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CD40 Signalling in Platelet Function

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CD40 Signalling in Platelet Function

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Résumé

Le CD40 est un membre de la famille des récepteurs du facteur de nécrose tumorale ("Tumour necrosis factor", TNF), initialement identifié sur des cellules de carcinome de la vessie. L'interaction du CD40 avec son ligand (CD40L) est d'une importance cruciale pour le développement des cellules B et de la commutation d'isotype au cours de la réponse immunitaire acquise. L'expression du complexe CD40/CD40L était initialement cru d'être limiter aux cellules du système immunitaire, mais aujourd'hui il est bien connu que ce complexe est également exprimé sur les cellules du système circulatoire et vasculaire, et est impliqué dans diverses réactions inflammatoires; de sorte que le CD40L est maintenant considéré comme une molécule thrombo-inflammatoire prédictive des événements cardiovasculaires. Les plaquettes expriment constitutivement le CD40, alors que le CD40L n'est exprimé que suite à leur l'activation. Il est ensuite clivé en sa forme soluble (sCD40L) qui représente la majorité du sCD40L en circulation. Il fut démontré que le sCD40L influence l'activation plaquettaire mais son effet exact sur la fonction plaquettaire, ainsi que les mécanismes cellulaires et moléculaires sous-jacents à son action demeurent inconnus. Ainsi, ce projet a été entrepris dans le but d'adresser les objectifs spécifiques suivants: 1) évaluer les effets *in vitro* du sCD40L sur l'activation et l'agrégation plaquettaire; 2) identifier les récepteurs plaquettaires impliqués dans l'action du sCD40L; 3) élucider les voies signalétiques intracellulaires induits par le sCD40L; 4) évaluer les effets du sCD40L sur la formation de thrombus *in vivo*.

Nous avons trouvé que le sCD40L augmente fortement l'activation et l'agrégation des plaquettes en réponse à de faibles concentrations d'agonistes. Les plaquettes humaines traitées avec une forme mutante du sCD40L qui n'interagit pas avec le CD40, et les plaquettes de souris déficientes en CD40 ne furent pas en mesure d'induire de telles réponses, indiquant que le récepteur principal du sCD40L au niveau des plaquettes est le CD40. En plus, nous avons identifié la présence de plusieurs membres de la famille du facteur associé du récepteur du TNF ("TNF receptor-associated factor", TRAF) dans les plaquettes et nous avons montré que seulement le TRAF2 s'associe avec le CD40 suite à la stimulation par le sCD40L. Nos résultats indiquent aussi que le sCD40L agisse sur les plaquettes au repos par l'entremise de deux voies signalétiques distinctes. La première voie implique l'activation de la petite GTPase Rac1 et de sa cible en aval, soit la protéine kinase p38 activée par le mitogène ("p38 mitogen-activated protein

kinase", p38 MAPK), menant au changement de forme plaquettaire et à la polymérisation de l'actine; alors que la deuxième voie implique l'activation de la cascade signalétique du NF-kB. Par ailleurs, à la suite d'une lésion artérielle induite par le chlorure de fer, le sCD40L exacerbe la formation de thrombus et l'infiltration leucocytaire au sein du thrombus dans les souris du type sauvage, mais pas chez les souris déficientes en CD40.

En conclusion, ce projet a permis d'identifier pour la première fois deux voies signalétiques distinctes en aval du CD40 plaquettaire et a permis d'établir leur implication dans l'activation et l'agrégation plaquettaire en réponse au sCD40L. De manière plus importante, ce projet nous a permis d'établir un lien direct entre les niveaux élevés du sCD40L circulant et la formation de thrombus *in vivo*, tout en soulignant l'importance du CD40 dans ce processus. Par conséquent, l'axe CD40/CD40L joue un rôle important dans l'activation des plaquettes, les prédisposant à une thrombose accrue en réponse à une lésion vasculaire. Ces résultats peuvent expliquer en partie la corrélation entre les taux circulants élevés du sCD40L et l'incidence des maladies cardiovasculaires.

Mots clés: plaquettes ■ thrombose ■ voies signalétiques ■ CD40 ■CD40L

Abstract

CD40 is a member of the tumour necrosis factor (TNF) receptor family, originally identified on human bladder carcinoma cells. Interaction of CD40 with its ligand (CD40L) is of crucial importance for B cell development and immunoglobulin isotype switching during the adaptive immune response. Expression of the CD40/CD40L dyad was initially thought to be restricted to cells of the immune system, but today it is known to be also expressed on cells of the circulatory and vascular systems, and have important implications in various inflammatory reactions, such that CD40L is now regarded as a thrombo-inflammatory molecule and a reliable predictor of cardiovascular events. Platelets constitutively express CD40, whereas CD40L is expressed upon activation and subsequently cleaved into its soluble form (sCD40L), accounting for the majority of circulating sCD40L. Soluble CD40L has been shown to influence platelet activation but its precise effect on platelet function, and the underlying cellular and molecular mechanisms remain undefined; hence the purpose of this project. The specific aims of this study are: 1) to evaluate the *in vitro* effects of sCD40L on platelet activation and aggregation; 2) to determine the receptor(s) on platelets involved in the action of sCD40L; 3) to elucidate the intracellular signalling pathways induced by sCD40L; and 4) to evaluate the *in vivo* effects of sCD40L on thrombus formation.

We have showed that sCD40L strongly enhances activation and aggregation of washed human platelets in response to sub-threshold concentrations of agonists. Human platelets treated with a mutated form of sCD40L that lacks CD40 binding, and platelets from CD40 deficient mice failed to elicit such responses, indicating that CD40 is the major platelet receptor for sCD40L. Moreover, we identified the presence of multiple members of the TNF receptor-associated factor (TRAF) in platelets and showed that only TRAF2 associates with CD40 after sCD40L stimulation. Interestingly, sCD40L primes resting platelets through two distinct signalling pathways. The first pathway involves activation of the small GTPase Rac1 and its downstream target p38 mitogen-activated protein kinase, leading to platelet shape change and actin polymerization; whereas the second pathway involves activation of the NF-κB signalling cascade. Furthermore, sCD40L exacerbates thrombus formation and leukocyte infiltration within the thrombus mass in wild-type mice but not in CD40 deficient mice following ferric chloride-induced arterial injury.

In conclusion, we have identified for the first time two distinct signalling pathways downstream of platelet CD40, and established their implication in platelet activation and aggregation in response to sCD40L. Noticeably, we established a direct link between elevated levels of sCD40L and *in vivo* thrombus formation, while emphasizing the requirement of CD40 in this process. Therefore, the CD40/CD40L dyad plays an important role in platelet priming that predisposes platelets to enhanced thrombus formation in response to vascular injury. These results may partly explain the correlation between elevated circulating levels of sCD40L and the incidence of cardiovascular diseases.

Key words: platelets ■ thrombosis ■ signal transduction ■ CD40 ■ CD40L

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List of abbreviations

5-HT:	5- hydroxytryptamine (a.k.a. serotonin)
ACS:	Acute coronary syndrome
ADAM-10:	A disintegrin and metalloproteinase domain-containing protein-10
AP1:	Activator protein 1
APC:	Antigen presenting cell
ApoE:	Apolipoprotein E
Bcl:	B-cell leukemia
BCR:	B cell antigen receptor
cAMP:	Cyclic adenosine monophosphate
Cbl-b:	Casitas B-lineage lymphoma b
c-Cbl:	Casitas B-lineage lymphoma
CD40L:	CD40 ligand
cFLIP:	Cellular homolog of viral Fas-associated via death domain-like IL-1β converting-enzyme inhibitory protein
CHO:	Chinese hamster ovary
cIAP:	Cellular inhibitor of apoptosis
COX-1:	Cyclooxygenase-1
CRP:	Collagen-related peptide
CTL:	Cytotoxic T lymphocyte
DAG:	Diacylglycerol
DC:	Dendritic cell
EAE:	Experimental autoimmune encephalomyelitis
EGF:	Endothelial growth factor
Erk:	Extracellular signal-regulated protein kinase
FasL:	Fas ligand
FcRγ :	Fc receptor γ-chain
FGF:	Fibroblast growth factor
GM-CSF:	Granulocyte/monocyte growth stimulating factor
GP:	Glycoprotein

GPCRs:	G protein coupled receptors
HIGM:	X-linked hyper-IgM syndrome
ICAM:	Intercellular adhesion molecule
Ig:	Immunoglobulin
IKK:	Inhibitor κB kinase
IKK:	IκB kinase
IL:	Interleukin
INF:	Interferon
IP_3:	Inositol-1,4,5- triphosphate
ITAM:	Immunoreceptor tyrosine-based activation motif
IκB:	Inhibitor κB
JAK3:	Janus kinase 3
JNK:	c-jun N-terminal kinase
LDL:	Low-density lipoprotein
LFA:	Lymphocyte function-associated antigen
LPS:	Lipopoplysaccharide
LT-α:	Lymphotoxin-α
Mac-1:	Macrophage antigen-1
MAPK:	Mitogen-activated protein kinase
MCP-1:	Macrophage chemotactic protein-1
MDC:	Macrophage derived chemokine
MEF:	Mouse embryonic fibroblast
MEKK:	Mitogen-activated protein kinase kinase kinase
MIDAS:	Metal ion-dependent adhesion site
MIP-1α:	Macrophage inflammatory protein-1α
MMP:	Matrix metalloproteinase
NEMO:	Nuclear factor-κB essential modulator
NF-IL-6:	Nuclear factor of interleukin-6
NF-κB:	Nuclear factor-κB
NIK:	Nuclear factor-κB-inducing kinase
NK:	Natural killer

NO:	Nitric oxide
NOD:	Non-obese diabetic
NSF:	N-ethylmaleimide sensitive factor
NTPDase-1:	Nucleoside triphosphate diphosphohydrolase-1
OCS:	Open canalicular system
oxLDL:	Oxidized low-density lipoprotein
PAF:	Platelet activating factor
PARs:	Protease activated receptors
PDGF:	Platelet derived growth factor
PECAM:	Platelet/endothelial cell adhesion molecule
PF4:	Platelet factor 4
PI3K:	Phosphoinositide 3-kinase
PIP_2:	Phophatidylinositol 4,5-bisohosphate
PKC:	Protein kinase C
PLC:	Phospholipase C
PLCβ:	Phospholipase Cβ
PS:	Phosphatidylserine
PSGL-1:	P-selectin glycoprotein ligand-1
PSI:	Plexin-semaphorin-integrin
PSP:	Platelet Sec1 protein
RA:	Rheumatoid arthritis
RANK:	Receptor activator of nuclear factor-κB
RANTES:	Regulated upon activation normal T-cell expressed and released
Rho GEFs:	Rho guanine-exchange factors
RIP1:	Receptor-interacting protein kinase 1
ROS:	Reactive oxygen species
sCD40L:	Soluble CD40 ligand
SCR:	Short consensus repeat
siRNA:	Small interfering RNA
SLE:	Systemic lupus erythematosus
SMC:	Smooth muscle cells

SNAP:	Soluble N-ethylmaleimide sensitive factor-associated protein
SNARE:	Soluble N-ethylmaleimide sensitive factor receptor
STAT5:	Signal transducer and activator of transcription 5
TAD:	Transcription activation domain
TAP:	Antigen peptide transporter
TCR:	T-cell receptor
TF:	Tissue factor
TGF-β:	Transforming growth factor β
TLR:	Toll-like receptor
TNF:	Tumour necrosis factor
TRAF:	Tumour necrosis factor receptor associated factor
TxA_2:	Thromboxane A_2
VASP:	Vasodilator-stimulated phosphoprotein
VCAM:	Vascular cell adhesion molecule-1
VEGF:	Vascular endothelial growth factor
vWF:	von Willebrand factor

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Chapter 1

Platelets

1.1 Introduction

Platelets were considered as "red cell dust" mere fifty years ago, but now they are viewed as sentinels of the vascular system, where they react to damage to the vascular wall by forming a haemostatic plug. Beyond their role in haemostasis, platelets have emerged as active players in inflammation as well as modulators of both the innate and adaptive immunity. Their involvement in all facets of atherosclerosis, which is now considered as a chronic inflammatory disease, is probably the most relevant example in which platelets are viewed as true thrombo-inflammatory cells. The initiation, development, and progression of the atherosclerotic plaque depend largely on the interaction of platelets with the endothelium and subsequent recruitment of leukocytes to the lesion site, which is mediated by a plethora of molecules expressed and secreted by platelets. The thrombotic events resulting from atherosclerotic plaque rupture rely mainly on the activation and aggregation of platelets, and their subsequent involvement in mediating activation of the coagulation cascade.

CD40 ligand (CD40L) is among the important inflammatory molecules expressed by activated platelets. This inflammatory mediator was initially thought to be exclusive to activated T lymphocytes, but now it is known to be expressed on a wide array of cells, including endothelial cells, macrophages, smooth muscle cells, and platelets.[1] Interaction of CD40L with its cognate receptor, CD40, on B lymphocytes is of major importance in the immune response. The pioneering work by Henn et *al*.[2, 3] demonstrated the presence of the CD40 and CD40L dyad in platelets, as well as the role of platelet CD40L in inducing an inflammatory reaction in endothelial cells. Platelet CD40L has since been viewed as a key player in atherosclerosis development, and now a plethora of evidence point to multiple roles of platelet CD40L, including the stabilization of thrombus formation by sustaining platelet/platelet aggregates, activation of antigen presenting cells (APC), and differentiation and Ig isotype class switching in B cells.[4-6]

Platelets are estimated to account for more than 95% of circulating soluble CD40L (sCD40L).[7] In addition, there is a close link between elevated levels of circulating sCD40L and clinical complications related to multiple vascular diseases, including acute coronary syndromes (ACS), peripheral arterial occlusive disease, hypercholesterolemia, and diabetes.[8-12] Indeed, elevated levels of sCD40L are now considered as a risk factor for future cardiovascular events.[13]

Therefore, a better understanding of the interplay between CD40L and platelets could shed light on future diagnostic and preventive cardiovascular disease treatments.

1.2 Origin and structure

Platelets were first described by Osler in 1873 for their disk-like structure,[14] then in 1881 Bizzozero identified platelets anatomically and associated them with a haemostatic and experimental thrombotic role.[15, 16] Bizzozero was also the first to identify bone marrow megakaryocytes without recognizing them as the precursors of platelets, which was later discovered by Wright in 1906.[17, 18] Actually, platelets are not only recognized for their physiological haemostatic role, but also for their contribution in multiple pathological conditions such as vascular thrombosis, atherosclerosis, inflammation, immunity, oncology, coronary and cerebral-vascular diseases, diabetes and psychiatric diseases.

Platelets are derived from highly specialized cells called megakaryocytes, which have the sole purpose of generating platelets. Megakaryocytes develop from pluripotent hematopoietic stem cells that are characterized by their surface expression of CD34 and CD41, and have become committed to the megakaryocyte lineage as indicated by expression of CD61 (integrin β_3, GPIIIa) and CD41 (integrin α_{IIb}, GPIIb) according to the model in Figure 1.1.[19]

Although it is well established that platelets originate from megakaryocytes, the mechanisms by which they are formed and released remains controversial. Three models of platelet formation have been proposed: 1) cytoplasmic fragmentation, 2) platelet budding, and 3) pro-platelet formation. The cytoplasmic fragmentation model describes the formation of mature platelets within the megakaryocytes. These platelets are then released following the defragmentation of the megakaryocytic membrane.[20, 21] However, this model lost support because of several inconsistent observations. For instance, the platelet territories within the megakaryocytes do not exhibit structural characteristics of platelets. In the platelet budding model, platelets are formed by blebs pinching off from the megakaryocyte surface.[22, 23] This model was also rejected because examination by electron microscopy revealed that these blebs lack platelet organelles. Accumulating evidence now support the current proposed pro-platelet formation model, in which megakaryocytes in the bone marrow form long cytoplasmic extensions through junctions in the lining of blood sinuses, thereby releasing barbell shaped pro-platelets into the circulation that undergo further fragmentation into individual platelets.[24, 25]

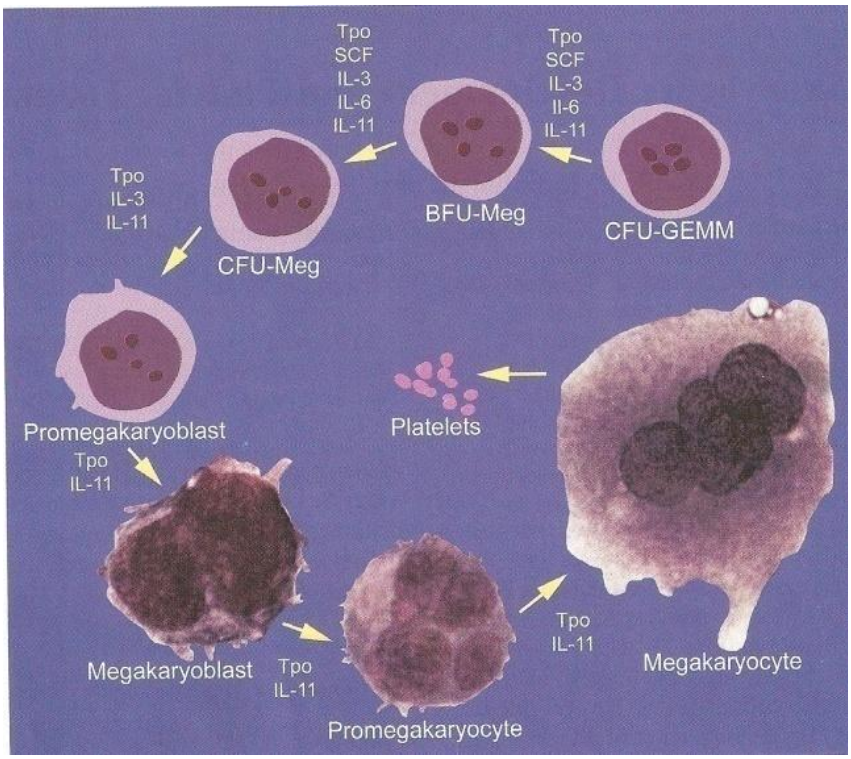

Figure 1.1: Megakaryocytopoiesis. (CFU-GEMM: Colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte; BFU-Meg: burst-forming unit-megakaryocyte; CFU-Meg: colony-forming unit-megakaryocyte. Michelson A. D. (2007). *Platelets*. Second Edition. Burlington: Academic Press. p. 24.

Platelets circulate in the blood at a concentration of 150-450 x 10^6 per mL in adults and have a life span averaging 10 days. Senescent platelets are removed from the circulation in the spleen, liver and the mononuclear phagocyte system. Platelets are disc shaped with an average dimension of 3 µm x 0.5 µm. They are anucleated cells and their cytoplasmic organelles, such as mitochondria, granules, lysosomes and residual membranes of the endoplasmic reticulum originate from megakaryocytes.[26] The platelet structure is divided into three essential components: the plasma membrane, the cytoskeleton and the secretory granules.

The platelet plasma membrane is a smooth surface containing invaginations that form the open canalicular system (OCS). This system forms an elaborate circuit of plasma membrane allowing entry of certain molecules inside the cell. In addition, during platelet activation, the granules secrete their contents to the cell surrounding through fusion with the membrane of the OCS. Moreover, the OCS provides additional plasma membrane reserve that is used for platelet shape change and spreading. Finally, as for other cells, the plasma membrane is an anchoring site for multiple receptors and adhesion molecules.[27]

The platelet cytoskeleton is formed by a monolayer of microtubule filaments underlining the plasma membrane, a spectrin membrane cytoskeleton and a cytoplasmic cytoskeleton formed by actin filaments (Figure 1.2).[28-30] The microtubule filament confers the discoid shape to resting platelets while the spectrin membrane cytoskeleton, as well as the actin filaments, helps in maintaining the shape of resting platelets. In addition, the actin filaments constitute anchoring sites for different membrane glycoproteins (GP).[31] As a whole, the platelet cytoskeleton harbours a site where multiple biochemical reactions, involved in platelet activation, occur.[32] During platelet activation, there is reorganization of the cytoskeleton that allows platelets to change from their discoid structure into a spherical one containing multiple filopodia protrusions.[33, 34]

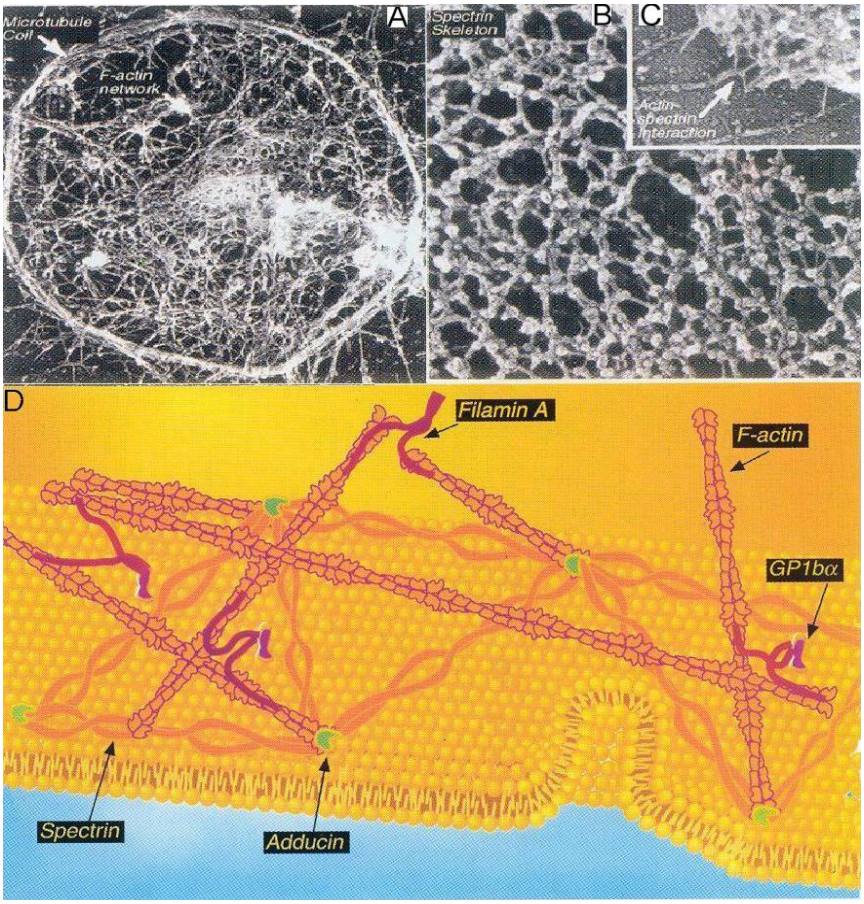

Figure 1.2: Structure of platelet cytoskeleton. A) A monolayer of microtubule filaments surrounding the cytoplasmic cytoskeleton, which is formed by actin filaments. B) Structure of the membrane cytoskeleton formed by spectrin. C) Interaction between spectrin of the membrane cytoskeleton and actin filaments. D) Schematic of the membrane and cytoplamic cytoskeletons of platelets. Michelson A. D. (2007). Platelets. Second Edition. Burlington: Academic Press. p. 76.

The cytoplasm of platelets is occupied by a large number of secretory granules (see section 1.4.3). Platelets contain three types of granules; alpha granules, dense granules and lysosomes, which contain a plethora of molecules that play an important role in platelet activation, adhesion and aggregation.

1.3 Platelet receptors and adhesion molecules

Platelets play a pivotal role in haemostasis by maintaining the integrity of the blood vessels. In order to accomplish this task, platelets contain multiple receptors and adhesion molecules that allow them to respond to external stimuli and to interact with the endothelium, the sub-endothelial matrix and other platelets. Extensive research on the understanding of platelet receptors, adhesion molecules and the underlying signalling cascades shed light into the mechanisms by which platelets contribute not only to their physiological role in haemostasis but also to their pathological involvement in thrombosis.

