production and function [77]. Also, they exhibited significant macrothrombocytopenia with a functional defect, GPVI and GPIbα shedding. As a result, G6b-B was proposed to be an important megakaryocyte function and platelet formation regulator [77].

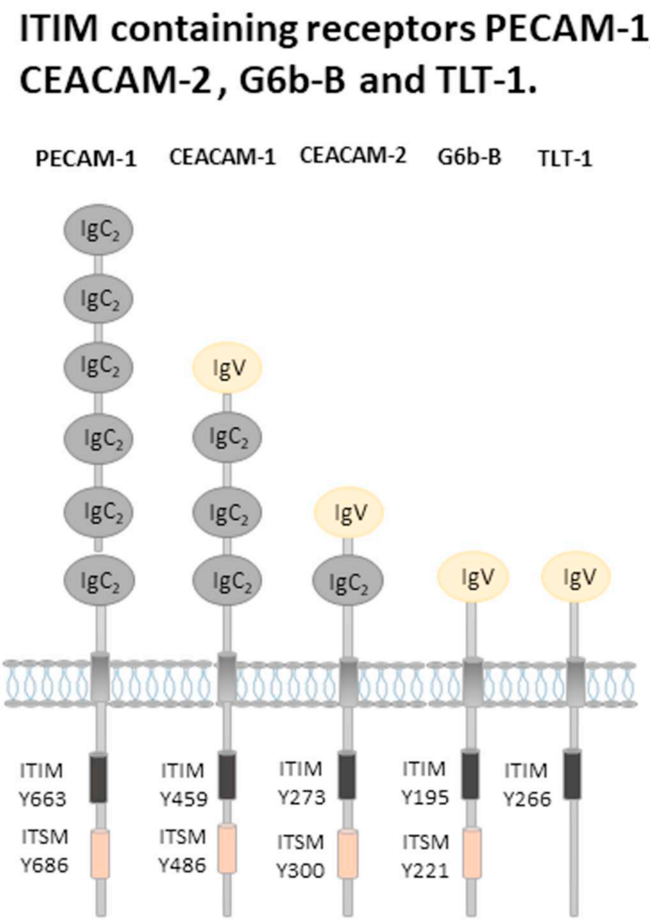

Fig. 3. Schematic diagram of ITIM containing receptors PECAM-1, CEACAM-1, CEACAM-2, G6b-B and TLT-1. The main structural features including the extracellular constant-region-type-2-like (IgC2-like) domains and/or variable Ig like (IgV) domains. The intracellular tail contains ITIM and ITSM or a single ITIM in TLT-1.

3.5. *TLT-1*

Triggering receptor expressed on myeloid cells (TREM) is an immunoreceptor that is involved in cell activation within the immune system. TREM contains a single V-type extracellular immunoglobulin domain, a short cytoplasmic tail and a trans-membrane domain (Fig. 3) [78]. TERM-like Transcript 1 (TLT-1) is a type 1 single IgV-containing protein that is localised exclusively in megakaryocytes and platelet α–granules [78]. TLT-1 is redistributed on the platelet surface during thrombin-induced platelet activation [79]. Moreover, two TLT-1 isoforms are expressed, the first isoform has a cytoplasmic tail with a potential ITIM, and the second isoform is characterised by a cytoplasmic tail lacking an ITIM [80].

TLT-1 is the only TREM family member that contains an ITIM in its cytoplasmic domain which acts as a docking site for the SHP family proteins. SHP-2, SHP-1 and SHIP1 are recruited to the ITIM (at Y281) to negate the ITAM-mediated signalling (Fig. 2) [81,82].

Pharmacologically inhibited TERM-1 using LR12 has been found to reduce platelet activation induced by ADP, thrombin and collagen in human platelets [83]. In addition, Trem-1 −/− mice have reduced platelet aggregation to ADP, collagen and thrombin. Trem-1 inhibition reduces thrombus formation in a carotid artery model and protects mice from pulmonary embolism [83].

4. Conclusion

Megakaryocytes and platelets are very complex and express a number of immunoreceptors that participate in haemostasis and thrombosis control. Knockout mice models have been invaluable for determining the role of ITAM and ITIM bearing receptors in the context of platelet haemostasis and thrombosis. Ig-ITIM bearing receptor knockout cleat a unique phonotype indicating they are redundant collectively, and the combined Ig-ITIM bearing receptors is only to play a potential role in regulating platelet-collagen interaction as a natural platelet inhibitor in vivo. Furthermore, questions have been raised about whether ITIM-containing receptors can regulate other signalling pathways other than GPVI signalling pathway. One unanticipated finding was that, ITIM-containing receptors have a positive regulatory effect on integrin αIIbβ3 activation, but the underlying mechanisms have not yet been clearly elucidated.

Acknowledgements

Shaqra University, Saudi Arabia.

Conflict of interests

Authors declare that there is no conflict of interest.

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