Among the most important platelet receptors are the seven transmembrane domain receptors also known as G protein coupled receptors (GPCRs). Platelets express five types of GPCRs: the protease activated receptors (PARs), the purinergic receptors, the thromboxane receptors, the serotonin receptor (also known as the 5-hydroxytryptamine receptor; 5-HT receptor), and the adrenergic receptor. Human platelets contain PAR1, PAR4 and traces of PAR3, while mouse platelets contain PAR4 and PAR3.[35-37] The purinergic receptors expressed on platelets are the ADP receptors $P2Y_2$ and $P2Y_{12}$, and the ATP receptor $P2X_1$.[38-40] The thromboxane receptors expressed by platelets are the two TP splice variants TPα and TPβ.[41, 42] The 5-HT and adrenergic receptors expressed by platelets are the $5\text{-}HT_{2A}$ and α_{2A}, respectively.[43, 44]

In addition to the GPCRs, platelets also contain four types of adhesion molecules: the integrins, the immunoglobulins, the selectins and the sialomucins. The integrins include the integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa), the integrin $\alpha_2\beta_1$ (GPIa/IIa), the integrin $\alpha_v\beta_3$ (vitronectin receptor), the integrin $\alpha_5\beta_1$ (fibronectin receptor) and the integrin $\alpha_6\beta_1$ (laminin receptor). Among the immunoglobulins, platelets contain the GPVI, the Fc receptors (FcγRIIA and FcεRI), the intercellular adhesion molecule-2 (ICAM-2) and the platelet/endothelial adhesion molecule-1 (PECAM-1). Among the selectins, platelets contain only P-selectin (CD62P) and among the

sialomucins, platelets express only GPIb/IX/V. In this section, only the most relevant receptors and adhesion molecules will be discussed.

1.3.1 Protease activated receptors

Activation of platelets by thrombin was first discovered by Wright and Minot in 1917.[45] Now it is well established that thrombin is the most potent platelet activator.[46] Platelets contain two types of thrombin receptors: GPIb/IX/V (see section 1.3.4) and PARs (PAR1, PAR3, and PAR4). There are four known ubiquitously expressed members of the PAR family (PAR1, PAR2, PAR3, and PAR4).[47] PAR1 was first discovered in human platelets following cloning of a mRNA encoding this receptor in platelets.[48] The presence of PAR3 in murine platelets was put into evidence by the delayed thrombin response of platelets derived from PAR3 deficient mice. The PAR4-activating peptide (AYPGKF-NH_2) was shown to induce activation and aggregation of human platelets as well as platelets derived from PAR3 deficient mice, indicating the presence of PAR4 as a second PAR receptor in platelets.[37] This section will focus on PAR1 and PAR4, since PAR3 does not induce signalling but is rather a cofactor for PAR4 in murine platelets, and PAR2 is absent from platelets.[49]

PARs are seven transmembrane GPCRs that are irreversibly activated by serine proteases such as thrombin and trypsin, which cleave the receptors' amino terminal extracellular domain at a specific arginyl site, leading to unmasking of a previously cryptic tethered ligand domain that binds to a region in the second extracellular loop of the receptor, thereby inducing self activation of the receptor.[48] Activation of PAR1 by thrombin rapidly transmits the signal across the plasma membrane to internally located G proteins. $G_{12/13}$ activation by PAR1 allows them to bind Rho guanine-exchange factors (Rho GEFs) which are responsible for the change in platelet shape.[50, 51] Activation of G_i by PAR1 induces a rapid rise in intracellular Ca^{2+} due to inhibition of cyclic adenosine monophosphate (cAMP), while activation of G_q stimulates phospholipase Cβ (PLCβ)-induced hydrolysis of membrane phosphoinositides (such as phophatidylinositol 4,5-bisohosphate [PIP_2]), thereby releasing inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG).[52, 53] DAG in turn activates protein kinase C (PKC), which causes phosphorylation of proteins such as the vasodilator-stimulated phosphoprotein (VASP).[54] IP_3 increases cytosolic levels of Ca^{2+} by binding to the IP_3 receptor on the endoplasmic reticulum and triggering the

release of intracellular Ca^{2+} stores, and inhibits adenylyl cyclase, leading to decreased cAMP levels.[52, 55]

PAR4, on the other hand, is also coupled to $G_{12/13}$ and G_q, but signalling through this receptor is different from that of PAR1. Following cleavage by thrombin, PAR4 generates a slower response than PAR1, therefore, providing the majority of the intracellular calcium flux.[46, 56] Moreover, unlike PAR1, PAR4 does not require additional signals from the $P2Y_{12}$ receptor to sustain stable platelet-platelet aggregates.[57, 58]

It is now clear that thrombin acts on human platelets through both PAR1 and PAR4. It seems that thrombin cleavage of PAR1 initiates the signals necessary for platelet activation, while subsequent activation of PAR4 may be necessary to sustain the signalling events that optimize propagation of platelet activation. In addition, it has recently been shown in human platelets that PAR1 and PAR4 form a stable complex, which enables thrombin to act as a bivalent functional agonist.[59]

1.3.2 Purinergic receptors

ADP released form damaged blood vessels, red blood cells and secreted from platelet granules (in addition to ATP secreted from platelet granules) (see section 1.4.3) induces platelet activation through the purinergic receptors. These extracellular nucleotide receptors are subdivided into two groups: the P2X ligand-gated cation channels and the GPCR P2Y receptors.[60] Up to date, seven members of the P2X receptors ($P2X_1$ - $P2X_7$) have been identified, and each member exhibits a distinct agonist profile.[61] The P2Y group includes twelve members ($P2Y_1$ - $P2Y_{12}$).[62, 63] It was thought that platelets express a single ADP receptor that was designated as the P2T receptor (thrombocyte P2), but now it is well known that platelets express three distinct purinergic P2 receptor subtypes ($P2Y_1$, $P2Y_{12}$ and $P2X_1$).[64, 65]

The $P2Y_1$ is expressed in a wide range of tissues, including the heart, blood vessels, smooth muscle cells (SMC), neural tissues and platelets.[66] About 150 copies of this receptor are expressed per platelet, which probably explains why ADP is a weak platelet agonist. Nonetheless, the $P2Y_1$ receptor is crucial for the initiation of platelet activation induced by ADP or collagen.[67, 68] The $P2Y_1$ receptor is a 373 amino acid protein that exhibits the classical seven transmembrane GPCR structure. Given that $P2Y_1$ is coupled to G_q, it triggers mobilization of calcium from internal stores resulting in platelet shape change and a weak, transient aggregation

in response to ADP, which were put into evidence through pharmacological inhibition and in a murine $P2Y_1$ genetic deficient model.[69-71] In addition, $P2Y_1$ deficient mice exhibit a prolonged bleeding time, which is due to a defect in *in vivo* thrombus formation.[72]

The identity of the $P2Y_{12}$ receptor on platelets was elusive for a long time, and it was designated as the $P2Y_{ADP}$, $P2Y_{AC}$, $P2T_{ac}$, $P2_T$ and $P2Y_{cyc}$. Molecular cloning confirmed this receptor to be the novel $P2Y_{12}$ receptor, which is uniquely and abundantly expressed on platelets, and to a minor extent in brain tissue.[73] The $P2Y_{12}$ receptor is a GPCR coupled to G_i and its activation by ADP induces inhibition of adenylyl cyclase. The important role of the $P2Y_{12}$ receptor is in the amplification of platelet aggregation induced by all known platelet agonist such as thrombin, collagen, thromboxane A_2 (TxA_2) and ADP.[74] The $P2Y_{12}$ is also involved in platelet dense granule secretion and TxA_2 generation.[75, 76]

In platelets, it was initially thought that the $P2X_1$ receptor was activated by ADP, but experiments with αβ-meATP (a specific $P2X_1$ and $P2X_3$ agonist) demonstrated an antagonistic effect of ADP in human platelets.[77] As $P2X_1$ is a ligand-gated cation channel, activation of this receptor by ATP induces a transient Ca^{2+} influx that is responsible for platelet shape change but not for aggregation.[78]

In summary, co-activation of the $P2Y_1$ and $P2Y_{12}$ receptors is necessary for normal ADP-induced platelet aggregation.[71] Hence the targeting of the $P2Y_{12}$ receptor by the thienopyridine compounds ticlopidine and clopidogrel, which are used as antithrombotic drugs.[79] On the other hand, the $P2X_1$ receptor participates in collagen-induced platelet aggregation under shear conditions, which are a requirement for this receptor to fully play its role in thrombus formation.[77]

1.3.3 Thromboxane receptors

Thromboxane A_2 is generated in platelets through the sequential enzymatic conversion of arachidonic acid, from the phospholipid bilayer, by the cyclooxygenase (COX-1 in platelets) and thromboxane synthase enzymes following activation by agonists, such as thrombin, collagen or ADP.[80] Thus, TxA_2 is considered as a secondary mediator that is involved in the second wave of platelet activation as well as in amplifying the response to more potent platelet agonists.[81] In contrast to TxA_2, prostacyclin (PGI_2), which is also generated through the sequential enzymatic conversion of arachidonic acid (mainly by endothelial cells in the vasculature), inhibits platelet

function through the prostagladin I_2 (IP) receptor.[82] There is much discrepancy regarding the TxA_2 receptors on platelets. Although cDNAs for both receptors TPα and TPβ splice variants have been shown to be present in platelets, only the TPα variant was demonstrated to be expressed on platelets using a specific splice variant antibody, which is probably due to a low level of expression of the TPβ variant.[41, 42]

The TPα receptor cDNA was cloned from human placenta from which a 343 amino acid sequence revealed that it belongs to the GPCR superfamily.[83] The TPβ receptor cDNA was recently cloned from human endothelial cells from which a 407 amino acid sequence revealed that it is a splice variant of the TPα receptor.[84] Therefore, the TPα and TPβ receptors differ only in their C-terminal domains where the 15 amino acid sequence in the carboxyl end of TPα is replaced by a 79 amino acid sequence in TPβ. The G proteins that are coupled to either TPα or TPβ in platelets remain unclear. Reports suggest that both TP receptors are coupled to G_q, G_i, G_{12} or G_{13}, and that signals from either TP receptor result in activating PLC, thereby releasing IP_3 and DAG.[52, 85, 86]

1.3.4 GPIb/IX/V complex

The importance of the GPIb/IX/V complex in haemostasis was put into evidence with the discovery of a rare but often severe bleeding disorder, Bernard-Soulier syndrome, where the complex is either absent, expressed at low levels or dysfunctional.[87] The GPIb/IX/V complex is the second most abundant receptor on platelets, as compared to the $\alpha_{IIb}\beta_3$ integrin, with approximately 25 000 copies per platelet.[88] The complex consists of GPIbα (CD42b) disulfide linked to GPIbβ (CD42c), non-covalently complexed with GPIX (CD42a) and GPV (CD42d) at a ratio of 2:2:2:1.[87] The GPIb/IX/V complex does not have a built-in tyrosine kinase activity. It is not directly coupled to G proteins, and it does not contain phosphorylatable tyrosine residues that recruit signalling molecules. Nevertheless, it utilizes all these avenues to transmit signals by associating with other signalling molecules. Indeed, the cytoplasmic tail of GPIbα associates with 14-3-3ζ, calmodulin, phosphoinositide 3-kinase (PI3K), the Src family tyrosine kinases and filamin A (actin-binding protein).[89-93] Interaction of the GPIb/IX/V complex with other proteins is mostly due to the globular domain of GPIbα, where it binds von Willebrand factor (vWF), thrombin, integrin $\alpha_M\beta_2$ (CD11b/CD18, macrophage antigen-1 [Mac-1]) on leukocytes, P-selectin, coagulation factors XI and XII, and high molecular weight kininogen.

Platelet adhesion under high shear depends on the interaction of GPIb/IX/V with multimeric vWF deposited on the sub-endothelial matrix. vWF is a large glycoprotein synthesized by endothelial cells and megakaryocytes and stored in platelet α-granules and Weibel-Palade bodies of endothelial cells.[94, 95] Upon a damage to the vessel wall, vWF is secreted into the circulation in a form incapable of interacting with GPIb/IX/V. vWF then forms multimers on exposed collagen of the sub-endothelial matrix, thereby allowing its binding with the GPIb/IX/V complex, which then favours platelet adhesion, activation, secretion and aggregation. As mentioned earlier, the GPIb/IX/V complex can also interact with thrombin. In fact, it has been demonstrated that this complex is actually the high affinity receptor for thrombin on platelets. However, it is still unclear if the GPIb/IX/V complex induces signals that activate platelets following its interaction with thrombin.[96] On the other hand, interaction of GPIbα with thrombin allows it to act as a cofactor that localizes thrombin to the platelet surface to support thrombin cleavage of PAR1.[97] Moreover, through interaction with Mac-1, the GPIbα subunit of the GPIb/IX/V complex favours recruitment of leukocytes to the site of injury where platelets are already adhered.[98] Finally, the GPIb/IX/V complex seems to favour platelet rolling on the activated endothelium through its interaction with endothelial P-selectin.[99]

1.3.5 GPVI

GPVI is a valuable adhesion molecule that is uniquely expressed on platelets, and in addition to $\alpha_2\beta_1$, is a major collagen receptor. Because of the low level of expression of GPVI on platelets as well as the difficulties in finding a suitable agonist (due to the second collagen receptor $\alpha_2\beta_1$), it was difficult to determine the structure of this receptor. However, with the discovery of a specific GPVI agonist, the collagen-related peptide (CRP), it was possible to identify the structure of the receptor, which revealed that GPVI belongs to the immunoglobulin (Ig) superfamily with two Ig-like domains.[100] GPVI was also shown to form a complex with the Fc receptor γ-chain (FcRγ) through a salt bridge at the Arg^{252} residue in the transmembrane domain of GPVI, which is responsible for stabilizing the complex in two Ig-like domains and one FcRγ configuration (Figure 1.3).[101] Moreover, the two Ig-like domains of GPVI harbour the collagen binding site. Although the exact region that interacts with collagen is still unclear, it seems that there are more than one interaction site. The Ig-like domains also contain a high level of

glycosylation that allows GPVI to extend over the polysaccharide layer of the platelet surface, in a structure similar to GPIbα, thereby increasing its accessibility to collagen.[102]

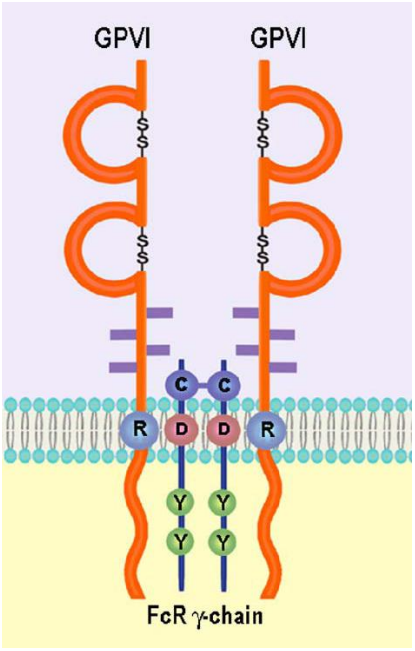

Figure 1.3: Schematic representation of the GPVI receptor. Mori M. et *al*. *Thrombosis Research*. 2004; 114:221-233.

The FcRγ is essential for both expression and function of GPVI. Upon activation of GPVI by collagen, the immunoreceptor tyrosine-based activation motif (ITAM) within the FcRγ becomes phosphorylated by the Src kinases, Fyn and Lyn that are constitutively bound to the cytoplasmic tail of GPVI.[103] In turn, the phosphorylated ITAM phosphorylates the tyrosine kinase Syk, which then induces the activating downstream signalling events, including activation of PKC and increase in intracellular Ca^{2+} by PLCγ2.[102]

It is now clear that GPVI is a major collagen receptor on platelets, but its physiological role is still unclear. There are two common haplotypes of GPVI known as *GP6a* and *GP6b* with observed frequencies of 0.85 and 0.13, respectively, that differ by 5 amino acid substitutions, 3 in the extracellular domain (Ser199Pro, Lys217Glu, Thr229Ala) and 2 in the cytoplasmic domain (Gln297Leu and His302Asn).[104] Although there is no difference in the expression level between the low- and high-frequency alleles, homozygosity for *GP6b* is associated with decreased functional responses of the receptor, as well as a reduced risk for recurrent cardiovascular events and mortality.[104, 105] Moreover, it has been shown that in GPVI-deficient

patients and GPVI-depleted mice, there is a moderate increase in bleeding time and an impairment of platelet aggregation in response to collagen.[106, 107] In addition, GPVI-deficient platelets exhibit decreased adhesion to collagen under flow conditions, which is abolished upon $\alpha_2\beta_1$ blockade.[108] Therefore, it seems that GPVI and $\alpha_2\beta_1$ make each their contribution to the platelet response to collagen.

1.3.6 $\alpha_2\beta_1$

The $\alpha_2\beta_1$ is the first collagen receptor to be identified on platelets and serves mainly as an adhesion molecule.[109] The crystal structure of the $\alpha_2\beta_1$ integrin allowed identification of the structures of the α_2 and β_1 subunits. The extracellular domain of the α_2 subunit has a β propeller-like structure and a collagen-binding αI domain. The extracellular domain of the β_1 subunit has an I domain-like fold, but it does not directly participate in collagen binding.[110, 111] Interaction of collagen with the αI domain of the α_2 subunit is dependent on the Mg^{2+} metal coordination site, named metal ion-dependent adhesion site (MIDAS), located in the bottom groove of the αI domain, while the αC helix in the αI domain seems to guide the collagen molecule into the groove in the right position, which is necessary for proper binding (Figure 1.4).[112, 113] Signalling through the $\alpha_2\beta_1$ integrin is similar to that induced by GPVI stimulation, which includes phosphorylation of the Src family kinases and the subsequent activation of PLC.[114] Moreover, as for most integrins, the $\alpha_2\beta_1$ integrin exists in two conformational configurations, an inactive conformation and an active one capable of bind collagen.[111]

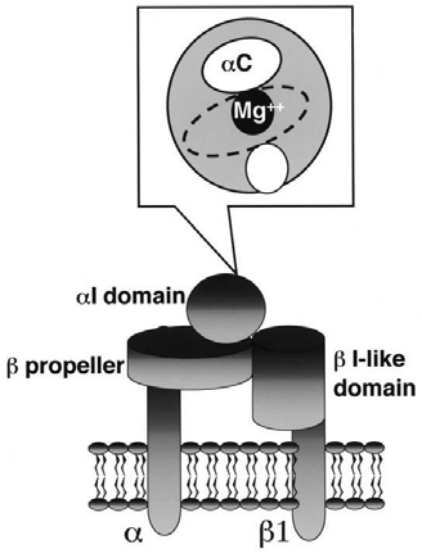

Figure 1.4: Schematic representation of the $\alpha_2\beta_1$ integrin. Heino J. *Matrix Biology*. 2000; 19:319-323.

It is now evident that $\alpha_2\beta_1$ and GPVI are the two collagen receptors on platelets. However, their relative function in collagen-mediated platelet response is still unclear. The GPVI induced collagen response seems to be the most important. On the other hand, the $\alpha_2\beta_1$ integrin is responsible for platelet adhesion to the exposed collagen surface in the sub-endothelium and in β_1-deficient mice, the thrombi consist of loose platelet aggregates.[115, 116] In addition, genetic or pharmacological depletion of GPVI highlight the capacity of $\alpha_2\beta_1$ to mediate collagen signalling and haemostasis.[108]

1.3.7 $\alpha_{IIb}\beta_3$

The $\alpha_{IIb}\beta_3$ integrin is the most important and most abundant integrin on platelets, with approximately 80 000 copies per resting platelet and with important $\alpha_{IIb}\beta_3$ reserves within platelet α-granules and the OCS, which increase the integrin's surface expression by 25-50% upon platelet activation.[117-119] The crucial importance of $\alpha_{IIb}\beta_3$ in primary haemostasis is evidenced by the bleeding disorder of patients with Glanzmann's disease, where genetic disorders in the α_{IIb} and β_3 gene result in functional abnormalities and/or prevention of surface expression of the $\alpha_{IIb}\beta_3$ integrin.[120] The bleeding disorder is due to absence or improper haemostatic plug formation because of lack of platelet aggregation, which is mediated by the cross-linking of $\alpha_{IIb}\beta_3$ on adjacent platelets by soluble fibrinogen at low shear rate or vWF at high shear rate.[121, 122]

The α_{IIb} and β_3 subunits of the $\alpha_{IIb}\beta_3$ integrin are both products of a single gene located on chromosome 17, giving rise to a 1008 and a 762 amino acid α_{IIb} and β_3 subunits, respectively.[123] The α_{IIb} is proteolytically processed into a heavy and a light chain. The light chain contains a 20 amino acid cytoplasmic tail, a transmembrane helix, and an extracellular segment that is disulfide linked to the heavy chain, which is entirely extracellular. Within the heavy chain, a large domain composed of a series of 60 amino acid repeats, which are arranged to form the seven blades of the β-propeller, extend outward from a central core. At the base of the β-propeller there are four divalent cation (Ca^{2+}) binding motifs.[124-126] The β_3 subunit contains a 48 amino acid cytoplasmic tail and three major extracellular domains: the A domain, the PSI (plexin-semaphorin-integrin) domain, and four endothelial growth factor (EGF)-like domains (Figure 1.5).[127] The PSI domain seems to be involved in integrin activation, because mutation of the cysteine linking it to the EGF-like domain, results in a constitutively active integrin.[128] The A domain contains 2 or 3 divalent cations (Ca^{2+}) sites, including a MIDAS motif that is highly involved in ligand binding. In addition, the A domain of the β_3 subunit also contains two recognition sites, one for the γ-chain sequence and another for the RGD (arginine-glycine-aspartic acid) sequence, which is present in ligands of the $\alpha_{IIb}\beta_3$ integrin, including fibrinogen, fibrin, vWF, vitronectin, fibronectin and autotaxin.[121, 129-133] Finally, the EGF-like domains are formed by four cysteine rich loops (EGF-1 to EGF-4), which seem to have a regulatory function because a cysteine mutation within this region causes activation of the $\alpha_{IIb}\beta_3$ integrin.[134, 135]

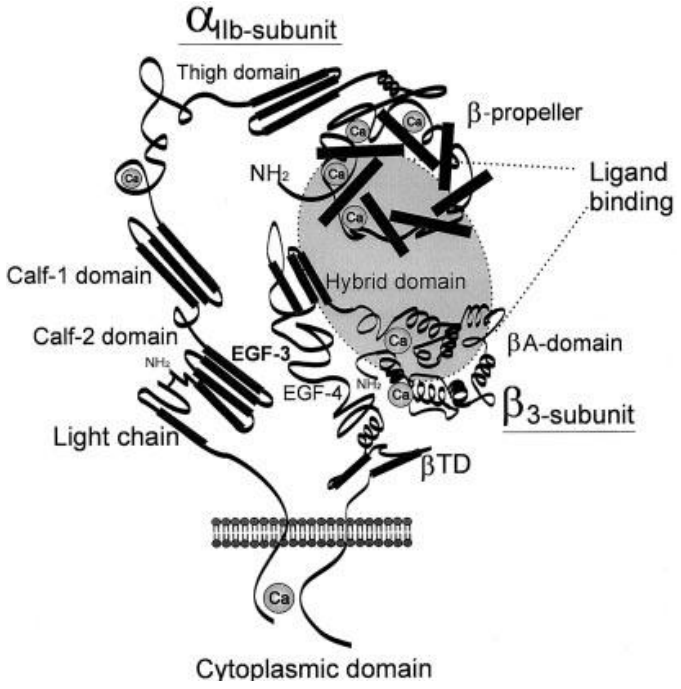

Figure 1.5: Schematic representation of the $\alpha_{IIb}\beta_3$ integrin. Quinn M. J. et *al*. Arteriosclerosis*, Thrombosis, and Vascular Biology*. 2003; 23:945-952.

As most integrins, the $\alpha_{IIb}\beta_3$ integrin exist in two conformations; an inactive low affinity ligand binding conformation, and an active high affinity ligand binding one. Activating signals mediated by platelet agonists such as collagen and thrombin induce "inside-out" signals, which shifts the $\alpha_{IIb}\beta_3$ integrin from its inactive to active conformation. In the active conformation, ligand binding to $\alpha_{IIb}\beta_3$ stimulates "outside-in" signalling that promote firm platelet adhesion and spreading on the extracellular matrix, fibrin clot retraction, and development of platelet pro-coagulant activity and microparticle generation.[136-138] Moreover, there is a close association between $\alpha_{IIb}\beta_3$ and the platelet cytoskeleton, where the cytoskeleton is involved in regulating the structure and activation of the $\alpha_{IIb}\beta_3$ integrin. In resting platelets, the $\alpha_{IIb}\beta_3$ integrin is associated with the membrane cytoskeleton, which favours anchoring of the integrin to the plasma membrane, and upon platelet activation and ligation of the $\alpha_{IIb}\beta_3$ integrin with fibrinogen, the $\alpha_{IIb}\beta_3$ integrin associates with cytoplasmic actin, thereby stabilizing ligand/integrin interactions, which ultimately lead to stabilizing the platelet aggregates.[139] The exact role of the $\alpha_{IIb}\beta_3$ integrin in platelet adhesion, aggregation and signalling will be discussed in more detail in section 1.4 of this chapter.

1.3.8 P-selectin

P-selectin belongs to the selectin family, which is composed of three members that are named according to their main expression site: L-selectin is expressed in leukocytes, E-selectin is expressed on endothelial cells, and P-selectin is mainly found in platelets but also in endothelial cells.[140-142] The human selectin family is encoded by genes located on chromosome 1, and all the members share a conserved structure consisting of an N-terminal Ca^{2+}-dependent lectin recognition motif followed by an EGF-like motif, a series of short consensus repeats (SCRs), a transmembrane domain, and a short cytoplasmic tail.[143] The main structurally differentiating factor between all members of the selectin family is the variation in the number of SCRs, with L-selectin, E-selectin, and P-selectin having 2, 6 and 9 SCRs, respectively (Figure 1.6).

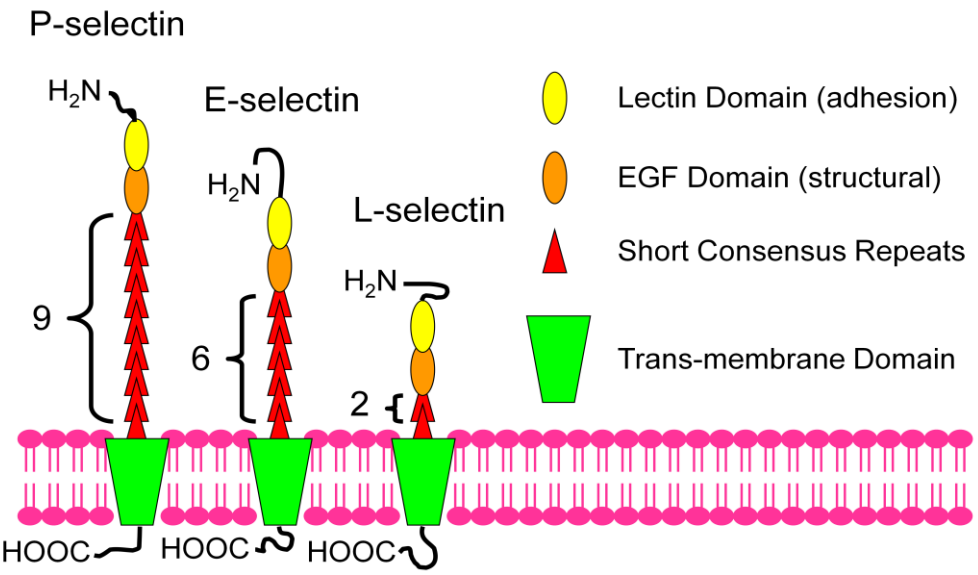

Figure 1.6: Schematic representation of the selectin family members. Image is from the laboratory of Dr. Merhi.

In platelets, P-selectin is stored in the α-granules, and upon activation it is translocated to the plasma membrane, where approximately 10 000 copies of the molecule are then expressed on the surface of an activated platelet. This translates to a density of 350 molecules/μm^2, which exceeds that of activated endothelial cells by approximately 10 fold.[144] One of the major roles of platelet P-selectin is mediating interactions between platelets and leukocytes through their constitutively expressed high affinity P-selectin ligand, the P-selectin glycoprotein ligand-1 (PSGL-1).[145] Therefore, with such a high density of P-selectin molecules expressed on activated platelets, this interaction might have an even more important role in recruiting leukocytes to the

site of injury/inflammation than the interaction between endothelial P-selectin and leukocyte PSGL-1. In fact, it has been demonstrated that in P-selectin deficient mice, there is lack of leukocyte recruitment onto the platelet monolayer at the site of vessel injury.[146] Moreover, the platelet/leukocyte interaction is of major importance not only in a physiological setting, but also in pathological ones such as in ischemia-reperfusion injury and in atherosclerosis.[147, 148]

P-selectin also has an important role in platelet/platelet interactions, as shown by its involvement in stabilizing platelet aggregates and thrombus formation; however, its ligands on platelets are still a matter of debate.[149, 150] Although platelets express three potential ligands for platelet P-selectin; PSGL-1, GPIbα and sulfatides, sulfatides and GPIbα seem to be the most probable ligands. P-selectin and GPIbα but not PSGL-1 blockade, inhibits platelet rolling onto an activated endothelium and affects the stability of platelet aggregates, indicating a possible role for GPIbα as a P-selectin ligand on platelets.[99, 149] By contrast, it has recently been shown that blocking antibodies against P-selectin and sulfatides, but not PSGL-1 or GPIbα inhibited platelet adhesion to P-selectin and platelet aggregation, indicating that sulfatides, and not PSGL-1 or GPIbα are the major P-selectin ligands on platelets.[149, 151, 152] Such discrepancy in identifying the platelet P-selectin ligand could be attributed to the experimental settings. Furthermore, P-selectin expressed on activated platelets promotes fibrin and thrombus formation by recruiting tissue factor (TF) bearing monocytes and monocyte derived microparticles to the site of vessel injury.[153, 154]

1.4 Platelet function

As mentioned earlier in this chapter, platelets play a pivotal role in haemostasis by maintaining the integrity of blood vessels through the formation of a haemostatic plug that prevents blood loss. In order to accomplish such task, platelets respond to external stimuli mediated by the interaction of their receptors with the respective ligands. These interactions lead to a sequence of events consisting of adhesion, activation, secretion, and aggregation. In brief, GPIb/IX/V, GPVI, $\alpha_2\beta_1$ integrin and $\alpha_{IIb}\beta_3$ integrin mediate platelet adhesion and activation, while $\alpha_{IIb}\beta_3$ is responsible for platelet aggregation, clot retraction and thrombus stability.

1.4.1 Platelet adhesion

Under normal circumstances, platelets circulate in the blood in an inactive state unable to adhere or become activated, a process mediated by an important function of the endothelium. The endothelial cells not only create a physical barrier preventing platelet and sub-endothelial matrix contact, but also actively participate in inhibiting platelet activation by secreting inhibitory molecules such as nitric oxide (NO), PGI_2, and nucleoside triphosphate diphosphohydrolase-1 (NTPDase-1, CD39).[82] On the other hand, damage to the vessel wall exposes the sub-endothelial matrix and activates endothelial cells, thereby initiating the haemostatic mechanism. Platelet adhesion at the site of vessel injury is initiated by platelet rolling on the activated endothelium, followed by firm adhesion of platelets to the components of the sub-endothelial matrix (collagen and vWF).[155]

Platelet rolling on the activated endothelium

Platelet rolling on the activated endothelium is the first step in initiating the haemostatic plug. Before adhering to the sub-endothelial matrix, circulating platelets undergo a deceleration process on activated, P-selectin (present in Weibel-Palade bodies) expressing endothelial cells neighbouring the injured vasculature.[156] This process is mediated by the interaction of endothelial P-selectin with its yet unresolved ligand on platelets, albeit platelet PSGL-1 and the GPIb/IX/V complex could be the two possible candidates.[99, 151]

Platelet adhesion to the sub-endothelial matrix

At the site of the injured vasculature, following the rolling process onto the activated endothelium, platelets come to an arrest upon contact with the sub-endothelial matrix. The arrest is made possible by the interaction of platelet GPIb/IX/V, GPVI, $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ with the components of the sub-endothelial matrix. The importance of the relative initial interactions of these receptors with their respective ligands is dependent on the shear force present at the site of injured vasculature. For instance, at high shear levels such as in stenotic arteries, initial tethering of platelets onto the sub-endothelial matrix fully depends on the weak interaction between the GPIb/IX/V complex and vWF, then followed by the firm adhesion of platelets to the exposed collagen matrix, which is GPVI and $\alpha_2\beta_1$-dependent.[157-159] By contrast, at low shear levels such as in the venous circulation, platelet adhesion to the sub-endothelial matrix depends on the

interactions of GPVI and $\alpha_2\beta_1$ with collagen fibers, even though the GPIb/IX/V and vWF interaction is still present, it is of less importance.[160] The $\alpha_{IIb}\beta_3$ integrin also participates in platelet adhesion by binding fibrinogen and vWF molecules present in the sub-endothelial matrix. Figure 1.7 summarizes the major events in platelet adhesion to the sub-endothelial matrix.

It is of interest to mention that circulating vWF molecules are not able to bind the GPIb/IX/V complex, but once in contact with collagen in the sub-endothelial matrix and in the presence of shear forces, the vWF molecule undergoes conformational change, which allows it to bind the GPIb/IX/V complex.[161, 162] Although the vWF/GPIb/XI/V interaction is of a weak nature, at high shear levels, it occupies a crucial role in slowing down circulating platelets, which then favours GPVI and $\alpha_2\beta_1$-dependent platelet arrest at the sites of injury.

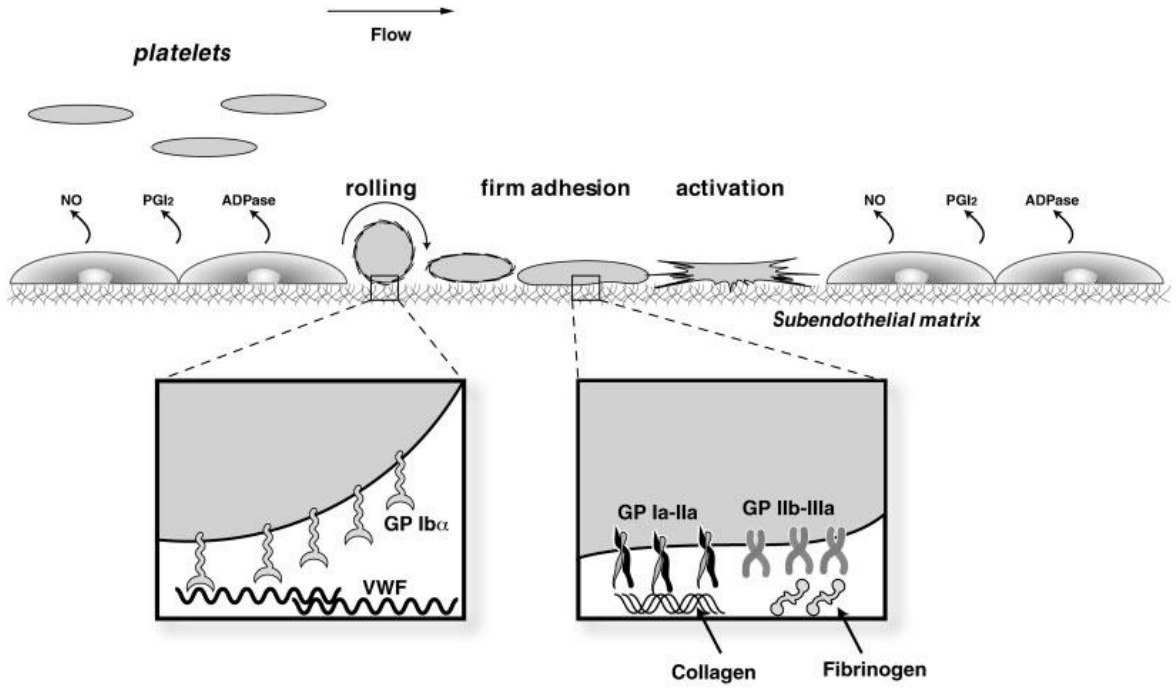

Figure 1.7: Platelet adhesion to the sub-endothelial matrix. Under normal conditions, platelet activation is prevented by the secreted endothelial mediators (NO, PGI_2 and NTPDase-1). At the site of vessel lesion, platelet rolling, tethering, adhesion and activation is mediated by the interaction of platelet receptors/integrins with the components of the sub-endothelial matrix (see above text). Conde I. D. et *al*. *Catheterization and Cardiovascular Interventions.* 2003; 60:236-246.

1.4.2 Platelet activation

Interaction of GPIb/IX/V, GPVI, $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ with components of the sub-endothelial matrix is not only responsible for platelet adhesion, but also mediates signals that induce platelet activation, secretion and aggregation. As mentioned in the previous section, ligation of GPIb/IX/V, GPVI and the $\alpha_2\beta_1$ integrin with their respective ligands mainly induce activation of Src family kinases which ultimately activate PLC, specifically PLCγ2, leading to the generation of DAG and IP_3, that activate PKCs and increase cytosolic Ca^{2+} levels, respectively. Furthermore, ligation of fibrinogen to the $\alpha_{IIb}\beta_3$ integrin also induces activation of the Src family kinases that activate PLCγ2, and the interaction of talin with the cytoplasmic tail of the β_3 subunit, which is involved in clot retraction.[163]

Following the initial wave of platelet activation induced by ligation of GPIb/IX/V, GPVI, $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ with the components of the sub-endothelial matrix, activated platelets secrete secondary mediators such as TxA_2 and ADP (see section 1.4.3) that recruit and activate additional circulating platelets at the site of vascular lesion. In addition, TF expressed on recruited monocytes, endothelial cells and activated platelets leads to the generation of thrombin at the site of injured vasculature, which also participates in recruitment and activation of additional platelets.[154, 164, 165] Activation of platelets by TxA_2, ADP and thrombin is mediated by ligation of these secondary mediators with their respective receptors; TPα and TPβ, the purinergic receptors, and PARs, which are coupled to G proteins (see section 1.3). Briefly, G_q and $G_{12/13}$ lead to the generation of DAG and IP_3 by PLCβ, and to the activation of the Rho GEFs, respectively. DAG in turn activates PKCs, which lead to protein phosphorylation, including VASP phosphorylation. VASP functions as an anti-capping protein, which is involved in crucial cellular functions, including shape change by directly modulating the actin ultra-structure.[166] IP_3 binds to its receptor on the endoplasmic reticulum thereby inducing release of Ca^{2+} from intracellular stores. The Rho GEFs; RhoA, Rac1, and Cdc42 are regulators of signalling pathways that control actin organization by the formation of stress fibers, lamellipodia and filopodia.[167] In summary, activation of platelets is mediated by two major signalling pathways, one including activation of PLC (PLCγ2 or PLCβ), and the second mediated by activation of G proteins through GPCRs. Figure 1.8 summarizes the major signalling pathways implicated in platelet activation.

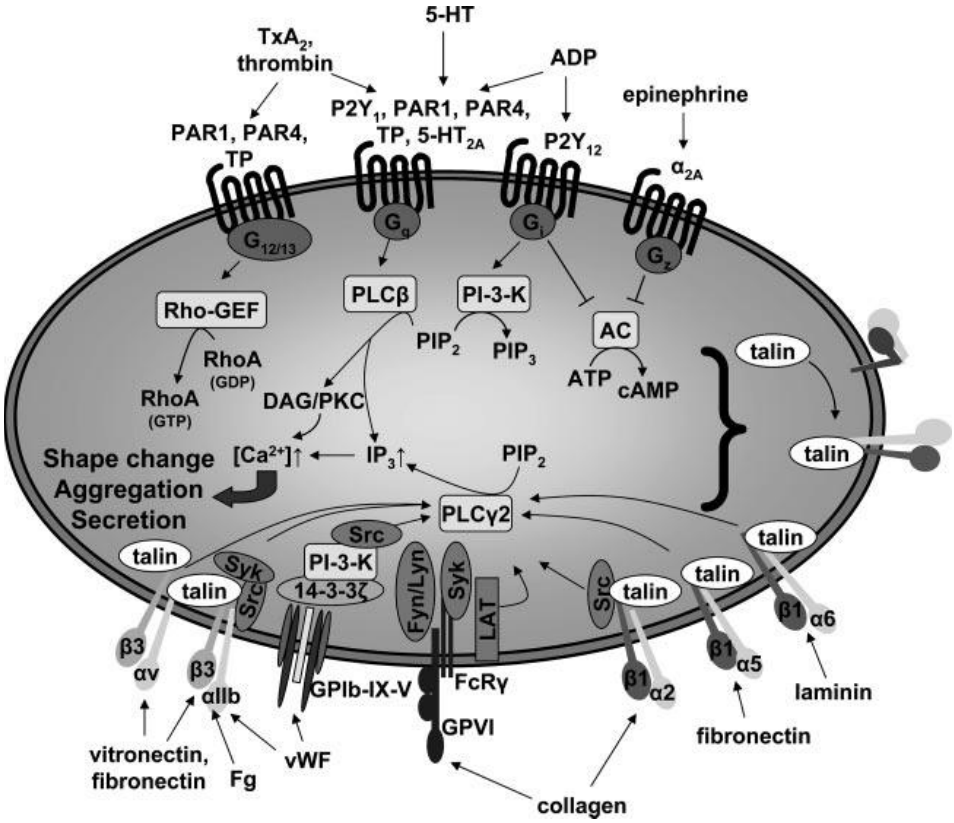

Figure 1.8: The major signalling pathways implicated in platelet activation. Varga-Szabo D. et *al*. Arteriosclerosis*, Thrombosis, and Vascular Biology*. 2008; 28:403-412.

One of the early hallmarks of platelet activation is the morphological shape change induced by the reorganization of the platelet actin cytoskeleton, which transforms platelets from their disk-like structure into a spherical one containing multiple filopodia protrusions (Figure 1.9).[33, 34] The change in platelet shape as well as the formation of filopodia allows platelets to expand and cover a larger surface area at the site of vessel injury. Platelet activation also leads to the expression of P-selectin and the activation of the $\alpha_{IIb}\beta_3$ integrin (through an "inside-out" signalling mechanism), where they each make their contribution to platelet aggregation.

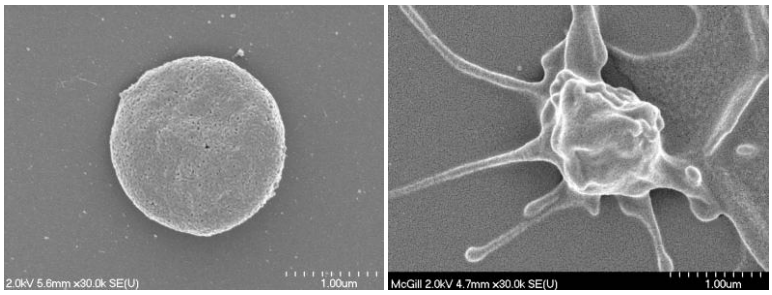

Figure 1.9: Scanning electron microscopy image of platelet shape change. Left: Resting platelet. Right: Activated platelet. Images are from the laboratory of Dr. Merhi.

1.4.3 Platelet secretion

Platelet activation is accompanied with secretion, where platelets secret a plethora of molecules stored in their granules, as well as newly enzymatically synthesised compounds, such as TxA_2. Platelet secretion is an indispensible process linked to their pathophysiological role, including recruitment and activation of additional circulating platelets, initiation of thrombus formation, mediating intracellular adhesion and triggering cell proliferation and migration. Platelets contain three types of secretory granules; α-granules, dense granules and lysosomes, which are formed, pre-packaged, and sorted into pro-platelets during platelet formation from megakaryocytes.[168]

Megakaryocytes synthesise most of the protein contents present in platelet granules, and during the platelets' life span, platelets incorporate proteins into their existing granules through endocytosis. For instance, incorporation of fibrinogen is mediated by its interaction with the $\alpha_{IIb}\beta_3$ integrin, and its subsequent internalization and sorting into α-granules.[169] The α-granules are the largest and most abundant granules in platelets with approximately 80 α-granules per platelet, and they contain coagulation proteins (e.g. fibrinogen, factor V), soluble adhesion molecules (e.g. vWF), growth factors (e.g. platelet derived growth factor [PDGF]), protease inhibitors (e.g. plasminogen activator inhibitor-1), and membrane adhesion molecules (e.g. P-selectin and $\alpha_{IIb}\beta_3$).[170] The dense granules are less abundant and less voluminous than α-granules, with about 3-8 dense granules per platelet; however, they contain substances indispensible for platelet activation, such as ADP, serotonin, Ca^{2+}, and magnesium.[171, 172] Although the contents of the α and dense granules differ from each other, recent studies have shown the presence of P-selectin, $\alpha_{IIb}\beta_3$, and GPIb in both granules.[173, 174] Finally, lysosomes contain enzymes that have a role in degrading and digesting multiple proteins.[175, 176] Table 1.1 summarizes the major contents of platelet granules.[177]

Table 1.1: Platelet granule contents.

α-granules	Dense granules	Lysosomes
P-selectin	GPIb	Cathepsin D
$\alpha_{IIb}\beta_3$ integrin	$\alpha_{IIb}\beta_3$ integrin	Cathepsin E
GPIb/IX/V	P-selectin	Carboxypeptidase A
GPVI	CD107a (LAMP-1)	Carboxypeptidase B
$\alpha_v\beta_3$ integrin	CD107b (LAMP-2)	Proline carboxypeptidase
PECAM-1	CD63 (LAMP-3)	CD107a (LAMP-1)
Stomatin	Serotonin	CD107b (LAMP-2)
PDGF	Histamine	CD63 (LAMP-3)
EGF	ATP	Acid phosphatase
VEGF	ADP	Arylsulphatase
Transforming growth factor β (TGF-β)	GTP	β-D-glucuronidase
Albumin	GDP	β-D-galactosidase
Fibrinogen	Pyrophosphate	β-D-fucosidase
Fibronectin	Calcium	β-D-glucosidase
Vitronectin	Magnesium	α-D-mannosidase
Osteonectin		α-D-galactosidase
vWF		α-L-arabinofuranosidase
von Willebrand antigen II		α-L-fucosidase
Thrombospondin		
Platelet factor-4 (PF4)		
IgG, IgA, IgM		
C1 inhibitor		
Plasminogen		
Plasminogen activator inhibitor-1		
Platelet-derived collagenase inhibitor		
High molecular weight kininogen		
Protein S		
α_2-antitrypsin		
α_2-macroglobulin		
α_2-antiplasmin		
Multimerin		
Platelet basic protein		
β-thromboglobulin		
Histidine-rich glycoprotein		
Connective tissue-activating protein III		
Neutrophil-activating protein II		
Coagulation factor V		
Coagulation factor VIII		

Activation of platelets by physiological agonist such as thrombin, ADP, TxA_2 and collagen leads to intracellular signalling events that mediate platelet secretion, where platelets discharge their granule contents into the extracellular environment by exocytosis.[172] Although most of the platelet physiological agonists can mediate α and dense granule secretion, it seems that only thrombin, the most potent agonist, is capable of inducing lysosome secretion.[178] As mentioned earlier in this chapter, ligation of platelet agonists with their cognate receptors induces activation of two major signalling pathways (PLC and G protein activation), of which PLC

activation seems to be of significant importance for platelet secretion. The increase in cytosolic Ca^{2+} levels by IP_3, as well as the activation of DAG following PLC mediated degradation of PIP_2, leads to the activation of PKCs, which are crucial signalling molecules involved in platelet secretion. Platelets contain multiple PKCs (α, β_1, β_{11}, δ, ζ, η, and θ) and it seems that each of them has a specific role in platelet secretion, where they induce phosphorylation of intracellular signalling molecules implicated in the exocytotic machinery.[172, 179-183]

The molecular machinery involved in platelet secretion, which mediates granule fusion with surface connected membranes of the OCS or the plasma membrane, remained obscure for a long time.[184, 185] However, insights into similarities between neuronal and platelet exocytosis, revealed molecular components of the secretory mechanism involved in both cell types.[186] The molecular machinery responsible for membrane fusion during exocytosis is composed of the core soluble N-ethylmaleimide sensitive factor (NSF) associated protein receptor (SNARE) complexes. The SNAREs associated with granules are termed vesicular SNAREs (vSNARE), while those associated with target membranes (e.g. OCS and plasma membrane) are termed tSNAREs. In platelets, the identified vSNAREs include the vesicle-associated membrane proteins (VAMP) -2, -3, -7, and -8, while the identified tSNAREs include the syntaxins 2, 4, 7, and 11, and the soluble NSF-associated proteins (SNAP) -23, -25, and -29.[187-193] Platelets also contain the Sec1 (platelet Sec1 protein, [PSP]) and Rab proteins that regulate SNARE function.[183, 194] Platelet exocytosis is mediated by orchestrated steps involved in granule and plasma membrane fusion. Briefly, in resting conditions, PSP binds to tSNAREs and prevents formation of the SNARE complex required for membrane fusion. Upon platelet activation, the increase in cytosolic Ca^{2+} levels, as well as the activation of the PKCs, induces phosphorylation of PSP, which in turn relieves its inhibitory effects on SNARE complex formation.[183] Concurrently, NSF disassembles the *cis*-conformation of SNAREs on the same membrane thereby allowing formation of the *trans*-conformation of the SNAREs so that they are able to interact with their respective SNAREs on opposing membranes.[195] Finally, the association of the vSNAREs and tSNAREs, in addition to the modulating role of Rab proteins, which facilitates docking of opposing membranes and modifies SNARE protein function, allow fusion of granule and plasma membranes thereby releasing the granule contents into the extracellular milieu.[196-198]

In addition to granule exocytosis, platelets also synthesize and secrete new molecules such as TxA_2, upon activation. Platelet activation by physiological agonists such as thrombin,

ADP, and collagen induce activation of phospholipase A_2, which is responsible for liberating arachidonic acid from the membrane lipid bilayer. Arachidonic acid is then converted into the prostaglandin TxA_2 through sequential enzymatic modification by COX-1 and thromboxane synthase enzymes.[199]

1.4.4 Platelet aggregation

Platelet aggregation is the final step in primary haemostasis, where platelets cluster together to form a stable haemostatic plug following their adhesion and activation. Platelet aggregation is mediated by the cross-linking of $\alpha_{IIb}\beta_3$ integrins on adjacent platelets by soluble fibrinogen. However, in circulating quiescent platelets, the $\alpha_{IIb}\beta_3$ integrin is in an inactive conformation with a low affinity for fibrinogen. The subsequent activation of platelets by physiological agonists, such as thrombin, collagen, ADP, induces intracellular signals, which change the $\alpha_{IIb}\beta_3$ integrin from a low affinity inactive conformation to a high affinity active one through an "inside-out" signalling mechanism. Although activation of the $\alpha_{IIb}\beta_3$ integrin is a crucial step in determining platelet aggregation, the exact signalling mechanisms involved in its conformational changes have not been completely elucidated. Reconstruction of the $\alpha_{IIb}\beta_3$ activation pathway in Chinese hamster ovary (CHO) cells revealed the importance of talin (a major cytoskeleton actin binding protein) in the integrin's activation, and furthermore linked talin to upstream signalling partners.[200] Platelet activation by physiological agonists leads to the activation of PKCs (see section 1.3), which in turn activate the Rap1 small guanosine triphosphatase. Activated Rap1 in turn interacts with talin and recruits it to the cytoplasmic tails of the $\alpha_{IIb}\beta_3$ integrin where it mediates their separation and hence activation of the integrin (Figure 1.10).[200, 201]

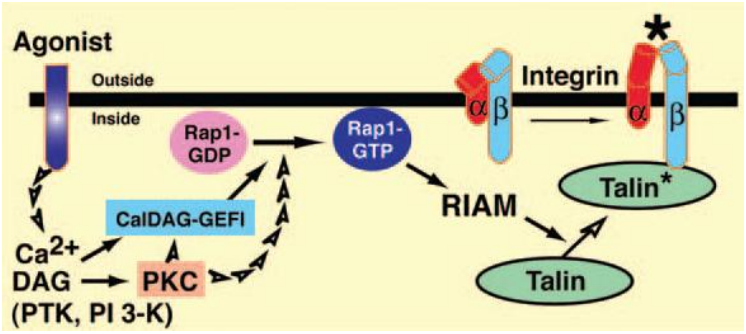

Figure 1.10: Schematic representation connecting agonist stimulation to $\alpha_{IIb}\beta_3$ activation in platelets. Kasirer-Friede A. et *al. Immunological Reviews*. 2007; 218:247-264.

As mentioned earlier in this chapter, following platelet activation and the subsequent activation of the $\alpha_{IIb}\beta_3$ integrin, ligand binding to $\alpha_{IIb}\beta_3$ stimulates "outside-in" signalling that promote firm platelet adhesion and spreading on the extracellular matrix, stabilization of platelet aggregates, fibrin clot retraction, and development of platelet pro-coagulant activity. Studies conducted on human platelets and CHO cells transfected with the $\alpha_{IIb}\beta_3$ integrin revealed three distinct "outside-in" signalling pathways derived from $\alpha_{IIb}\beta_3$ ligation, where the first is mediated by activation of the tyrosine kinase Syk, the second originates from the activation of the focal adhesion kinase ($pp125^{FAK}$), and the third is initiated by phosphorylation of tyrosine residues in the cytoplasmic tail of the β_3 subunit.[202, 203]

The cytoplasmic tail of the β_3 subunit contains two conserved tyrosine residues (Y747 and Y759), both of which become phosphorylated, probably by the Src kinase Fyn, following ligand binding to the $\alpha_{IIb}\beta_3$ integrin.[203] The N-terminal tyrosine residue is within a conserved NPXY motif that mediates binding to proteins with phosphotyrosine binding domains, such as the adapter Dok2, which undergoes tyrosine phosphorylation downstream of the integrin.[204, 205] The second tyrosine falls within a similar NXXY motif, and has been shown to bind multiple signalling proteins, including Shc.[206] The functional importance of these two conserved tyrosine residues is evidenced by the recurrent bleeding phenotype and the impaired clot retraction as seen in transgenic mice that have these two tyrosine residues of the β_3 subunit mutated to phenylalanine.[207] The molecular basis for this disorder was later attributed to the loss of myosin binding to the phosphorylated β_3 tail; however, the contribution of Shc or Dok2 to this response is still unclear.[208]

Activation the Syk and $pp125^{FAK}$ signalling pathways result from clustering of the $\alpha_{IIb}\beta_3$ integrin, albeit activation of the $pp125^{FAK}$ signalling pathway requires additional platelet stimulation by the secondary mediators, TxA_2 and ADP.[209, 210] The Syk signalling pathway leads to activation of the Rac exchange factor Vav-1 and the molecular adaptor SLP-76, that are implicated in cytoskeletal regulation downstream of the $\alpha_{IIb}\beta_3$ integrin, which leads to lamellipodia formation and platelet spreading.[211, 212] In addition, Syk also activates PLCγ2, through a pathway that is likely to be dependent on SLP-76 and Vav, which also mediates platelet spreading.[213, 214] Finally, little is known about the $pp125^{FAK}$ signalling pathway, but it seems that activation of $pp125^{FAK}$ mediates its interaction with Hic-5 protein, which is an adapter molecule implicated in platelet spreading.[209]

1.5 Pathological role of platelets

It was previously thought that the major platelet role was in physiological haemostasis, in which they maintain the integrity of the blood vessels by limiting blood loss following vascular injury. Now, a plethora of evidence points to their involvement in multiple pathological conditions. As mentioned above, platelets are endowed with multiple molecules either present on their surface or secreted following platelet activation, which modulate the development and progression of a wide variety of diseases, including vascular thrombosis, atherosclerosis, inflammation, rheumatoid arthritis, lupus, cancer, coronary and cerebral-vascular diseases, diabetes and psychiatric diseases. This section will focus on the platelet contribution to development and progression of the following relevant pathologies: atherosclerosis, thrombosis and inflammation.

1.5.1 Atherosclerosis

Beyond their role in haemostasis and thrombosis, platelets are critically involved in the onset of atherosclerosis, which is now considered as a chronic inflammatory disease.[215, 216] Their involvement in the initiation, progression and stability of the atherosclerotic plaque has recently been put into evidence thanks to new intra-vital microscopy techniques and to the development of appropriate atherosclerosis animal models. For instance, it has been shown that in hypercholesterolemic rabbits and apolipoprotein E (ApoE) deficient mice (an atherosclerosis mouse model characterized by high levels of circulating cholesterol), platelets adhere to atherosclerotic-prone sites before lesions are detectable, suggesting that platelets are among the first (if not the first) vascular cells to be recruited to the lesion site.[217, 218]

Although the first steps that initiate formation of the atherosclerotic plaque are still unclear, it seems that activation of endothelial cells could be the culprit. Mechanoreceptors on endothelial cells respond to variations in shear force that is generated by flowing blood at atherosclerotic-prone sites (arterial branching points), thereby mediating a change in cell morphology, gene expression profiles and increases in adhesiveness.[219-221] Therefore, activation of endothelial cells increases surface expression of adhesion molecules, specifically P-selectin and $\alpha_v\beta_3$, which have been shown to be required for platelet recruitment to atherosclerotic-prone sites. Recruitment of platelets to atherosclerotic sites is mediated by a two step process, where the initial contact between platelets and activated endothelial cells is mediated by the weak interaction of platelet GPIb/IX/V or PSGL-1 and endothelial P-selectin. This weak interaction is

then followed by a firm adhesion of platelets on the activated endothelium through the cross-linking of $\alpha_{IIb}\beta_3$ on platelets with $\alpha_v\beta_3$ on endothelial cells by fibrinogen.[222-225]

Following adhesion to the activated endothelium, platelets favour atherosclerotic plaque development by three different processes. The first is inducing an inflammatory reaction in the endothelium through the release of the pro-inflammatory compounds interleukin-1β (IL-1β) and CD40L, which induce a pro-atherogenic phenotype of endothelial cells. This is characterized by expression of adhesion molecules (P-selectin, E-selectin, ICAM-1, vascular cell adhesion molecule-1 [VCAM-1] and $\alpha_v\beta_3$), and secretion of chemokines (macrophage chemotactic protein-1 [MCP-1]) and matrix degrading enzymes (matrix mettaloproteinases-9 [MMP-9]).[2, 3, 226-228] Secondly, platelets also contribute to the pro-atherogenic environment by releasing growth factors (PDGF, EGF, TGF-β, and fibroblast growth factor [FGF]), chemokines (PF4, platelet activating factor [PAF], macrophage inflammatory protein-1α [MIP-1α], and regulated upon activation normal T-cell expressed and released [RANTES]), and matrix degrading enzymes (MMP-2).[216, 229-233] Finally, adherent platelets favour recruitment, activation, secretion and transmigration of leukocytes at the atherosclerotic site.[229, 234] Effectively, as mentioned earlier in this chapter (see section 1.3.8) platelets have a much higher level of expression of P-selectin than activated endothelial cells, which has been shown to greatly influence leukocyte recruitment to the atherosclerotic site. In the absence of adherent platelets, leukocyte recruitment to the lesion site is markedly reduced.[235] Moreover, secreted chemokines (RANTES and MCP-1) by adherent platelets favour leukocyte homing toward the lesion site.[2, 236] Figure 1.11 summarizes the platelet involvement in atherosclerotic plaque formation.

Development of coronary artery diseases, such as unstable angina, acute myocardial infarction, and stable coronary-artery disease, as well as peripheral vascular diseases, are tightly linked to the progression and complications related to atherosclerosis.[237-239] Moreover, rupture of the atherosclerotic plaque exposes pro-thrombotic substances, including components of the extracellular matrix and components of the necrotic plaque core, which induce platelet activation and thrombus formation. In summary, platelets are implicated in all aspects of atherosclerosis; hence the development of multiple anti-platelet agents, such as aspirin (COX-1 inhibitor), $\alpha_{IIb}\beta_3$ inhibitors (abciximab, eptifibatide, and tirofiban), $P2Y_{12}$ inhibitors (clopidogrel and ticlopidine), and thrombin inhibitors (heparin), in order to dampen or limit platelet activation.[240]

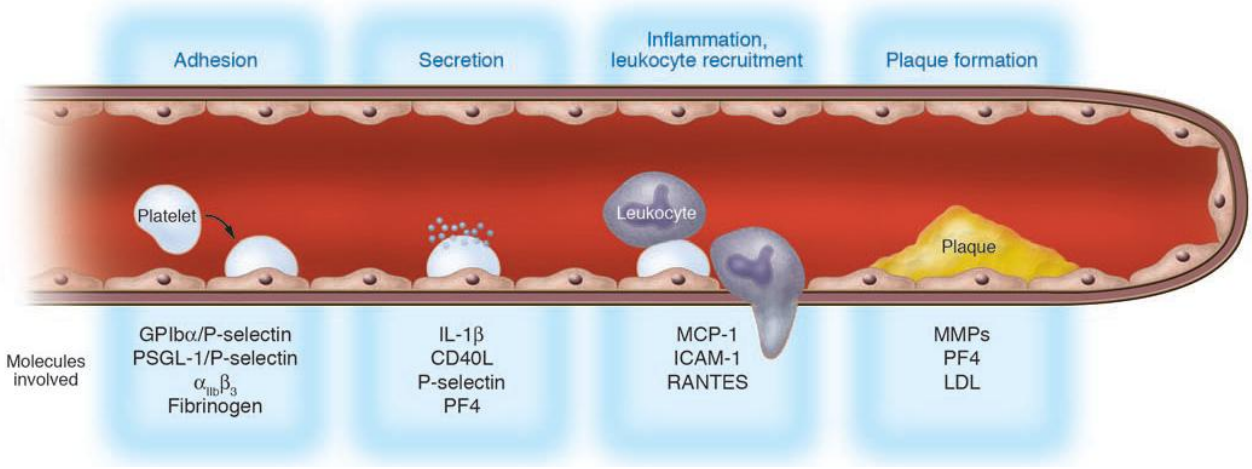

Figure 1.11: Model of the role of platelets in atherosclerotic plaque formation. Platelets adhere to the activated endothelium by a two step mechanisms, the first is mediated by the weak interactions of platelet GPIb/IX/V or PSGL-1 with endothelial P-selectin, then followed by the firm adhesion mediated by $\alpha_{IIb}\beta_3$. Activated adherent platelets then secrete pro-inflammatory compounds that trigger an inflammatory reaction in the endothelium. Moreover, adherent platelets favour recruitment and transmigration of leukocytes at the lesion site. Gawaz M. et *al*. *The Journal of Clinical Investigation*. 2005; 115:3378-3384.

1.5.2 Thrombosis

One of the major complications in the circulatory system is thrombosis, which is the formation of a blood clot (thrombus) within the blood vessel. Partial or complete occlusion of a blood vessel due to a thrombus leads to reduction or blockage of the blood flow, which ultimately causes ischemia or infarction of the irrigated organ because of lack of nutrients to meet its metabolic needs. Thrombosis is divided into two categories, depending on the location where the thrombus is formed, giving rise to arterial or venous thrombosis. Despite similarities between arterial and venous thrombosis, in that they both contain a fibrin mesh (the end product of the coagulation cascade) and platelet aggregates, there are differences in the initiating cause and the composition of the two thrombus types. For instance, there is a body of evidence suggesting that venous thrombosis is caused by a disturbance in blood flow, such as stagnation, while in arterial thrombosis disturbance in blood flow has a role in initiating thrombus formation but atherosclerotic plaque rupture is of more significance.[241, 242] In addition, venous thrombi are also designated as "red clots" because they contain more fibrin and trapped red blood cells, as well as less platelets than arterial thrombi, which are referred to as "white clots".[243] This section will focus on the mechanisms underlying arterial thrombosis, specifically atherothrombosis, and the platelet contribution to the disease.

Atherothrombotic diseases are the leading cause of mortality, and account for more than 25% of deaths worldwide.[244] Development of a clot, following atherosclerotic plaque rupture or erosion, in the coronary or cerebral circulation (causing acute myocardial infarction or ischemic stroke, respectively) is now the single most common cause of morbidity and mortality globally, and involves both aggravated platelet and coagulation cascade activation. Although the endothelium actively secretes a range of molecules that inhibit platelet activation (PGI_2, NO, NTPDase-1) and thrombin generation (thrombomodulin), plaque rupture in combination with a severe pre-existing arterial stenosis (abnormal narrowing of blood vessel) leads to an exaggerated platelet aggregation response.[245, 246] Exposure of the sub-endothelial matrix as well as the highly thrombogenic necrotic core to the circulation, following plaque rupture, leads to platelet adhesion and activation onto the components of the sub-endothelial matrix (vWF and collagen), and the subsequent recruitment and activation of circulating platelets by secreted secondary mediators (ADP and TxA_2). In addition, at the surface of the growing thrombus, blood flow is accelerated, thereby subjecting platelets and immobilized ligands, such as vWF, to extensional drag forces.[247] Thus platelet recruitment onto the thrombus surface becomes extensively dependent on vWF and platelet GPIbα interactions, which is then followed by the stable platelet-platelet aggregates mediated by the $\alpha_{IIb}\beta_3$ integrin interactions with its ligands, such as vWF, fibrinogen, fibrin, and/or fibronectin, and P-selectin interactions with its cognate ligands on platelets (see section 1.4.4).[87]

Another important function of activated platelets in thrombus formation is their ability to support the assembly of coagulation complexes on their plasma membrane, either through the direct recruitment of TF to phosphatidylserine (PS) expressed on the membrane or through the indirect binding of TF bearing microparticles (derived from activated or apoptotic monocytes/macrophages and T lymphocytes) and leukocytes mediated by P-selectin/PSGL-1 interactions.[153, 248] TF on its turn favours formation of thrombin, which is not only the most potent platelet activator, but also converts fibrinogen to fibrin during the coagulation process, both of which are important for thrombus growth and stability.[164] Moreover, disturbance in blood flow, which is an important but yet incompletely understood aspect of thrombogenesis, has an impact on platelet adhesiveness and the blood clotting process. Specific alterations in blood flow, including flow acceleration at the apex of the stenosis (narrowing caused by growing thrombus) followed by flow deceleration, promote platelet deposition onto the thrombogenic surfaces.[247]

The flow deceleration downstream from the site of plaque injury also induces recirculation and stagnation of the blood, which favours blood coagulation, leading to the propagation of a fibrin and red blood cell rich thrombus that is known as the "fibrin tail".[249] Figure 1.12 schematizes thrombosis following atherosclerotic plaque rupture.

Thrombosis is of a more threat for individuals with hyperactive platelets, such as diabetic patients, which are also resistant to regular anti-platelet therapy and therefore require more intensive anti-platelet regimens, and in thrombosis-prone groups, including individuals with hypertension, hypercholesterolemia, and cigarette smokers and the elderly.[250-256] In addition, platelet deposition onto reactive surfaces induced by shear gradients is not prevented by antiplatelet therapy, such as aspirin, clopidogrel or thrombin inhibitors.[247] Therefore, a more comprehensive understanding of the role and the possible inhibition avenues of the platelet involvement in thrombus formation could be of benefit for atherothrombotic disease management.

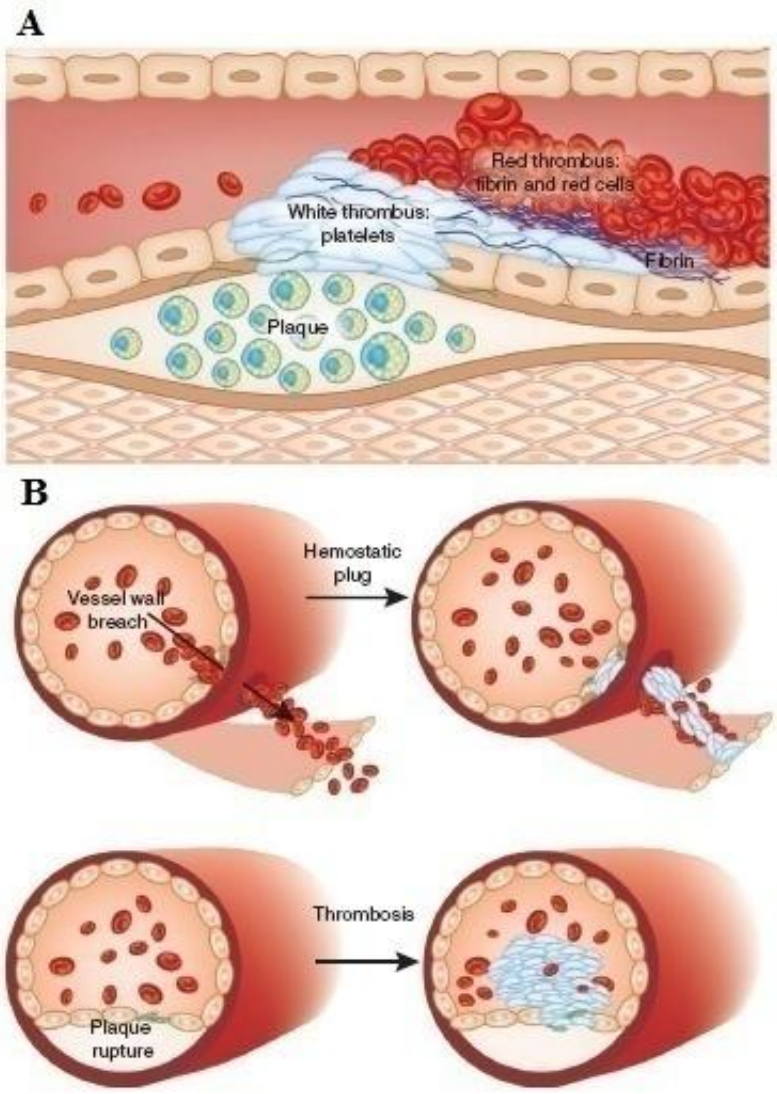

Figure 1.12: Cartoon representation of thrombosis. A) Representation of the ruptured atherosclerotic plaque covered by a "white thrombus" formed by platelet aggregates, followed by a "fibrin tail" formed by a fibrin mesh entrapping red blood cells. B) Representation of the differences between haemostatic plug formation and thrombosis. Jackson S. P. *Nature Medicine*. 2011; 17:1423-1436.

1.5.3 Inflammation and immunity

The platelets' role was thought to be restricted to haemostasis, but it is now clear that platelets actively participate in inflammation and the host's defence against foreign pathogens. The active contribution of platelets to the initiation, development and progression of the atherosclerotic plaque (see section 1.5.1), was among the first signs showing their pathological pro-inflammatory functions.[257] Now a plethora of evidence shows that platelets actively interact with bacteria, viruses and fungi, and help modulate the immune system.

Toll-like receptors (TLR), which are probably the most important receptors of the innate immune system, are pattern recognition receptors that recognize broadly shared molecules by pathogens, such as lipopoplysaccharide (LPS), lipoproteins, and other bacterial wall constituents.[258] Platelets contain TLR1, 2, 4, 6, and 9, which enable platelets to bind bacteria, thereby favouring either platelet mediated killing of the bacteria through secretion of thrombocidins (antibacterial proteins within platelet α-granules, including thombocidin 1 and 2) or by aggregating around the bacteria and "trapping" them for elimination by professional phagocytes.[259-263] Interestingly, platelet derived thrombocidins have also been shown to kill fungi, such as *Cryptococcus neoformans*. Moreover, LPS activated platelets via TLR4, such as in severe sepsis, induce neutrophil activation and their subsequent release of inflammatory compounds, including tumour necrosis factor-α (TNF-α) and IL-6 as well as the formation of neutrophil extracellular traps consisting of extracellular DNA and nuclear proteins that trap and kill free bacteria.[261, 264, 265] Platelets have also been shown to drive pathological events during infection with *Plasmodium spp*. parasites (the causative agents of malaria) by promoting sequestration and killing of infected red blood cells.[262, 266] Platelets also actively aid in combating viruses, through phagocytosis of viral particles, which is mediated by either the indirect internalization of virus/IgG complexes by the FcγRIIA or through the direct capture of viruses by platelet receptors, such as the capture of HIV-1 by platelet DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, CD209) and CLEC-2 (C-type lectin receptor) receptors.[267, 268]

An abundant amount of evidence now points to the role of platelets in modulating both the innate and adaptive arms of the immune system. Besides their above mentioned participation in killing foreign pathogens, platelets and platelet derived microparticles focus the complement system (consisting of a series of proteases and inhibitors that are activated in a cascade-like fashion during host defence) to sites of vascular injury and inflammation.[269] In addition, secreted chemokines and cytokines (RANTES, IL-1β and MCP-1), as well as P-selectin expression by platelets, favour recruitment, adhesion and transmigration of leukocytes at sites of vascular injury. On the other hand, platelets promote cytotoxic T lymphocyte (CTL) mediated antiviral immune response during infection with the hepatitis B virus, where platelet activation has been shown to be necessary for the accumulation of virus-specific CTLs at the site of hepatic inflammation.[270] Moreover, CD40L expressed on activated platelets not only induces an

inflammatory response in the endothelium, but also mediates activation of antigen presenting cells (dendritic cells [DC] and macrophages), leading to enhanced antigen presentation to T lymphocytes. CD40L on activated platelets can also mediate B cell differentiation and Ig class-switching as demonstrated in virus infected CD40L deficient mice.[5, 6] Furthermore, patients with immune thrombocytopenia have deficiencies in regulatory T (T_{Reg}) cells, and therapeutical increase in platelet counts restores T_{Reg} cell numbers and functions in these individuals.[271-274] Whether this is due to TGFβ secreted by platelets is still unclear, but given that T_{Reg} cell differentiation requires TGFβ, it is possible that platelets contribute to T_{Reg} formation.

Finally, platelets also have important inflammatory functions. This is not only reflected by their contribution to the local inflammatory environment during atherosclerotic lesion formation, but also to the development of different pathological diseases, such as rheumatoid arthritis. Platelet derived microparticles have been shown to be present in joint fluid from patients with rheumatoid arthritis.[275] This was demonstrated to be dependent on the interaction of platelet GPVI with fibroblast-like synoviocytes in the joint, leading to platelet shedding of microparticles. In turn, activated platelets as well as platelet derived microparticles induce an IL-1 dependent inflammatory response in fibroblast-like synoviocytes that is characterized by the release of pro-inflammatory cytokines and chemokines, such as IL-8, which promote neutrophil recruitment and activation within the joint.[275]

Chapter 2

The CD40/CD40L Axis

2.1 The CD40/CD40L dyad

Since its initial discovery more than two decades ago, the CD40/CD40L dyad has gained much attention in the scientific community. The pivotal role of CD40/CD40L dyad in immunity was initially evidenced by the finding that patients suffering from the X-linked hyper-IgM syndrome (HIGM) are characterized by mutations in their CD40L gene, resulting in loss of function in the CD40L protein. The resulting inactive CD40L protein is unable to induce T cell dependent B cell responses, which are characterized by severe defects in humoral immunity, as well as the absence of IgG, IgA, and IgE antibodies due to a lack of B cell Ig isotype switching.[276] Now it is clear that aside from its importance for appropriate immune responses, the CD40/CD40L dyad has a much broader cell expression pattern and it is associated with diverse physiological and pathological processes.

2.1.1 Structure of CD40

Human CD40 is a type I transmembrane protein of 48 kDa belonging to the TNF receptor superfamily.[277, 278] It was initially identified on B lymphocytes by antibody binding, then cloned in the Burkitt lymphoma Raji cell line, revealing a cDNA encoding for a 1.5 kb mRNA.[279, 280] The gene encoding CD40 was then localized to region q12-13.2 of human chromosome 20, which includes 9 exons.[281, 282] Transcription of the human CD40 gene results in a 277 amino acid membrane-bound protein that consists of a 22 amino acid leader sequence, a 171 amino acid extracellular domain, a single 22 amino acid transmembrane domain, and a 62 amino acid cytoplasmic tail.[1] A homology exists between CD40 and other members of the TNF receptor family, in which the C-terminal domain of CD40 is intracellular, while its N-terminal domain is extracellular. In addition, the extracellular domain of CD40 is characterized by a cysteine-rich repetitive pattern consisting of 20 cysteines, which form four subdomains composed of a combination of two cysteine modules (A1, A2, B1, and B2) (Figure 2.1).[278, 283]

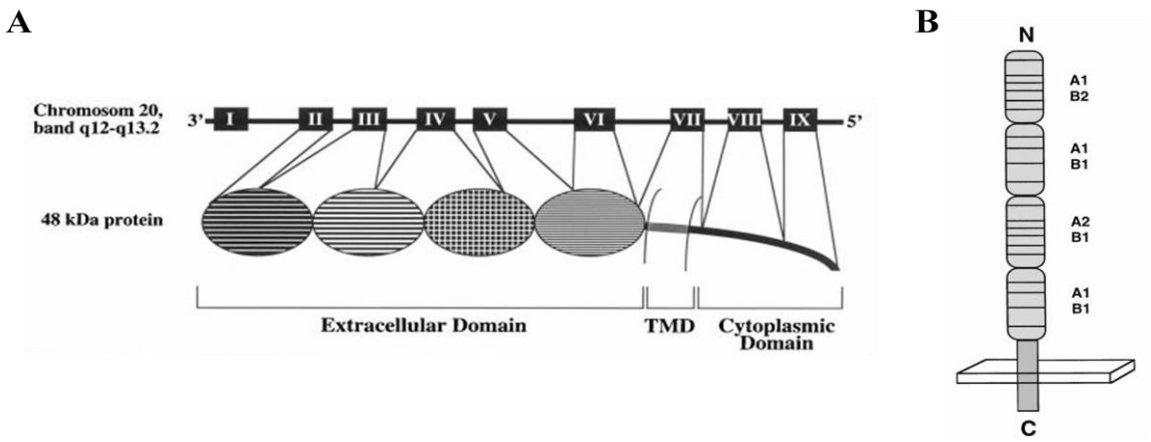

Figure 2.1: Human CD40 gene and protein structure. A) Structure of the CD40 on the human chromosome 20, region q12-q13.2, including the 9 exons, which encode for the leader sequence (exon I), the extracellular domain (exons II - VI), transmembrane domain (TMD; exon VII), and cytoplasmic domain (exons VIII - IX). Schonbeck U. et *al*. *Cellular and Molecular Life Sciences*. 2001; 58:4-43. B) Schematic representation of the extracellular domain of CD40 containing the 20 cysteine residues (horizontal lines) forming four cystein-rich subdomains composed of a combination of two cysteine modules (A1, A2, B1, and B2). The N-terminal region of CD40 is extracellular, while the C-terminal region is intracellular. Van Kooten C. et *al*. *Journal of Leukocyte Biology*. 2000; 67:2-17.

It remains unclear whether CD40 molecules are arranged as monomers or multimers on the surface of the cell membrane. Certain reports indicate the formation of CD40 dimers on the cell surface, while others point to the constitutive trimer composition of the receptor, which probably favours its interaction with its trimeric ligand, CD40L.[284-286] It seems that the monomeric form of CD40 on the cell surface, which then forms trimers following ligation with CD40L is probably the most credible arrangement, given that trimeric CD40L molecules exhibit a higher potency than the monomeric or dimeric forms (discussed in more detail in section 2.1.3).[287, 288]

2.1.2 Structure of CD40L

Human CD40L (also known as CD154, gp39, TBAM, and TRAP) is a type II transmembrane protein belonging to the TNF superfamily.[289] The human CD40L cDNA was initially identified by screening activated human peripheral blood T lymphocytes with the murine CD40L probe, which revealed a 13 kb DNA sequence that was later mapped to the region q26.3-q27.1 of chromosome X.[289-291] This DNA sequence contains five exons, of which exons II - V mainly encode the extracellular domain, while exon I encodes the transmembrane and the cytoplasmic domains of CD40L (Figure 2.2).[287, 292] Transcription of the human CD40L gene results in a 261 amino acid membrane-bound protein that consists of a 215 amino acid extracellular domain, a

single 24 amino acid transmembrane domain, and a 22 amino acid cytoplasmic tail. Unlike CD40, the N-terminal of the CD40L protein is intracellular, while its C-terminal is extracellular. On the other hand, the amino acid backbone of CD40L predicts a protein of 29 kDa; however, CD40L is identified as a 39 kDa protein, which suggests that posttranslational modifications convey its actual molecular mass.[278] In addition to the 39 kDa membrane bound full-length form of CD40L, soluble forms of the ligand have been described with molecular weights of 31, 18, and 14 kDa, of which the 18 kDa form is mainly derived from enzymatic cleavage of CD40L expressed on activated platelets.[7, 293-295]

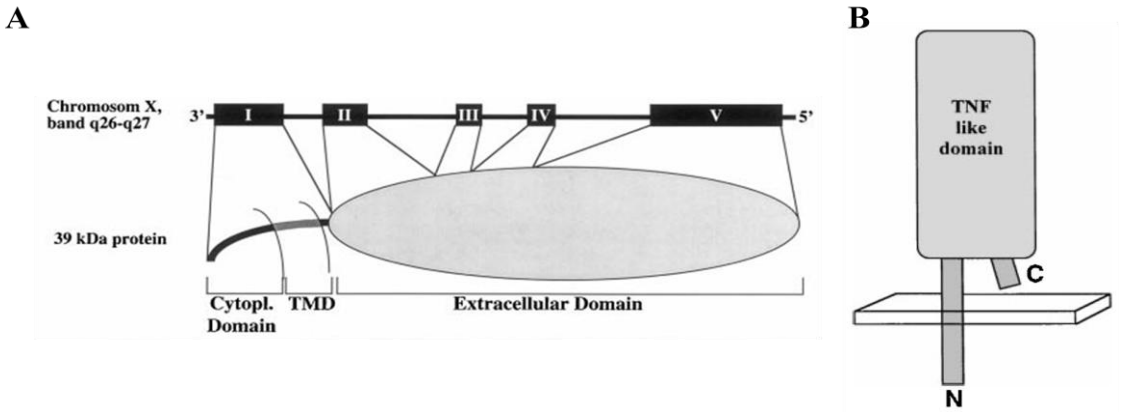

Figure 2.2: Human CD40L gene and protein structure. A) Structure of the CD40L on the human chromosome X, region q26.3-q27.1, including the 5 exons, which encode the extracellular domain (exons II - V), transmembrane domain (TMD; exon I), and cytoplasmic domain (exon I). Schonbeck U. et *al*. *Cellular and Molecular Life Sciences*. 2001; 58:4-43. B) Schematic representation of CD40L with its extracellular C-terminal region and intracellular N-terminal region. Van Kooten C. et *al*. *Journal of Leukocyte Biology*. 2000; 67:2-17.

Despite being a type II transmembrane protein, CD40L is expressed on the cell surface as a trimeric complex.[296] This multimeric conformation of CD40L is of crucial importance for its effective interaction with CD40 and the subsequent intracellular signalling induced by the latter.[286] Moreover, the soluble forms of CD40L also retain their ability to form trimers, which could bind CD40 and deliver biological signals.[293, 294, 297]

2.1.3 CD40/CD40L interactions

The X-ray crystal structure of CD40L, as well as mutagenesis analysis of both CD40 and CD40L, allowed the identification of the residues implicated in stabilizing the interaction between these two molecules. The polar interaction between the charged residues, with CD40L presenting the basic chains K143, R203, and R207, while CD40 presenting the acidic side chains

D84, E114, and E117, favour ligation of the receptor/ligand dyad. In addition, the CD40/CD40L complex is further stabilized by a wall of hydrophobic residues surrounding the interacting groups.[298-300]

As mentioned above, there is a discrepancy regarding the arrangement of CD40 on the cell surface. Whether CD40 is present as a monomer or trimer on the cell surface gave rise to two hypothetical models of CD40/CD40L interactions. The first model, in which trimers of CD40L interact with constitutive CD40 trimers that form the pre-ligand-binding assembly domains (PLAD) (Figure 2.3B), gained much attention within the scientific community. Given that CD40 is a member of the TNF receptor family, which are pre-assembled in a trimeric composition on the cell membrane, supports this model of interaction.[285] On the other hand, a second school of thought favours the ligand-induced receptor trimerization model, in which trimeric molecules of CD40L induce oligomerization of the CD40 receptor (Figure 2.3A). This model is probably the most logical scenario of CD40/CD40L interactions, since the CD40 intracellular adapter proteins have been shown to form homotrimers or heterodimers and to associate with the receptor following its oligomerization, which is due to their increased avidity for the cytoplasmic domain of the trimeric receptor.[301-303] Moreover, different degrees of antibody-induced cross-linking of CD40 have been shown to result in different cellular responses, which are probably due to different levels of affinity of the CD40 intracellular adapter proteins to the receptor.[288, 304] Finally, it has been shown that trimeric molecules of CD40L exert a more potent biological response than their monomeric or dimeric counter parts, which is probably due to different degrees of ligand-induced receptor oligomerization.[288] Thus, there is ample amount of evidence supporting the ligand-induced receptor trimerization model of CD40/CD40L interactions.

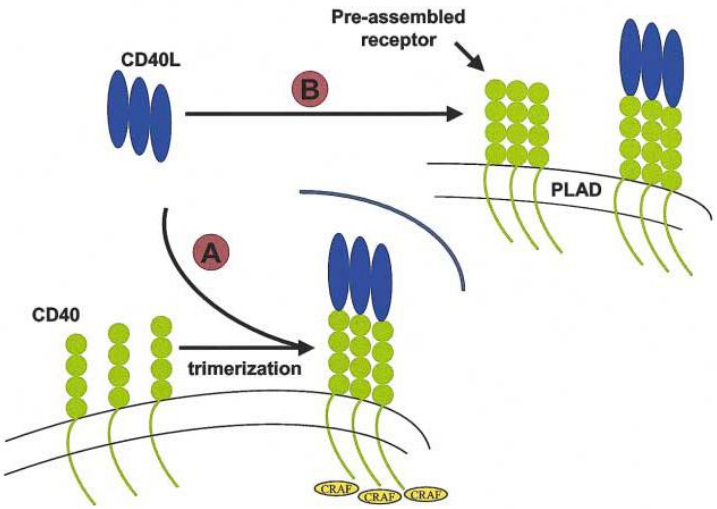

Figure 2.3: Models of CD40/CD40L interaction. A) Timeric CD40L-induced CD40 trimerization interaction model. (CRAF: CD40 associated factors. They signify the old nomenclature for the CD40 intracellular adapter proteins). B) Model of trimeric CD40L interaction with the pre-assembled CD40 trimer. (PLAD: pre-ligand-binding assembly domain). Anand S. X. et *al*. *Thrombosis and Haemostasis*. 2003; 90:377-384.

2.1.4 Alternative CD40L receptors

Besides CD40, the dedicated receptor, CD40L has been shown to bind three other receptors, namely the $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, and Mac-1 ($\alpha_M\beta_2$) integrins (Figure 2.4). Interaction of CD40L with the $\alpha_{IIb}\beta_3$ integrin was first identified in platelets. Although the $\alpha_{IIb}\beta_3$ integrin mainly binds ligands (such as fibrinogen) with a RGD motif, the discovery of a snake venom that is capable of binding the integrin via a KGD motif, shed light into the possibility that the KGD motif within CD40L could favour its interaction with $\alpha_{IIb}\beta_3$ on platelets.[305, 306] Indeed, CD40L was shown to stabilize *in vivo* thrombus formation by promoting platelet activation through an interaction with the $\alpha_{IIb}\beta_3$ integrin.[4] This interaction was later shown to induce tyrosine phosphorylation within the cytoplasmic tail of the β_3 subunit of the integrin, which ultimately mediates "outside-in" signals by the latter (see section 1.4.4 of chapter 1).[307] However, the CD40L/$\alpha_{IIb}\beta_3$ interaction requires an active conformation of the integrin, because $\alpha_{IIb}\beta_3$ is unable to bind its ligands in its inactive conformation.

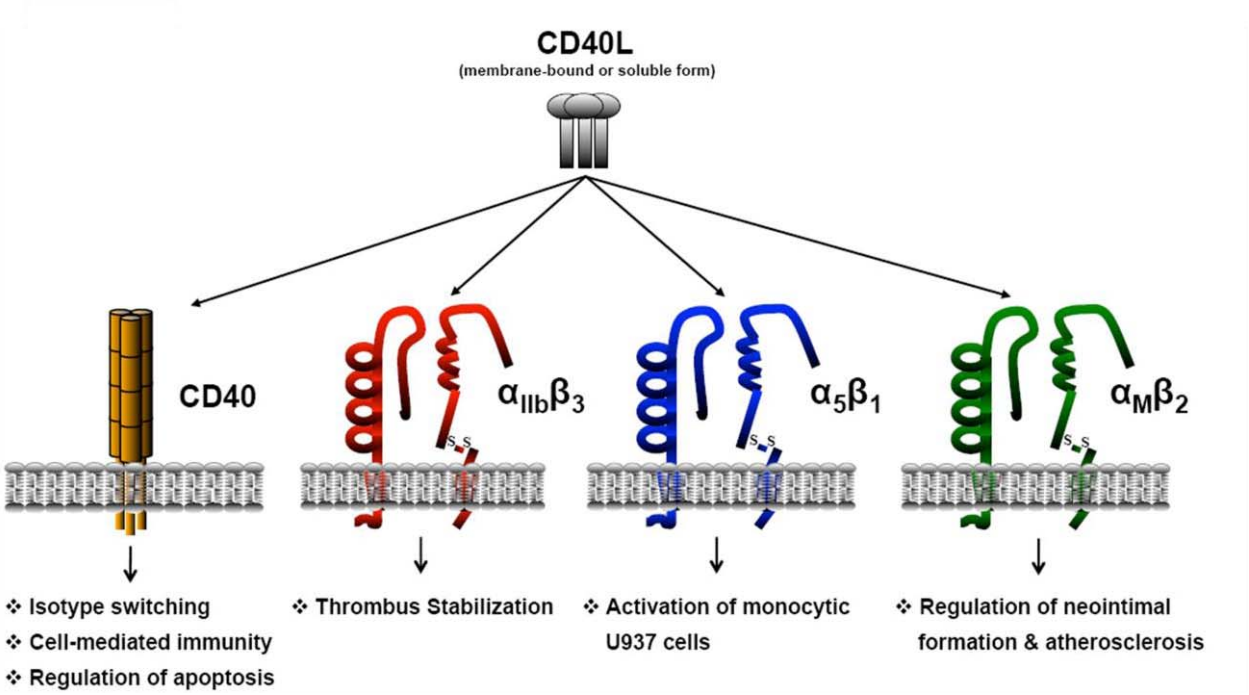

Figure 2.4: CD40L and its receptors. Binding of CD40L to CD40, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, or Mac-1 ($\alpha_M\beta_2$) induces different biological responses. Gaxiola E. (2012). *Atherothrombosis*. INTECH. p. 82.

The fibronectin receptor, $\alpha_5\beta_1$, has recently been shown to be one of the CD40L receptors. This was demonstrated in the U937 human monocytic cell line, which lacks both CD40 and $\alpha_{IIb}\beta_3$ expression, where inhibition of either CD40L or $\alpha_5\beta_1$ abolished sCD40L binding to the cell surface, as well as sCD40L-induced cell activation.[308] Interestingly, sCD40L was also shown to only interact with the $\alpha_5\beta_1$ integrin in its inactive conformation, and activation of the integrin prevents this interaction. Moreover, point mutations of residues within CD40L, which are involved in its interaction with either CD40 or $\alpha_{IIb}\beta_3$, revealed that CD40L is capable of binding $\alpha_5\beta_1$ independently of its interaction with either CD40 or $\alpha_{IIb}\beta_3$.[309] Therefore, it seems that CD40L is capable of simultaneously binding multiple receptors. Whether this is the case in cells that express more than one CD40L receptor, such as platelets (CD40, $\alpha_{IIb}\beta_3$, and $\alpha_5\beta_1$), remains to be explored.[4, 308, 310, 311]

Finally, Mac-1, also known as $\alpha_M\beta_2$, is a highly promiscuous receptor capable of binding multiple ligands, including GPIbα, fibrinogen, and vitronectin. It is also an important mediator of neutrophil and monocyte adhesion to the activated endothelium during inflammation. Recently, it has been demonstrated that Mac-1 is another receptor for CD40L, which is involved in mediating CD40L/Mac-1-dependent monocyte and neutrophil adhesion and transmigration at the

atherosclerotic lesion site, as well as neointimal formation during atherogenesis.[312, 313] Furthermore, binding of CD40L to Mac-1 requires an active conformation of the integrin.

2.2 Cellular expression and function of the CD40/CD40L dyad

As mentioned earlier, the CD40/CD40L dyad was initially identified on cells of the immune system, specifically on B and T lymphocytes. Now, it is well known that these two molecules have a much broader cell expression pattern, encompassing not only cells of the immune system, but also cells of the circulatory and vascular systems (Table 2.1). The intricate role of this dyad in these systems highlights its crucial function in inflammation and immunity (Table 2.2).

Table 2.1: Cell types expressing the CD40/CD40L dyad. (N/A: no documentation available, - : no expression, + : weak expression, ++ : average expression, +++ : strong expression). Modified from Lievens D. et *al*. *Thrombosis and Haemostasis*. 2009; 102:206-214.

Cell Type	CD40		CD40L		Forms of CD40L
	Resting	Activated	Resting	Activated	
CD4+ T lymphocytes	-	+++	-	+++	Membrane, soluble
B lymphocytes	+++	+++	++	+++	Membrane
Macrophages	+	+++	+	+++	Membrane
Platelets	++	+++	-	+++	Membrane, soluble
Dendritic cells (DCs)	-	+++	++	+++	Membrane
Neutrophils	+	+++	+	+	Membrane
Endothelial cells	+	+++	+	+	Membrane
SMCs	+++	N/A	+	N/A	Membrane

2.2.1 B lymphocytes

The CD40/CD40L dyad has a crucial role in B lymphocytes-mediated humoral immunity. B lymphocytes constitutively express CD40, and ligation of this receptor with CD40L expressed on activated T lymphocytes, in addition to secreted cytokines by the latter, induces proliferation, differentiation, and Ig production of B lymphocyte. CD40 activation guides B lymphocytes throughout their differentiation program, initiated by pre-B lymphocytes activation and differentiation into naive B lymphocytes, which in turn undergo a maturation process, thereby forming germinal center cells. Further engagement of CD40L on activated T lymphocyte with CD40 on germinal center cells induces their proliferation and differentiation into plasma cells

(antibody producing cells) and memory B lymphocytes (Figure 2.5).[278, 314] However, the presence of the IL-2 and IL-10 cytokines is also required for the differentiation of germinal center cells into memory B lymphocytes. In addition, ligation of CD40 on germinal center and memory B lymphocytes induces re-expression of telomerase activity, thereby contributing to the expanded life-span of these cells.[315] On the other hand, activation of CD40 on memory B lymphocytes induces Fas expression, which increases their susceptibility for Fas-mediated apoptosis (process of bystander B lymphocyte elimination), an effect that is prevented by engagement of the B cell antigen receptor (BCR) in the presence of IL-4.[316, 317] Interestingly, activated B lymphocytes have been shown to express CD40L, which is capable of inducing CD40 activation on adjacent B lymphocytes in a similar way as CD40L expressed on activated T lymphocytes.[318]

CD40 activation favours B lymphocytes proliferation and differentiation by increasing their expression of co-stimulatory molecules (VCAM-1, ICAM-1, lymphocyte function-associated angtigen-1 [LFA-1], CD23 [FcεRII], CD80 [B7.1], and CD86 [B7.2]) and their release of cytokines (IL-6, IL-10, TNF-α, TGF-β, and lymphotoxin-α [LT-α]).[1, 319-325] CD40 engagement also increases surface expression of the major histocompatibility complexes-I and -II (MHC-I and MHC-II) on B lymphocytes, thereby improving their antigen presenting function.[326]

Finally, CD40 activation is responsible for B lymphocyte Ig isotype class switching, a process absent in patient suffering from HIGM, in whom there is accumulation of circulating IgM antibodies.[276] Although activation of CD40 on B lymphocytes is sufficient to induce the switch to IgA and IgG, the switch to IgE and IgG4 requires the IL-4 and IL-13 cytokines.[1, 278, 327] Additional cytokines, namely IL-7, IL-2, IL-10, and TGF-β, fine-tune and enhance Ig isotype switching and production.[327-329]

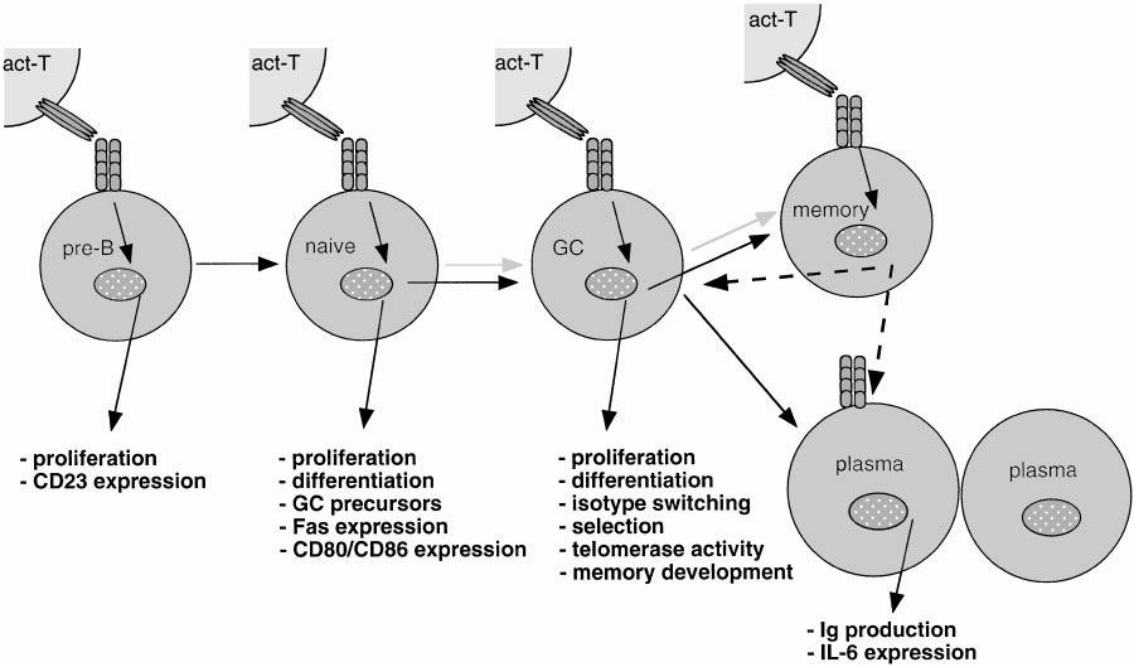

Figure 2.5: Effects of CD40 activation on different stages of B lymphocyte differentiation. CD40L-expressing T lymphocytes interact and modulate all stage of B lymphocyte differentiation. Activation of CD40 on memory B lymphocytes can either lead to a new round of germinal center reaction and/or an accelerated differentiation into plasma cells. (act-T: activated T lymphocyte; pre-B: pre-B lymphocyte; GC: germinal center cell). van Kooten C. et *al. Journal of Leukocyte Biology*. 2000; 67:2-17.

2.2.2 T lymphocytes

Efficient T lymphocyte activation requires two signals. The first antigen-specific signal is provided by the T-cell receptor (TCR), which interacts with a specific antigen presented on MHC molecules of APCs, such as dendritic cells, B lymphocytes, and macrophages. This first signal induces the expression of the co-stimulatory molecules CD28 and CD40L on T lymphocytes, which in turn interact with B7.1 or B7.2 and CD40 on APCs, respectively. This provides the second antigen non-specific signal that is required for effective T lymphocyte activation and maturation into effector T lymphocytes.[1, 330] This second co-stimulatory signal is necessary for T lymphocyte proliferation, differentiation, and survival, since its absence leads to T lymphocyte anergy, T lymphocyte deletion or the development of immune tolerance.[331-334] Moreover, interaction of CD40L on T lymphocytes with CD40 on APCs induces proliferation, differentiation, and activation of the APCs, as well as their up-regulation of the MHC-I and MHC-II, and the co-stimulatory molecules B7.1 and B7.2 (see section 2.2.3). Therefore, the CD40/CD40L dyad provides a bidirectional signalling mechanism that not only activates, but also amplifies the activation of both T lymphocytes and APCs. Furthermore, T lymphocyte

membrane-bound CD40L is cleaved by a disintegrin and metalloproteinase domain-containing protein-10 (ADAM-10), generating sCD40L, which accounts for a minor proportion of circulating sCD40L, given that platelets generate most of the sCD40L within the circulation.[7, 335]

Interestingly, T lymphocytes also express CD40 upon activation; however, its exact role remains unclear.[336] It seems that, like B lymphocytes, engagement of CD40 on $CD8^+$ T lymphocytes with CD40L on $CD4^+$ lymphocytes favours $CD8^+$ memory T lymphocytes generation.[337]

2.2.3 Dendritic cells

Dendritic cells are APCs by profession, which have a central role in linking innate and adaptive immunity. They are sentinels of the innate immune system that immediately react to potential pathogens and modulate the adaptive immune system by capturing, processing, and presenting antigens on MHCs to antigen-specific T lymphocytes. Immature DCs are capable of efficiently up-taking antigens, but they express low levels of MHC and co-stimulatory molecules, such as CD40, B7.1 and B7.2. However, upon microbial challenge-induced maturation, DCs up-regulate their expression of the MHC/antigen complexes, as well as the co-stimulatory molecules CD40, B7.1, and B7.2, thereby increasing their ability to stimulate naive T lymphocytes.[330, 338, 339] Ligation of CD40L on activated T lymphocytes with CD40 on mature DCs provides a bidirectional signalling mechanism that stimulates efficient T lymphocyte activation, proliferation, and differentiation, and amplifies DC function by increasing their surface expression of co-stimulatory and MHC molecules.[338, 340] Moreover, continuous stimulation of CD40 on DCs induces their release of IL-12, which in combination with CD40L stimulation on T lymphocytes drives a potent T helper 1 (T_{h1}) immune response.[341-343]

DCs also express CD40L at levels comparable to those on activated T lymphocytes. In addition, ligation of CD40 on DCs further increases their surface expression of CD40L.[344] Although the function of CD40L on DCs is not as well characterized as that of CD40, it seems to be involved in mediating activation of CD40 on B lymphocytes and adjacent DCs. This activation favours B lymphocyte activation and differentiation, as well as IL-12 production by DCs.[345, 346]

2.2.4 Monocytes/macrophages

Monocytes constitutively express CD40, and given their APC nature, they modulate T lymphocyte activation through a CD40/CD40L interaction. Ligation of CD40L on activated T lymphocytes with CD40 on monocytes provides a bidirectional signalling mechanism that stimulates efficient T lymphocyte activation and proliferation, as well as monocyte differentiation into macrophages. CD40 ligation on monocytes/macrophages also induces their up-regulation of co-stimulatory molecules (ICAM-1, LFA-3, B7.1 and B7.2), and their release of cytokines (IL-1β, IL-6, IL-8, IL-12, and TNF-α), MMPs (MMP-1, MMP-2, MMP-3, and MMP-9), and TF.[347-353] All of which are main mediators involved in the inflammatory, immune, and angiogenic functions of monocytes/macrophages.

Aside from CD40, monocytes also express Mac-1 and $\alpha_5\beta_1$, which are also CD40L receptors. Recruitment and transmigration of monocytes at the site of vascular lesion has been shown to be dependent on Mac-1/CD40L interactions, which also induce myeloperoxidase secretion by monocytes.[313] On the other hand, $\alpha_5\beta_1$/CD40L interactions induce monocyte activation and their subsequent IL-8 production.[308] Finally, upon activation, monocytes not only up-regulate CD40 expression, but also that of CD40L.[354] Although the exact function of CD40L expressed on monocytes is still unclear, it seems to be involved in the up-regulation of B7.2 expression on B lymphocytes, as well as atherosclerotic plaque progression and allograft rejection.[354, 355]

2.2.5 Neutrophils

Neutrophils are the most abundant leukocytes within the circulation, and they are among the first immune cells to be recruited to the site of inflammation, where they occupy an indispensable role in host defence.[356, 357] CD40 was initially identified on neutrophils through assessment of neutrophil priming by sCD40L within stored blood pools.[358] Neutrophil CD40 was later shown to mediate platelet/neutrophil interactions by interacting with platelet CD40L. This CD40/CD40L interaction influences both cell types, where activated platelets release sCD40L, which binds CD40 on neutrophils and induces their production of ROS. In its turn, ROS mediates stimulation of additional platelets, thereby creating a positive feedback loop.[359] Platelet sCD40L also enhances Mac-1 expression on neutrophils, and elevated levels of sCD40L (mainly

platelet derived) have been shown to promote platelet/neutrophil aggregates and neointima formation following arterial injury.[312]

2.2.6 Platelets

As mentioned in the previous chapter, platelets are not only recognized for their physiological haemostatic role, but also for their contribution in inflammation, as well as their modulation of both the innate and adaptive branches of the immune system. Since the discovery of the CD40/CD40L dyad in platelets, platelet CD40L has gained significant attention within the scientific community, given the platelet origin of 95% of circulating sCD40L. However, the amount of information regarding the role of platelet CD40 is scarce. Chapter three is dedicated to describe the role of the CD40/CD40L dyad in platelet function; therefore, this section will provide a brief overview.

In resting platelets CD40 is constitutively expressed on the cell surface, while CD40L is translocated to the surface following activation.[2] CD40L expressed on activated platelets has been shown to interact with CD40 on endothelial cells and monocyte, and to mediate an inflammatory reaction in these cells, which is characterized by the up-regulation of adhesion molecules (E-selectin, ICAM-1, and VCAM-1), and the secretion of chemokines (MCP-1), cytokines (IL-6, and IL-8), and MMP-9.[2, 3] Platelet CD40L has also been shown to stabilize *in vivo* thrombus formation by promoting platelet activation through an interaction with the $\alpha_{IIb}\beta_3$ integrin.[4, 307] On the other hand, ligation of CD40 on platelets leads to the generation and secretion of ROS and RANTES, in addition to being a requirement for CD40L cleavage and release to its soluble form.[3, 360, 361]

2.2.7 Endothelial cells

The CD40/CD40L dyad plays an important role in the inflammatory, angiogenic, and pro-coagulant functions of endothelial cells. Although both CD40 and CD40L are expressed on endothelial cells, the biological repercussions of this dyad on these cells predominantly stem from CD40 activation following leukocyte (monocyte, lymphocyte, and neutrophil) and platelet adhesion to the activated endothelium. CD40 activation mediates an inflammatory phenotype in endothelial cells by inducing the expression of adhesion molecules (E-selectin, VCAM-1, and ICAM-1), and the secretion of chemokines and cytokines (IL-1, IL-6, IL-8, MCP-1, MIP-1α, and

RANTES), all of which promote monocytes and lymphocytes recruitment at the site of lesion. [2, 354, 362-367]

Endothelial cells actively participate in the angiogenic process, which is greatly influenced by the CD40/CD40L dyad. Interaction of CD40L with CD40 on endothelial cells induces the synthesis and release of extracellular matrix degrading enzymes (MMP-1, MMP-3, and MMP-9), which are essential for the infiltration of endothelial cells through the extracellular matrix during angiogenesis.[368] Moreover, CD40 activation on endothelial cells has been shown to favour their proliferation by inducing the secretion of endothelial growth factors, including VEGF, FGF, and PAF, which are potent angiogenic agents.[369-371]

Finally, activation of CD40 on endothelial cells favours their pro-coagulant activity, which is manifested by TF generation and secretion.[372, 373] TF on its turn promotes activation of the coagulation cascade, and the subsequent platelet activation.

2.2.8 Smooth muscle cells

Vascular smooth muscle cells express both CD40 and CD40L; however, little information is available regarding the role of this dyad in the function of these cells.[354] Ligation of CD40 on vascular SMCs has been shown to mediate their release of the IL-8 and MCP-1 chemokines through the activation of mitogenic signalling pathways, namely the Src family-kinase pathway, which lead to the activation of p38 mitogen-activated protein kinase (MAPK), and the extracellular signal-regulated protein kinases (Erk) 1 and 2.[374] In addition, ligation of CD40 on vascular SMCs activates the IL-1β converting enzyme (caspase-1), and induces IL-6 release.[354, 375] Finally, CD40 activation promotes SMC proliferation, migration and neointimal formation, as well as the pro-atherogenic and pro-thrombotic functions of vascular SMCs by favouring the release of matrix degrading enzymes (MMP-1, MMP-3, and MMP-9), and TF.[376-380]

Table 2.2: Cell type specific CD40/CD40L-induced signalling.

Receptor	Induced by	Cell type	Effect
CD40	CD40L	B lymphocyte	- Isotype switching - Expression of MHC-I, MHC-II, VCAM-1, ICAM-1, LFA-1, FcεRII, B7.1, B7.2, and Fas - Cytokine production (IL-6, IL-10, TNF-α, TGF-β, and LT-α)
		Monocyte/ Macrophage	- Up-regulation of co-stimulory activity (ICAM-1, LFA-3, B7.1, and B7.2) - Myeloperoxidase secretion - Cytokine secretion (IL-1β, IL-6, IL-8, IL-12, and TNF-α) - MMP secretion (MMP-1, MMP-2, MMP-3, and MMP-9) - TF secretion
		Platelet	- Platelet activation - RANTES and ROS secretion
		Neutrophil	- ROS production - Enhanced Mac-1 expression
		DC	- Cytokine secretion (IL-10 and IL-12) - Up-regulation of co-stimulory activity (CD40, CD40L, MHC-I, MHC-II, B7.1, and B7.2)
		Endothelial cell	- Up-regulation of adhesion molecules (E-selectin, VCAM-1, and ICAM-1) - Chemokines and cytokines secretion (IL-1, IL-6, IL-8, MCP-1, MIP-1α, and RANTES) - ROS production - MMP secretion (MMP-1, MMP-3, and MMP-9) - Growth factor secretion (VEGF, FGF, and PAF) - TF secretion - Up-regulation of COX-2
		SMC	- Chemokines and cytokines secretion (IL-1β, IL-6, IL-8, and MCP-1) - MMP secretion (MMP-1, MMP-3, and MMP-9) - TF secretion - Up-regulation of COX-2
		T lymphocyte	- $CD8^+$ memory T lymphocyte generation
CD40L	CD40	T lymphocyte	- Activation of T lymphocyte

2.3 CD40 intracellular signalling

The CD40/CD40L dyad exerts its biological function on the above mentioned different cell types by activating specific intracellular signalling pathways. Although both members of the dyad are capable of inducing intracellular signals, CD40 has been regarded as the main signalling receptor following its interaction with CD40L. Despite the extensive amount of work performed on understanding the signalling mechanisms downstream of CD40, there is much controversy and the generated information is still incomplete. The discrepancies result from differences in CD40 mediated signal transduction among different cell types, as well as between the different stages of differentiation within the same cell type. Nonetheless, culmination of the knowledge regarding CD40 mediated signals lead to the identification of TNF receptor associated factor (TRAF)-dependent and -independent signalling mechanisms. The cytoplasmic domain of CD40 lacks intrinsic kinase activity, and therefore utilizes members of the TRAF family as adaptor proteins to mediate signalling events, which account for the majority of the signals generated by CD40 activation. Following their recruitment to the cytoplasmic domain of CD40, TRAFs thereafter induce activation of multiple signalling pathways, including the canonical and non-canonical nuclear factor-κB (NF-κB) (see section 2.3.2), the MAPKs (p38 MAPK, Erk1/2, Akt, and c-jun N-terminal kinases [JNKs]), the PI3K, and the PLCγ pathways.[381] On the other hand, the TRAF-independent signalling pathway induces activation of the signal transducer and activator of transcription 5 (STAT5) through the direct interaction of the janus kinase 3 (JAK3) with the cytoplasmic tail of CD40 following its ligation.[382, 383] Figure 2.6 summarizes the main CD40-mediated signalling pathways induced by the different TRAF members, as well as JAK3.

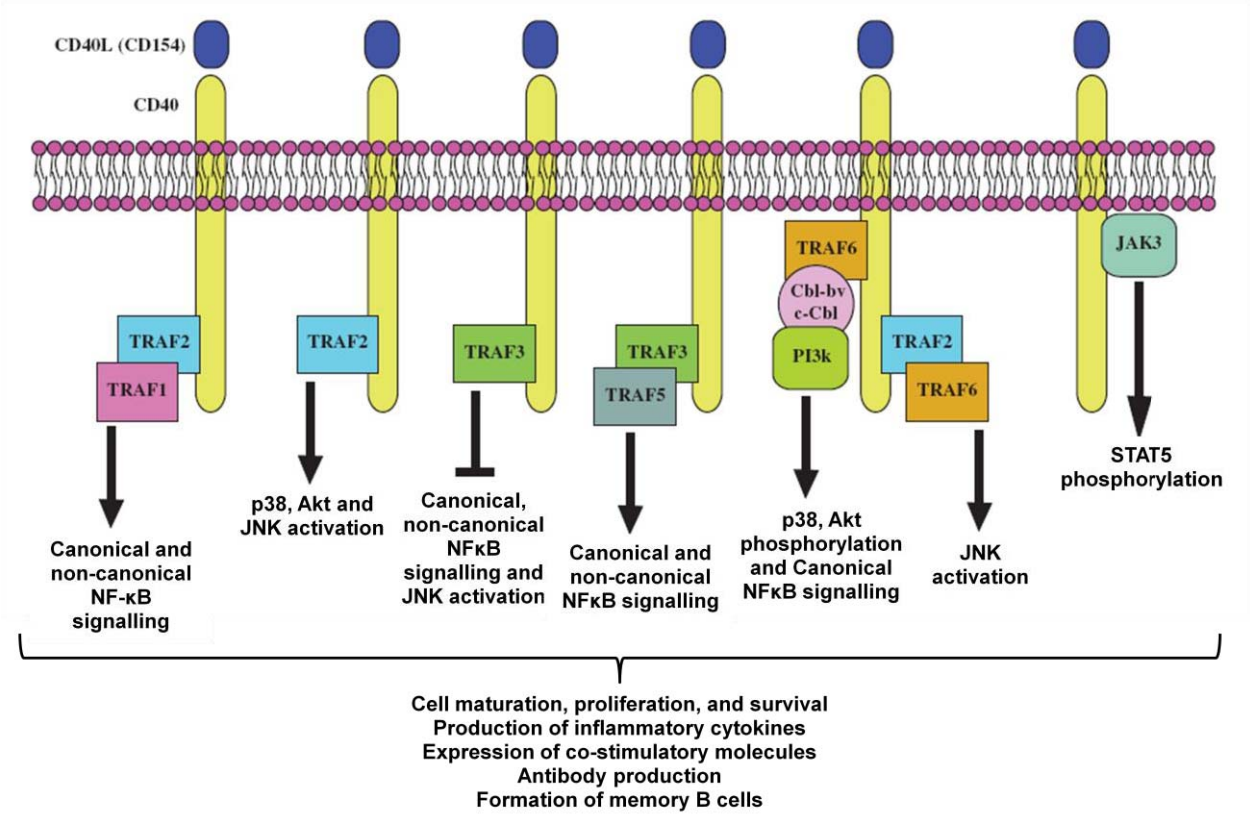

Figure 2.6: The different CD40-mediated signalling pathways. Following CD40 activation, TRAF members are recruited to its cytoplasmic tail. Each TRAF member then induces activation of a distinct signalling pathway; however, some signalling pathways are activated by multiple TRAF members. JAK3 interacts with the cytoplasmic tail of CD40, and induces activation of the TRAF-independent pathway. These signals regulate different cellular processes. Modified from Elgueta R. et *al*. *Immunological Reviews*. 2009; 229:152-172.

2.3.1 Structure and function of TRAFs

Up to date seven members of the TRAF family have been identified, including TRAF1 to TRAF7. These TRAF members have similar structures, which are formed by a TRAF-C domain (WD40 domain repeats in TRAF7) at the C-terminal, followed by a leucine rich coiled-coil domain, one or multiple zing-finger domains, and a RING domain (except TRAF1) at their N-terminal (Figure 2.7).[381, 384] The TRAF-C domain of TRAFs 1 to 6 is involved in their oligomerization, as well as their interaction with members of the TNF receptor family, including CD40, while the WD40 domain repeats of TRAF7 are involved in its interaction with MAPK kinase kinase 3 (MEKK3). The coiled-coil domain (a.k.a. TRAF-N) is involved in the homo- or heterotrimerization of TRAF proteins, such as TRAF3/TRAF5 heterotrimerization.[385] The zinc-finger and RING domains are involved in the ubiquitination of TRAF proteins and their subsequent interactions with downstream signalling molecules, such as transcription factors and

kinases. Moreover, as mentioned above, following CD40 ligation, TRAFs are recruited to the cytoplasmic tail of the receptor. This is done either directly or indirectly through the aid of additional TRAF members, as is the case for TRAF5 recruitment, which requires its heteroligomerization with TRAF3. Although each TRAF member has a distinct interaction site on the cytoplasmic tail of CD40, it seems that the interaction site for TRAF1, TRAF2, and TRAF3 overlap.[385]

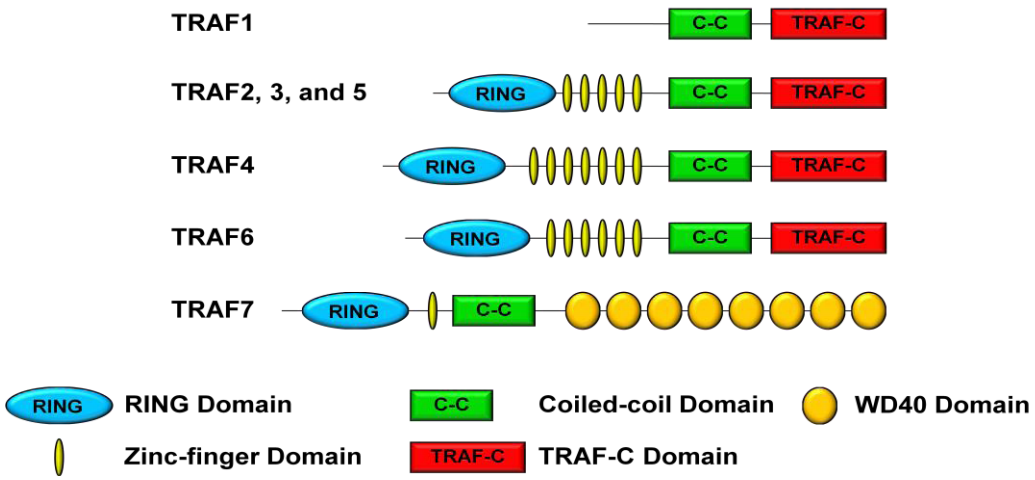

Figure 2.7: Structure of TRAF proteins. Image is produced by Hachem A.

2.3.1.1 TRAF1

TRAF1 was initially identified as a molecule interacting with CD120b (TNF receptor 2).[386] Unlike the other TRAFs, TRAF1 does not have zinc-finger and RING domains; therefore it has an important role in regulating other TRAF proteins, specifically TRAF2. Indeed, absence of TRAF1 in dendritic cells and B lymphocytes results in a reduced recruitment of TRAF2 to the cytoplasmic domain of CD40, as well as an increased TRAF2 degradation.[387, 388] This, suggests that TRAF1 may promote TRAF2-mediated CD40 signals by prolonging membrane localization of TRAF2. On the other hand, absence of TRAF2 results in a weak binding of TRAF1 to the cytoplasmic domain of CD40.[385] Moreover, TRAF1 deficiency in B lymphocytes results in a decrease of CD40-mediated IgM production, whereas TRAF1 and TRAF2 deficient B lymphocytes exhibit reduced canonical NF-κB pathway and JNK activation, and reduced IgM production as compared to cells deficient in either TRAF alone.[388] Interestingly, CD40 activation leads to the up-regulation of TRAF1 protein level, which probably plays a role in the feedback regulation of receptor signalling.[389]

2.3.1.2 TRAF2

TRAF2 was initially identified through its association with the TNF receptor 2.[386] Actually, TRAF2 is known to be responsible for the activation of the p38, Erk1/2, Akt, and JNK MAPKs, as well as the canonical and non-canonical NF-κB pathways following CD40 ligation. These signalling pathways were evidenced in B lymphocytes and embryonic fibroblasts deficient in TRAF2, which showed reduced activation of these pathways upon CD40 engagement.[390-394] Activation of the p38, Erk1/2, and JNK MAPKs does not occur directly by TRAF2, but rather through the indirect recruitment of the protein kinase MEKK1 by TRAF2 to the cytoplasmic tail of CD40. MEKK1 is an essential protein kinase involved in CD40-induced activation of the aforementioned MAPKs following CD40 engagement.[395]

Activation of the canonical NF-κB pathway by TRAF2 was put into evidence in TRAF2 deficient B lymphocytes. However, TRAF2 deficiency does not completely abolish activation of the canonical NF-κB pathway, given that TRAF6 can also activate it (Figure 2.8).[391, 393] Indeed, activation of the canonical NF-κB pathway following CD40 engagement has been shown to be completely abolished in B lymphocytes lacking both TRAF2 and TRAF6 binding to CD40.[391] On the other hand, the exact mechanism by which TRAF2 activates the canonical NF-κB pathway is still unclear. It was initially thought that the nuclear factor-κB-inducing kinase (NIK) was responsible for activating the IκB kinase (IKK), which ultimately mediates canonical NF-κB pathway activation. However, NIK deficiency does not result in a defective CD40 response.[396, 397] Therefore, TRAF2 probably activates IKK through its association with MEKK1, which has already been shown to activate IKK.[398, 399]

Despite its above mentioned activating roles, TRAF2 negatively regulates the non-canonical NF-κB pathway. In the absence of CD40 ligation, TRAF2 interacts with the cellular inhibitor of apoptosis (cIAP) 1 and 2, TRAF3, and NIK.[400, 401] Under these conditions, cIAP1/2 antagonizes the non-canonical NF-κB pathway by inducing degradation of NIK.[402, 403] Indeed, deficiency in either TRAF2 or cIAP results in NIK accumulation in mouse embryonic fibroblasts (MEF).[404] Furthermore, CD40 ligation destabilizes the TRAF2/TRAF3/NIK/cIAP1/2 complex, thereby favouring TRAF2 and TRAF3 recruitment to the cytoplasmic tail of CD40.[381] Once recruited, TRAF2 undergoes auto-degradation, whereas TRAF3 is degraded by cIAP1/2.[403, 405] Degradation of both TRAF2 and TRAF3 induces accumulation of NIK, which, in turn, mediates activation of the non-canonical NF-κB pathway.[381]

In summary, in the absence of CD40/CD40L interactions, TRAF2 acts as a negative regulator of the non-canonical NF-κB pathway activation, whereas TRAF2 mediates activation of both the canonical and non-canonical NF-κB pathways following CD40 ligation. Activation of the canonical and non-canonical NF-κB pathways is responsible for B lymphocyte activation and differentiation into plasma cells.[398, 400]

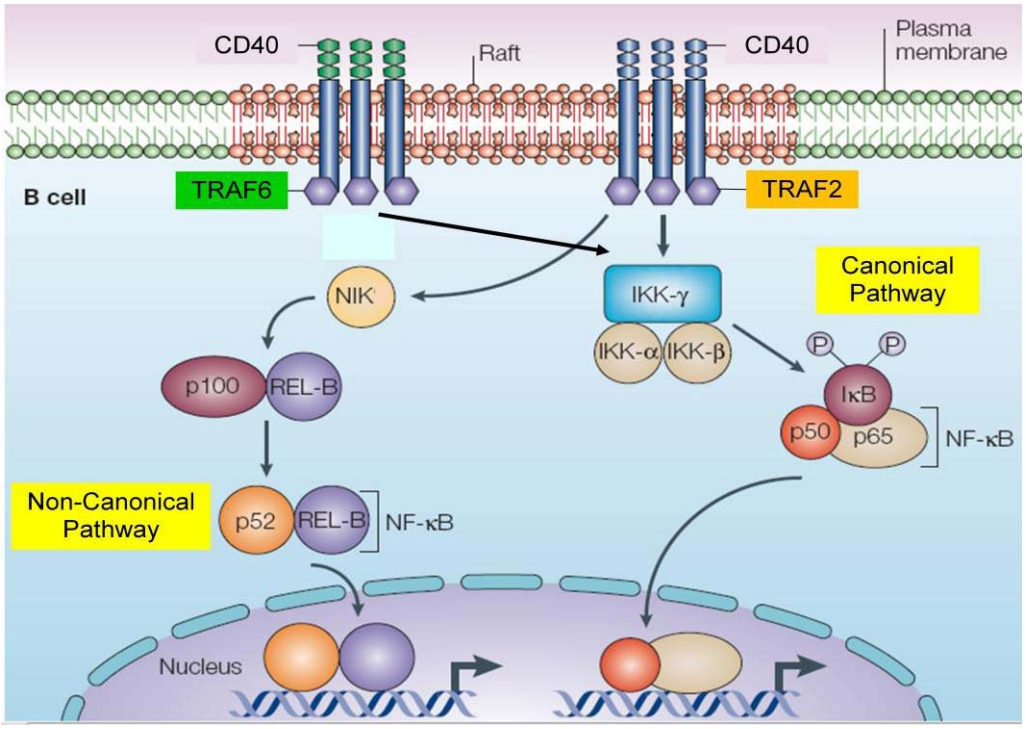

Figure 2.8: Activation of the canonical and non-canonical NF-κB pathways by CD40. TRAF2 interactions with the cytoplasmic domain of CD40 induce activation of both NF-κB pathways, whereas TRAF6 induces activation of the canonical NF-κB pathways. CD40-induced activation of the canonical NF-κB pathway results in nuclear translocation of NF-κB complexes, including the Rel-A/p50 NF-κB complex. Whereas the non-canonical NF-κB pathway results in nuclear translocation of predominantly the Rel-B/p52 NF-κB complex. Modified from Bishop G. A. *Nature Reviews Immunology*. 2004; 4:775-786.

2.3.1.3 TRAF3

TRAF3 was initially identified by its interaction with CD40 and the Epstein-Barr virus transforming protein LMP1.[406, 407] A controversy exists as to the role of TRAF3 in CD40-induced NF-κB pathway activation; given that TRAF3 has opposing effects in different cell types. As mentioned above, TRAF3 negatively regulates CD40 signalling in B lymphocytes in the absence of CD40/CD40L interactions. In fact, in B lymphocytes, expression of a TRAF3-dominant negative protein or deficiency in TRAF3 leads to the activation of the canonical NF-κB

and JNK signalling pathways, as well as the accumulation of NIK and an enhancement in TRAF2 recruitment to the cytoplasmic domain of CD40.[402, 403, 408, 409] In the presence of CD40/CD40L interactions, TRAF3 relieves its negative regulation of the CD40-induced signals through its degradation by cIAP1/2. In contrast, in epithelial cells, over-expression of TRAF3 induces activation of the canonical NF-κB pathway upon CD40 ligation.[410, 411] Therefore, additional investigations are required in order to better understand the different functions of TRAF3 in CD40 signalling.

2.3.1.4 TRAF4

The TRAF4 gene was initially discovered by screening of cDNA libraries for genes expressed in malignant breast cancers.[412] Its biological significance was later put into evidence in TRAF4 deficient mice, which exhibited tracheal malformations.[413] TRAF4 is mainly expressed in neuronal and epithelial cells, and plays a significant role in embryonic development.[413-415] Although TRAF4 does not interact with CD40, it has been shown to interact with other members of the TNF receptor family, namely the lymphotoxin-β receptor (LTβR) and the p75 nerve growth factor receptor.[414]

2.3.1.5 TRAF5

There is much discrepancy regarding the role of TRAF5 in CD40 signalling. Whether TRAF5 is capable of directly or indirectly binding CD40 remains a matter of controversy, given that early studies showed direct binding of TRAF5 to the cytoplasmic domain of CD40, whereas later studies showed that TRAF5 requires heterotrimerization with TRAF3 for interaction with CD40.[385, 416] Furthermore, it has been shown that B lymphocytes treated with small interfering RNAs (siRNAs) specific for TRAF5 and B lymphocytes of TRAF5 deficient mice exhibit a reduction in the canonical and non-canonical NF-κB pathways activation, which is manifested by a decrease in antibody production, proliferation, and co-stimulatory molecules expression.[417, 418] Therefore, the roles of TRAF5 and TRAF2 in activating CD40-mediated downstream signals seems to be redundant.[398] Further investigations are required for elucidating the exact role of TRAF5 in CD40 signalling.

2.3.1.6 TRAF6

TRAF6 was initially identified through its interaction with CD40 in a yeast two-hybrid system. Now TRAF6 is known to mediate CD40-induced activation of the canonical NF-κB pathway, as well as the p38, JNK, and Akt MAPK pathways, which were evidenced in TRAF6 deficient MEF and epithelial cells.[381] Interestingly, although TRAF6 has a dedicated binding site on the cytoplasmic domain of CD40, excision of this site in B lymphocytes does not affect TRAF6 induced JNK activation and enhanced B7.1 expression following CD40 engagement, as compared to TRAF6 deficiency in B lymphocytes.[381, 419] This is due to the interaction of TRAF6 with TRAF2, which subsequently mediates TRAF6 signals independently of its recruitment to its binding site on the cytoplasmic domain of CD40.[419, 420]

As mentioned above, TRAF6 induces activation of Akt, a process independent of NF-κB activation following CD40 ligation.[421] Akt activation occurs in a PI3K dependent manner, in which PI3K forms a complex with TRAF6, the Casitas B-lineage lymphoma b (Cbl-b), and the Casitas B-lineage lymphoma (c-Cbl) following CD40 engagement.[421] In turn Cbl-b induces phosphorylation of Akt through PI3K.[422] Activation of PI3K/Akt has a crucial role on cell survival following CD40 ligation, by mediating inhibition of the pro-apoptotic proteins, caspase 9 and B-cell leukemia (Bcl); in addition to inducing up-regulation of the anti-apoptotic proteins, caspase 8 and the cellular homolog of viral Fas-associated via death domain-like IL-1β converting-enzyme inhibitory protein p43 (cFLIPp43).[422-425]

2.3.1.7 TRAF7

TRAF7 is the most recent member of the TRAF family to be identified. It was first discovered as a protein associated with MEKK3, which is required for TNF-α-induced activation of NF-κB.[426] TRAF7 was later identified through the screening for protein-protein interactions around known and candidate components of the TNF-α/NF-κB pathway.[427] Up to date, there is still no evidence linking TRAF7 to any members of the TNF receptor family; however, it seems to be involved in the TLR-2 signalling pathway.[428] Moreover, TRAF7 seems to be involved in the activation of the JNK and p38 MAPKs, as well as the activation or inhibition of NF-κB, depending on the cellular model and biological setting.[384]

2.3.1.8 JAK3

Although most of the work has focused on the involvement of TRAFs as CD40 adaptor proteins, it has been shown in B lymphocytes that the cytoplasmic domain of CD40 contains a binding region for JAK3.[429] However, JAK3 activation following CD40 ligation is cell type dependent. For instance, in B lymphocytes, CD40 stimulation does not induce JAK3 phosphorylation, whereas in monocytes and APCs it does.[382, 383, 430] JAK3 induces activation of STAT5, thereby leading to gene expression of inflammatory cytokines, including TNF-α, interferon (INF)-γ, and IL-6, as well as maturation of DCs.[431]

2.3.2 Structure and function of NF-κB

NF-κB is a important transcription factor involved in the expression of a wide range of genes implicated in different biological processes, including immune responses, cell survival, stress responses, and maturation of various cell types (Table 2.3). In mammals, the NF-κB complexes are formed by homo- or heterodimerization of five transcription factors: Rel-A (p65), Rel-B (p68), c-Rel, and the precursors p105 (NF-κB1) and p100 (NF-κB2), which are processed into p50 and p52, respectively. These transcription factors contain an N-terminal DNA-binding/dimerization domain known as the Rel homology domain, which allows their dimerization, as well as their DNA binding (as dimers) at target sequences termed κB sites. The Rel-A, Rel-B, and c-rel transcription factors also contain C-terminal transcription activation domains (TADs), which favour co-activator recruitment and the subsequent expression of target genes. The p50 and p52 transcription factors lack TADs, and therefore activate transcription by forming heterodimers with Rel-A, Rel-B, or c-Rel. In contrast, p50 and p52 can inhibit transcription if they bind DNA as homodimers, given that they lack TADs.[432]

In the absence of receptor stimulation, the NF-κB complexes are maintained in the cytoplasm in an inactive state complexed with the inhibitory inhibitor κB (IκB) proteins (IκBα, IκBβ, IκBε, IκBζ, p100, p105, Bcl3, and IκBns), which sequester NF-κB complexes in a latent state through their ankyrin-repeat domains.[433] Upon receptor activation, and the subsequent TRAF recruitment, activation of the NF-κB signalling pathway leads to proteasomal degradation of the IκB proteins and the consequential release and nuclear translocation of the NF-κB complexes. Activation of the canonical NF-κB pathway leads to nuclear translocation of four transcriptional activator complexes (Rel-A/Rel-A, Rel-A/p50, c-Rel/c-Rel, and c-Rel/p50),

whereas activation of the non-canonical NF-κB pathway leads to nuclear translocation of predominantly Rel-B/p52 complexes.[432, 433] Figure 2.9 schematizes the NF-κB and IκB family members, in addition to the IκB kinase (IKK) members (discussed below).

Table 2.3: Genes induced by the NF-κB signalling pathway activation.[434, 435]

Cytokines and growth factors	- IL-1β - IL-2 - IL-6 - IL-8 - TNF-α - Lymphotoxin (TNF-β) - IP-10 - MIP-1α - MCP-1 - RANTES - INF-β - Macrophage colony-stimulating factor (M-CSF) - Granulocyte/macrophage colony-stimulating factor (GM-CSF) - Granulocyte colony-stimulating factor (G-CSF) - Melanoma growth stimulating activity (Gro-α, -β and -γ/MGSA) - Proenkephalin
Immunoreceptors	- Ig-κ light chain - TCR - MHC-I - MHC-II - MHC-II invariant chain - β2-microglobulin - Tissue factor-1 - IL-2 receptor α chain
Adhesion molecules	- ICAM-1 - VCAM-1 - Endothelial-leukocyte adhesion molecule-1 (ELAM-1)
Acute phase proteins	- Angiotensinogen - Serum amyloid A precursor - Complement factor B - Complement factor C4
Transcription factors and regulators	- c-Rel - p105 - IκBα - c-myc - Interferon regulatory factor 1 (IRF-1)
Others	- iNOS - Vimentin

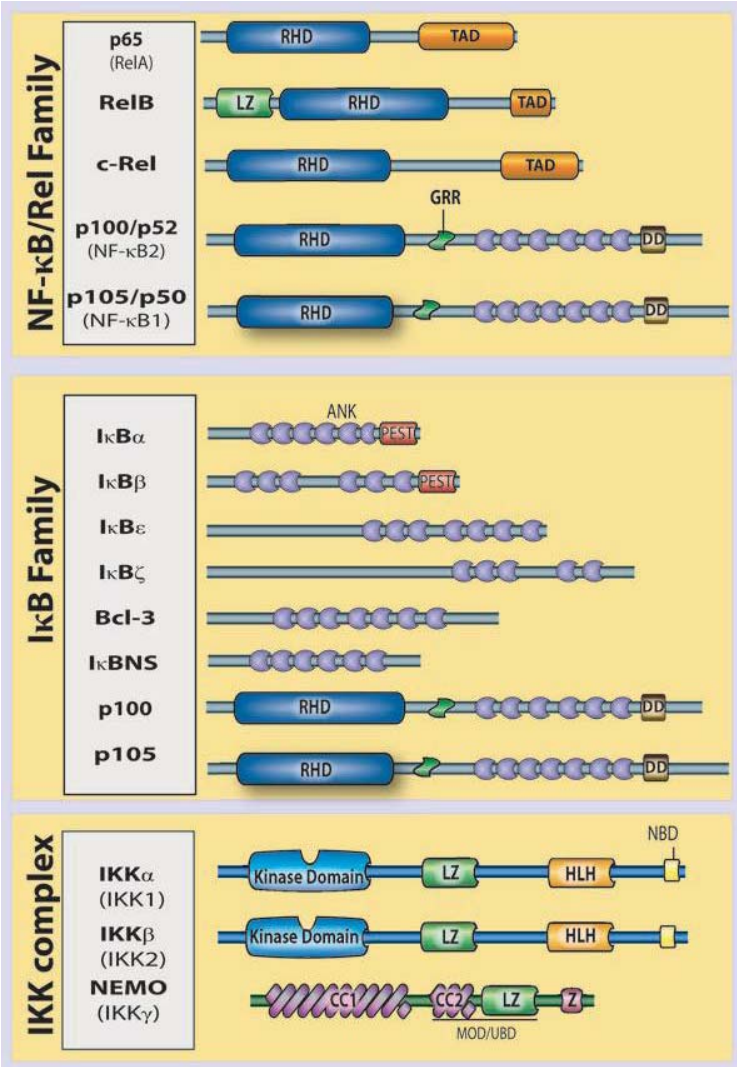

Figure 2.9: Schematic representation of the mammalian NF-κB, IκB and IKK family members. Alternative nomenclatures are in parenthesis. The precursors p100 and p105 act as IκB; however, following receptor activation and their subsequent proteasomal degradation into p52 and p50, respectively, they function as NF-κB family members. (ANK: ankyrin-repeat domain; DD: death domain; RHD: REL homology domain; TAD: transcription activation domain; LZ: leucine-zipper domain; GRR: glycin-rich region; HLH: helix-loop-helix domain; Z: zinc-finger domain; CC: coiled-coil domain; NBD: NEMO-binding domain; MOD/UBD: minimal oligomerization domain/ubiquitin-binding domain; PEST: proline, glutamic acid, serine, and threonine rich.) Modified from Hayden M. S. et *al*. *Cell Research*. 2011; 21:223-244.

2.3.2.1 Canonical pathway

As mentioned above, in the absence of receptor activation, the NF-κB complexes are sequestered in the cytoplasm in a latent state through their association with IκBs. In the canonical pathway, the classical IκB (IκBα, IκBβ, and IκBε) sequester the canonical NF-κB complexes (Rel-A/Rel-A, Rel-A/p50, c-Rel/c-Rel, and c-Rel/p50) in the cytoplasm, and the release of these complexes requires phosphorylation and proteasomal degradation of the IκBs following receptor

engagement.[433] Phosphorylation of IκBs in the canonical pathway is dependent on the activation of the IKK complex, which is formed by the two catalytic subunits IKKα (a.k.a IKK1) and IKKβ (a.k.a IKK2), and the regulatory subunit NF-κB essential modulator (NEMO; a.k.a. IKKγ). Activation of the IKK complex, specifically phosphorylation of IKKβ on serines 177 and 181, leads to IκBα phosphorylation at serines 32 and 36.[436, 437] Once phosphorylated, IκBα is targeted for degradation by the 26S proteasome, thereby releasing the canonical NF-κB complexes, which then translocate into the nucleus and initiate transcription of target genes (Figure 2.8). The IκBβ and IκBε are also phosphorylated by the IKK complex, and thereafter targeted for proteasomal degradation, and the consequential release and nuclear translocation of canonical NF-κB complexes.[433]

Since the canonical NF-κB pathway induces expression of genes involved in the inflammatory and immune response, its activation must be tightly controlled, given that improper regulation of NF-κB activation has been shown to be implicated in pathological conditions, such as chronic inflammation and cancer.[433, 438, 439] Indeed, IκBα and IκBε have been shown to be important negative regulators of the canonical NF-κB pathway activation, as their absence results in lethality due to hyperinflammation and increased cytokine expression.[439, 440] Additionally, NF-κB has been shown to induce transcription of the IκBα gene, thereby forming a negative feedback loop that limits activation of the canonical NF-κB pathway.[441]

2.3.2.2 Non-canonical pathway

In contrast to the canonical pathway, activation of the non-canonical NF-κB pathway is dependent on NIK and IKKα. As mentioned in section 2.3.1.2, following degradation of TRAF3, there is an accumulation of cytoplasmic NIK. In turn, NIK activates IKKα, which then phosphorylates the precursor p100.[442] Phosphorylation of p100 targets it for degradation by the 26S proteasome. However, unlike IκBα, proteasomal degradation of p100 results in degradation of only its C-terminal containing ankyrin-repeat domains.[443] Removal of these domains generates the p52 transcription factor containing the Rel homology domain, which, in turn, preferentially associates with Rel-B (Figure 2.8). The formed heterodimer subsequently translocates to the nucleus and mediates transcription of the non-canonical NF-κB pathway target genes, which are involved in the development and maintenance of secondary lymphoid organs.[443, 444]

Unlike the canonical pathway, there is limited information regarding the negative regulation of the non-canonical pathway. It has been reported that IKKα induces phosphorylation and the subsequent destabilization of NIK, which probably provides a mean to down-regulate activation of the non-canonical NF-κB pathway.[445]

2.3.3 Other CD40 intracellular signalling molecules

Members of the NF-κB family have been the main focus of most of the work on CD40/TRAFs-induced transcriptional activity. However, NF-κB family members are not the only transcription factors downstream of TRAFs. Activation of CD40 in B lymphocytes stimulates activation of a wide array of immuno-regulatory proteins, including cytokines, chemokines, Ig, and adhesion molecules; however, inhibition of CD40-induced activation of the NF-κB pathways ablates most, but not all CD40-mediated gene expression.[446, 447] For instance, IL-6 gene expression following CD40 activation does not require NF-κB nuclear translocation. The transcription factors activator protein 1 (AP1) and nuclear factor of IL-6 (NF-IL-6; a.k.a C/EBPβ) have been shown to mediate CD40-induced transcriptional activity alongside NF-κB family members in a TRAF6 dependent manner.[448] NF-IL-6 and AP1 have an important role in CD40-mediated IL-6 production, as well as the subsequent IgM production by B lymphocytes.

2.4 Physiological role of the CD40/CD40L axis

Section 2.2 covered the biological function of the CD40/CD40L dyad in a cell type specific manner, while section 2.3 described the signalling pathways ensued by CD40 following its interaction with CD40L. The role of this dyad in the interactions among the different cell types in a physiological setting, such as in humoral immunity, cell-mediated immunity and apoptosis, will be the main focus of this section.

2.4.1 Humoral immunity

Humoral immunity, which is also known as thymus-dependent humoral immune response, is a branch of immunity that is mediated through antibody production by B lymphocytes. The intricate cell-cell contact interactions between APCs (specifically DCs), T and B lymphocytes, as well as the crucial role of the CD40/CD40L dyad in these interactions culminate in B

lymphocyte activation and differentiation into antibody producing cells, also known as plasmocytes.

The importance of the CD40/CD40L dyad in the development of humoral immunity was initially evidenced in individuals with HIGM, who have a mutation in the gene encoding CD40L resulting in a non-functional protein.[276] The importance of this dyad was later confirmed by genetic deletion of either the CD40 or CD40L gene, and by antibody blockade of the CD40L protein.[449, 450] Under such conditions, there is a severe impairment in the humoral immune response, which is manifested by the lack of germinal center formation and progression, antibody isotype switching and affinity maturation, and memory B cells and plasma cells formation.[276, 449, 450]

The intricate interactions between DCs, T and B lymphocytes in mounting an effective humoral immune response are initiated by the interaction of DCs with pathogens. Upon a pathogenic challenge, DCs acquire and present pathogenic antigens to naive $CD4^+$ T lymphocytes, which recognize the antigens through the TCR and become fully activated following engagement of CD40L and CD28 with their cognate receptors (CD40 and B7.1 or B7.2, respectively) on DCs.[1, 330] On the other hand, ligation of CD40 on DCs induces their up-regulation of co-stimulatory (CD40 and B7.2 or B7.1) and MHC molecules (MHC-I and MHC-II), in addition to inducing secretion of the IL-12, IL-10 and IL-6 cytokines.[330, 338, 339, 341, 451, 452] In turn, IL-12, IL-10 and IL-6 favour T lymphocytes differentiation into effector T_{h1}, T_{Reg} and T_{h17} lymphocytes, respectively.[343, 452, 453] This bidirectional crosstalk favours clonal differentiation of antigen-specific T lymphocytes, as well as maturation and survival of DCs.[400] Activated T lymphocytes then home toward B lymphocyte follicles and position themselves at the border of the T lymphocyte zone and B lymphocyte follicles, where they encounter activated B lymphocytes expressing the cognate antigen.[454, 455] Activation of naive B lymphocytes prior to their interaction with activated T lymphocytes occurs through their direct uptake of pathogenic antigens or by their interaction with tissue-resident DCs that are activated by the pathogen and subsequently home toward the B lymphocyte zone in secondary lymphoid organs.[456, 457] Interaction of T and B lymphocytes at the border zone induces B lymphocyte differentiation into either plasmablasts or germinal center cells.[458, 459] Interestingly, the extent of CD40/CD40L interactions depicts the fate of B lymphocyte differentiation, where extensive CD40/CD40L interactions favour plasmablast differentiation.[400, 458] Further engagement of T lymphocyte

CD40L with CD40 on B lymphocytes stimulates B lymphocyte proliferation, isotype switching, and differentiation into plasma cells.[278, 314] In summary, pathogen-activated DCs induce activation of T lymphocytes, which in turn induce B lymphocyte proliferation, isotype switching, and differentiation, where CD40/CD40L interactions play a major role at each interaction step.

In contrast, DCs are capable of inducing T lymphocyte-independent B lymphocyte antibody production. CD40 activation on DCs by CD40L on activated T or B lymphocytes induces expression of the B lymphocyte stimulator protein (BLys or BAFF) and a proliferation induced ligand (APRIL) on DCs.[460] In turn, BAFF interacts with the BAFF receptor and the B cell maturation antigen (BCMA) on B lymphocytes, while ARPIL interacts with the transmembrane activator and calcium modulator and cyclophylin ligand interactor (TACI). These interactions in the presence of IL-2 and IL-4 secreted by T lymphocytes, favour B lymphocytes survival, isotype switching, and IgG and IgA secretion.[460-462]

2.4.2 Cell-mediated immunity

It was initially thought that the function of CD40/CD40L dyad was limited to humoral immunity; however, substantial amount of evidence now supports its role in cell-mediated immunity. This branch of immunity does not involve antibody production or activation of the complement system, but is rather mediated by a plethora of agents secreted and expressed by immuno-competent cells, including T and B lymphocytes, macrophages, natural killer cells, endothelial cells, SMCs, platelets, and fibroblasts.[1, 463] Ligation of CD40 induces a pro-inflammatory response in these cells, which is characterized by the up-regulated expression of adhesion molecules, as well as the induced secretion of pro-inflammatory mediators, namely cytokines, chemokines, extracellular degrading enzymes (MMPs), growth factors, and coagulation factors (TF). All of which are important mediators for both inflammation and immunity as they favour recruitment and activation of additional immuno-competent cells at the lesion/infection site. In addition, the various secreted growth factors and MMPs influence the wound repair process after the resolution of infection and inflammation.[1, 368, 463] Tables 2.2 and 2.3 summarize the aforementioned mediators influenced by the CD40/CD40L dyad in cell-mediated immunity.

2.4.3 Apoptosis

Aside from its role in immunity, the CD40/CD40L dyad has a role in modulating cell survival. Signals induced by CD40 following its ligation either induce or prevent apoptosis depending on the cell type in which they occur. For instance, CD40 signals rescue B lymphocytes from apoptosis, whereas in certain B lymphoma cell lines they induce apoptosis.[464-469]

CD40-induced inhibition of apoptosis is mediated through its modulation of mediators of the intrinsic and extrinsic apoptotic pathways. The intrinsic apoptotic pathway is triggered by mitochondrial stress resulting in cytochrome c and Smac/DIABLO release from the mitochondria. Association of cytochrome c with Apaf-1 and caspase 9 leads to caspase 3 activation and the subsequent cellular death by apoptosis.[470] Inhibition of the intrinsic apoptotic pathway by CD40 is mediated through the up-regulation of the anti-apoptotic Bcl-2 family members (Bcl-X_L, Mcl-1 and Bfl-1) and survivin that prevent cytochrome c release from the mitochondria and block caspase 3 activation, respectively.[464, 465, 470-475] On the other hand, the extrinsic apoptotic pathway is mediated by activation of Fas and TNF receptor 1, which lead to caspase 8 activation and the subsequent caspase 3 activation.[470] CD40 inhibits the extrinsic apoptotic pathway by up-regulating A20 and cFLIP expression, which respectively inhibit Fas and TNF receptor 1 mediated caspase 8 activation.[476, 477] Therefore, CD40 signals induce expression of anti-apoptotic proteins, which rescue B lymphocytes from apoptosis induced by either extrinsic factors (IgM, Fas ligand [FasL] and TNF) or intrinsic factors that induce mitochondrial damage.

In contrast, activation of CD40 on certain malignant and B lymphoma cell lines results in growth arrest and induced apoptosis.[470, 478, 479] The pro-apoptotic effect of CD40 activation in these cells is mediated through the up-regulation of the pro-apoptotic Bcl-2 family members (Bik, Bax and Bak), which favour cytochrome c release from the mitochondria, and the subsequent caspase 3 activation.[480, 481] CD40 also drives the extrinsic apoptotic pathway by inducing the up-regulation of FasL and TNF, thereby leading to caspase 8 activation following Fas/FasL and TNF/TNF receptor interactions.[482, 483]

2.5 Pathological role of the CD40/CD40L axis

In the previous sections, the physiological function of the CD40/CD40L dyad was discussed. The interactions between CD40 and CD40L were shown to be central for mounting an effective

inflammatory and immune response. However, these interactions must be tightly regulated, given that the improper expression of CD40 and CD40L or the unsuitable interactions between them are associated with the pathogenesis of multiple diseases, including autoimmune diseases, cancer and atherosclerosis.[484-486] This section will focus on the CD40/CD40L contribution to the development and progression of these pathologies.

2.5.1 Autoimmune diseases

An autoimmune disease arises when the body mounts an immune response against substances and tissues normally present in the body. In other words, the body attacks its own cells, thereby damaging a specific organ or tissue that is present in multiple parts of the body. Given its central role in modulating the immune response, the CD40/CD40L dyad significantly contributes to the development and progression of multiple autoimmune diseases, such as inflammatory bowel disease, type I diabetes, thyroiditis, multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis. This section will cover the general contribution of the CD40/CD40L dyad in the development of autoimmune diseases, as well as its implication in the aforementioned diseases.

2.5.1.1 Mechanism of action

Up to date, there are three proposed mechanisms for the contribution of the CD40/CD40L dyad to T lymphocyte-dependent autoimmune diseases. The first proposed mechanism is mediated through improper T lymphocyte selection in the thymus. Under normal circumstances, expression of tissue-restricted antigens by medullary thymic epithelial cells results in negative selection (deletion) of potentially auto-reactive T lymphocytes and the subsequent induction of self-tolerance. As CD40 has been shown to cooperate with the receptor activator of NF-κB (RANK) in promoting medullary thymic epithelial cells development, a disruption in CD40 activation in these cells could probably result in their inadequate development.[486, 487] Therefore, the disturbed development of medullary thymic epithelial cells potentially permits auto-reactive T lymphocyte clones to escape negative selection, leading to failure of central tolerance and the subsequent potential development of auto-immune diseases.

The second proposed mechanism of CD40/CD40L contribution to autoimmune diseases occurs in secondary lymphoid organs, where T lymphocytes are primed by APCs (B

lymphocytes or DCs) over-expressing CD40 either constitutively or transiently.[486] The over-expression of CD40 leads to increased interactions between CD40L on T lymphocytes and CD40 on APCs, which favour the activation of auto-reactive T lymphocytes, as well as the production of pro-inflammatory cytokines by APCs and auto-antibodies by B lymphocytes.[452] Among the secreted pro-inflammatory cytokines, IL-6 has been shown to drive T lymphocytes differentiation into T_{h17} cells.[452] In turn, T_{h17} cells induce cell-mediated tissue damage by secreting IL-17.[488] Therefore, the increase in CD40 expression and the subsequent exaggerated CD40/CD40L interactions potentially lead to the development of autoimmune diseases.

The third proposed mechanism results from abnormal expression of CD40 in tissues where it is normally undetectable. Under such conditions, the tissues themselves contribute to the initiation of the autoimmune disease. Indeed, elevated expression levels of CD40 in target tissues (thyroid and pancreatic islet cells) have been associated with the initiation of thyroiditis, and the production of inflammatory cytokines resulting in the failure of pancreatic islet cell transplants.[489, 490]

2.5.1.2 Inflammatory bowel disease

The two major subtypes of inflammatory bowel disease are Crohn's disease and ulcerative colitis. They are characterized by recurring inflammation of the small and large intestine. Infiltration of macrophages, T and B lymphocytes into the intestinal epithelium disrupts its barrier function, resulting in diarrhea, abdominal pain, rectal bleeding, and malnutrition in attained individuals.[491]

The initial evidences pointing at the contribution of CD40/CD40L to the autoimmune inflammatory bowel disease came from studies in mice. The results from these studies demonstrated that the CD40/CD40L interactions were crucial for the initiation of the disease, but were not necessary for the progression of the inflammatory responses. In fact, administration of a blocking CD40L antibody at the onset of colitis initiation inhibits lymphocytic infiltration into the intestinal epithelium, and disease occurrence, whereas blocking CD40L four weeks following colitis initiation only improves the disease symptoms.[492, 493] Additionally, in mice, over-expression of CD40L in T lymphocytes results in T lymphocyte infiltration in multiple organs and death caused by inflammatory bowel disease by 3-6 weeks of age.[494]

In patients with Crohn's disease, CD40 is over-expressed on microvascular endothelial cells in the inflamed mucosa, and there is an increase in $CD40^+$ DCs found within the intestinal mucosa.[495, 496] In contrast, genomic association studies did not show an association between the CD40 gene and inflammatory bowel disease incidences.[497] Nonetheless, in a small study, 77% of the patients with Crohn's disease that where treated with a chimeric antagonistic CD40 antibody showed a beneficial response to treatment, while the remaining 22% of the patients entered into remission.[498]

2.5.1.3 Type I diabetes

Type I diabetes, also known as insulin-dependent diabetes, is an autoimmune disease characterized by the complete absence of insulin production resulting from the destruction of the beta cells in the islets of Langerhans in the pancreas.[499] Much of the knowledge regarding type I diabetes development came from studies in animal models of this human disease, specifically non-obese diabetic (NOD) mice, which spontaneously develop diabetes following insulitis (inflammation of the islets of Langerhans), and leukocytic infiltration and destruction of the islets. In NOD mice, $CD8^+$ and $CD4^+$ T lymphocytes specific for beta cell antigens, as well as B lymphocytes, have been shown to be essential for the pathogenesis of the disease. Transfer of $CD8^+$ or $CD4^+$ T lymphocytes specific for beta cell antigens from NOD mice into normal mice induces diabetes in the latter, whereas depletion of these cells in NOD mice prevents the disease.[499] In addition, diabetes in NOD mice is prevented by B lymphocyte depletion.[500] Although B lymphocytes do produce auto-antibodies directed against beta cell antigens (insulin and glutamic acid decarboxylase), their role as APCs in promoting diabetogenic T lymphocytes activation and proliferation might be of more significance, given that the role of the produced auto-antibodies in the pathogenesis of the disease is still unclear.[499, 501] Given the central role of the CD40/CD40L dyad in the cross-talk between B and T lymphocytes, blocking this interaction with an antagonistic CD40L antibody has been shown to delay or prevent diabetes in NOD mice and bio-breeding diabetes-prone rats.[502, 503]

The role of $CD40^+$ T lymphocytes in diabetes has recently been examined. Triggering of the TCR and CD40 in T lymphocytes from NOD mice up-regulates the level of expression of CD40 on these cells. Additionally, the use of an antibody to block CD40 on diabetogenic T lymphocytes from NOD mice prevents diabetes induction in NOD SCID (severe combined

immunodeficiency) following T lymphocyte transfer.[504] In humans, the levels of $CD40^+$ T lymphocytes have been shown to be elevated in type I diabetes patients as compared to type II diabetes patients and controls.[505]

Finally, the CD40/CD40L dyad also contributes to the inflammatory environment within the islets of Langerhans. Pancreatic beta cells from human and mice constitutively express CD40, and activation of this receptor in these cells leads to the production of pro-inflammatory cytokines (IL-6, IL-8, MIP-1α, and MCP-1β); hence, promoting leukocyte recruitment and inflammation.[490, 506] Therefore, the CD40/CD40L dyad is a major component in diabetes development.

2.5.1.4 Thyroiditis

Autoimmune thyroid disease affects approximately 5% of humans, and includes Graves' disease and Hashimoto's thyroiditis. Graves' disease is characterized by thyrotoxicosis (excess circulating thyroid hormones due to an overactive thyroid), infiltration of lymphocytes into the thyroid gland, goiter, and presence of auto-antibodies directed against the thyroid stimulating hormone receptor. Hashimoto's thyroiditis differs from Graves' disease by a more intense infiltration of lymphocytes into the thyroid gland, a loss of thyroid function, and auto-antibodies directed against thyroglobulin and thyroid peroxidase.[507]

In Graves' disease patients, analysis of thyroid epithelial cells demonstrated an up-regulation of MHC-II and co-stimulatory (CD40 and B7.1) molecules, suggesting that these cells probably contribute to disease development by enhancing presentation of thyroid auto-antigens, as well as co-stimulatory molecules to T lymphocytes.[486, 508] Moreover, a single nucleotide polymorphism in the Kozak sequence (a consensus sequence in 5'-UTR of an mRNA) of the CD40 gene, which gives rise to an allele with a C polymorphism, has been associated with an increased risk for Graves' disease.[507] This increased risk is probably due to an augmented level of CD40 expression on resting B lymphocytes and thyrocytes, when both alleles of the CD40 gene harbour this C polymorphism.[486, 509]

In contrast, there are no polymorphisms in the CD40 gene that have been linked to Hashimoto's thyroiditis.[486] Nonetheless, the CD40/CD40L dyad seems to play a role in the development of this disease, since the use of a blocking antibody against CD40L averts experimental autoimmune thyroiditis induced in animal models through thyroglobulin

injection.[510] Additionally, B and T lymphocytes from experimental autoimmune thyroiditis mice treated with a CD40L blocking antibody are unable to induce the disease following their transfer into immuno-deficient mice, thus, confirming the involvement of the CD40/CD40L dyad in autoimmune thyroid disease development..[511]

2.5.1.5 Multiple sclerosis

Multiple sclerosis is an autoimmune disease characterized by demyelination of the brain and spinal cord due to an infiltration of T lymphocytes ($CD4^+$ and $CD8^+$), B lymphocytes, macrophages, and microglia (central nervous system resident macrophages) into the CNS and the subsequent destruction of myelin on nerve fibers, leading to motor and sensory dysfunction.[512] Most of the knowledge regarding multiple sclerosis pathogenesis came from analyzing human post-mortem brain lesions and experimental autoimmune encephalomyelitis (EAE) animal models that are featured by central nervous system infiltration by immune cells following immunization with myelin components.[512] Analysis of post-mortem brain lesions from patients with multiple sclerosis demonstrated that the majority of the cells within the lesion that express CD40L were $CD4^+$ T lymphocytes, whereas those expressing CD40 were B lymphocytes, macrophages, and microglia.[513] In addition, analysis of EAE mice demonstrated the expression of CD40 in the spinal cord during the acute and relapse phases of the disease, whereas CD40L expression only peaked during the relapse phase.[514]

The contribution of the CD40/CD40L dyad to multiple sclerosis development was evidenced in mice deficient in CD40L or mice treated with a CD40L blocking antibody on the onset of EAE induction, which prevented disease development probably by favouring a non-pathogenic T_{h2} immune response rather than a T_{h1} one.[513, 515, 516] Indeed, CD40L blocking antibody treatment in mice at the peak of the acute phase of the disease decreased the level of infiltration of inflammatory cells into the central nervous system, as well as T_{h1} cell differentiation.[517] Moreover, CD40 is expressed by microglia and it is up-regulated by INF-γ treatment.[518] Activation of CD40 in these cells induces their activation and secretion of IL-12 and TNF, which induce T_{h1} differentiation and the subsequent neuronal cell death.[518-520] CD40 expressed on microglia has also been shown to be an important contributor for EAE development, given that CD40 deficiency in microglia results in a less severe EAE due to a

decrease in T lymphocyte infiltration into the central nervous system, in addition to a reduction in demyelination.[520, 521]

Finally, there is accumulating evidence pointing toward a genetic background for multiple sclerosis; however, no correlation was found between the CD40 gene and incidence of the disease.[497, 512, 522]

2.5.1.6 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that affects multiple organs of the body, including skin, joints, lungs, kidneys, brain, and heart. It is due to circulating auto-antibodies directed against dsDNA (double stranded DNA) and other nuclear components, which form immune complexes (antibody and antigen) that deposit on small blood vessels and subsequently favour recruitment and assembly of the complement system, thereby causing vasculitis (inflammatory destruction of blood vessels).[523]

Patients with an active SLE disease have T lymphocytes ($CD4^+$ and $CD8^+$) that over-express CD40L, in addition to B lymphocytes and monocytes that abnormally express CD40L.[523-526] The implication of CD40L expressed on B lymphocytes in SLE pathogenesis was put into evidence by two approaches. The first approach showed that B lymphocytes from SLE patients produced antibodies in a CD40L-dependent manner, whereas the second approach demonstrated a lupus-like disease development in transgenic mice that harbour $CD40L^+$ B lymphocytes. [524, 527] Interestingly, in patients with SLE, there is a correlation between circulating levels of CD40L and disease severity.[528] Moreover, in these patients, there is a decrease in the number of $CD34^+$ haematopoietic progenitor cells in the bone marrow, which was shown to be due to CD40 activation and the subsequent Fas-mediated apoptosis of these cells.[529] The decrease in the number of $CD34^+$ haematopoietic progenitor cells in SLE patients probably correlates with the frequent pancytopenic (reduced number of circulating white and red blood cells, and platelets) phenotype in these patients.

In a mouse model of SLE, treatment with an anti-CD40L blocking antibody before any apparent SLE symptoms prevents complications associated with the disease, such as proteinuria (high urine protein levels), kidney disease, in addition to prolonging survival and decreasing the levels of circulating antibodies against dsDNA.[530-532] Interestingly, a long-term positive outcome

on kidney function, levels of anti-dsDNA antibodies and survival is observed albeit a short treatment period with an anti-CD40L antibody.[533]

Finally, SLE incidence has been shown to be associated with multiple genomic loci.[497, 523] The region q11.2-13.1 on chromosome 20, which harbours the CD40 gene, has been recognized as a site potentially linked to SLE occurrence.[523, 534] Moreover, a missense single nucleotide polymorphism within the CD40 gene, which gives rise to a CD40 protein with a higher signalling capacity, aggravates SLE symptoms in patients with a pre-established disease. However, there is no correlation between this polymorphism and SLE incidence.[535, 536]

2.5.1.7 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease affecting approximately 1% of humans and it is characterized by chronic inflammation within the joint due to the infiltration of immune cells and activation of fibroblast-like synoviocytes, which create a local inflammatory environment through the secretion of pro-inflammatory compounds, such as cytokines, chemokines and MMPs.[537, 538] In addition, joints of RA patients are characterized by thickening of the synovium and destruction of the cartilage and bone, which are mainly caused by fibroblast-like synoviocyte activation and proliferation.[537]

Fibroblast-like synoviocytes from RA patients have been shown to express CD40 that is additionally up-regulated following INF-γ and TNF-α treatment.[539] Activation of CD40 on these cells contributes to joint destruction by inducing the up-regulation of adhesion molecules (ICMA-1 and VCAM-1), as well as the secretion of pro-inflammatory cytokines (IL-6, IL-8, IL-15, IL-17 and TNF), chemokines (MIP-1α and MCP-1), and growth factors (GM-CSF).[537-542] Additionally, osteoclast-mediated bone resorption is stimulated by the RANK ligand, which is expressed by fibroblast-like synoviocytes following CD40 activation.[543] On the other hand, activation of CD40 expressed on DCs within the synovial tissue induces secretion of TNF, which has been shown to contribute to collagen degradation in *ex vivo* cultures.[544]

T lymphocytes from RA patients highly express CD40L as compared to healthy individuals and this level of expression correlates with higher disease severity and increased Ig production by B lymphocytes in these patients.[545-547] Moreover, T lymphocytes form these patients have been shown to induce IL-12 secretion by synovial DCs and macrophage, in a CD40L-dependent manner.[548] In a mouse model of arthritis, treatment with a CD40L blocking

antibody prevents the disease if given prior to disease induction; however, it does not reverse the disease if given once the disease has established.[549, 550] On the other hand, treatment with an agonistic CD40 antibody at the onset of arthritis induction aggravates the disease.[551] Therefore, it seems that the CD40/CD40L dyad has a main role only at the initial stages of arthritis.

Finally, as for most autoimmune diseases, RA has a strong genetic component. Multiple genomic loci have been associated with RA incidence, including the CD40 locus which has been associated with juvenile RA.[497, 552] Furthermore, a single nucleotide polymorphism in the CD40 gene, as well as single nucleotide polymorphisms in signalling molecules downstream of CD40, including A20 and TRAF1 have been associated with RA occurrence in the European population.[553]

2.5.2 Cancer

As mentioned earlier in this chapter, CD40 was initially cloned in Burkitt's lymphoma. It was later identified on multiple tumour cell types, including epithelial neoplasia, non-Hodgkin's lymphoma, melanoma, bladder carcinoma, and osteosarcoma.[279, 280, 554-558] Therefore, cancer is probably the first human disease to be associated with the CD40/CD40L dyad. Given the widespread expression of CD40 on multiple tumours, it is suggested to play a role in tumour pathogenesis. Indeed, accumulating evidence demonstrates that a constitutive low-level of CD40 engagement favours neoplastic cell growth by promoting tumour cell proliferation and protection against apoptotic cell death in an NF-κB dependent manner.[559-561] The constitutive low-level of CD40 activation is due to the low level of CD40L co-expression on tumour cells, including non-Hodgkin lymphoma, Burkitt lymphoma, chronic lymphocytic leukemia, and breast carcinoma, which mediates CD40 ligation in an autocrine manner. Disruption of this low-level of CD40/CD40L interactions by a CD40L blocking antibody has been shown to reduce tumour cell proliferation and favour tumour cell death.[559-562]

In contrast, the CD40/CD40L dyad has been shown to have a rather negative impact on tumour pathogenesis. Patients with HIGM syndrome, who lack a functional CD40L protein, have a severely compromised immune system, and are therefore presented with higher incidences of multiple leukemia and carcinoma.[563] Additionally, patients with chronic lymphocytic leukemia, who display a lack of CD40L on activated $CD4^+$ T lymphocytes, show characteristics comparable to those of HIGM patients, including immunodeficiency and increased tumour

incidences.[564] In mice, CD40L deficiency results in a lack of a protective anti-tumour immune response against fibrosarcoma, melanoma, bladder carcinoma, and adenocarcinoma, as compared to wild-type mice.[565, 566] Moreover, treatment of tumour-bearing mice with an agonistic CD40 antibody results in a reduction of tumour growth and metastasis due to an increase in tumour specific CTLs and natural killer (NK) cell activation.[567, 568]

The CD40/CD40L dyad exerts its anti-tumour function through indirect and direct mechanisms. The indirect mechanism involves activation of NK cells and tumour-specific CTLs that mediate tumour eradication.[569] The direct mechanism is mediated through the activation of CD40 on tumour cells, which induces their up-regulation of pro-apoptotic mediators, thereby driving apoptotic cell death. The direct mechanism also involves the up-regulation of adhesion and MHC molecules on tumour cells following CD40 activation, which favours their recognition by anti-tumour immune cells.[569]

2.5.3 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arterial wall, and it is the major pathology at the root of cardiovascular diseases, including myocardial infarction, stroke and peripheral arterial disease.[215] The atherosclerotic plaque is characterized by the accumulation of lipids, cells, cellular debris, calcium and extra-cellular matrix within the arterial wall.[570] Development and progression of an atherosclerotic lesion involve the contribution of immune (T lymphocytes, B lymphocytes, monocytes/macrophages, neutrophils, and mast cells) and non-immune cells (vascular SMCs, endothelial cells, and platelets). Both CD40 and CD40L are expressed on these different cell types in the atherosclerotic lesion.[463, 571] Interestingly, CD40 and CD40L are detected in the atherosclerotic lesions in the early stages of atherosclerosis, and their expression levels increase with plaque progression reaching highest expression levels in advanced, rupture-prone and ruptured plaques.[571]

The involvement of the CD40/CD40L dyad in atherosclerotic plaque development is supported by a substantial amount of evidence. For instance, treatment of hyperlipidemic low-density lipoprotein (LDL) receptor deficient mice with an anti-CD40L blocking antibody has been shown to considerably decrease the size and lipid content of the atherosclerotic lesions.[572, 573] Moreover, CD40L deficiency or inhibition with a blocking antibody in $ApoE^{-/-}$ mice results in a 5.5 fold decrease in atherosclerotic plaque area, as well as an increase in the level of collagen

and SMCs, and a decrease in the lipid levels and the number of inflammatory cells within the atherosclerotic plaque.[573, 574] Moreover, treatment of $ApoE^{-/-}$ mice with an anti-CD40L blocking antibody at the onset of atherosclerosis or after atherosclerosis establishment results in the formation of lipid-poor and collagen rich plaques, thereby demonstrating the involvement of CD40L in the initiation and progression of atherosclerotic plaques.[575] Therefore, it seems that lack of CD40L favours the development of stable atherosclerotic plaques by removing its influence on the initiation, progression, and stability of the plaques.

2.5.3.1 Plaque initiation

Initiation of the atherosclerotic plaque is characterized by the retention and modification of LDLs in the arterial wall, which is subsequently followed by chemokines and adhesion molecules expression. Expression of these molecules favours monocytes, neutrophils and T lymphocytes recruitment at the lesion site and their consequential trans-endothelial migration.[576] The CD40/CD40L dyad plays a substantial role in mediating this process. However, the initial factors involved in the expression of members of this dyad on macrophages, endothelial cells, SMCs and T lymphocytes in the developing atherosclerotic lesion are still unclear. Oxidized LDLs (oxLDLs), pathogens (e.g. *Chlamydia pneumonia*), disturbed mechanical forces within the arterial lumen, and heat shock proteins (HSPs) are among the candidates driving CD40 and CD40L expression on these cell types at the onset of atherosclerotic plaque formation.[463, 577] Ligation of CD40 on endothelial cells by CD40L expressed on activated T lymphocytes or platelets induces pro-inflammatory and pro-atherogenic phenotypes of endothelial cells, which are characterized by the expression of adhesion molecules (P-selectin, E-selectin, ICAM-1, VCAM-1, and LFA-1) and secretion of cytokines and chemokines (MCP-1, MCP-3, MIP-1α, MIP-1β, MIP-3α, RANTES, IL-6, IL-8, and IL-15).[367, 463, 578, 579] Thereby, favouring additional leukocyte recruitment, adhesion, and trans-endothelial migration at the lesion site.

2.5.3.2 Plaque progression

Progression of an atherosclerotic plaque into a complex lesion is distinguished by the formation of foam cells in the sub-endothelial space, which derive from phagocytosis of oxLDL particles by monocyte-derived macrophages. This is accompanied by the migration and proliferation of SMCs into the intima, ultimately forming a fibrous cap overlying a lipid-rich necrotic core, as

well as the formation of neovessels that sustain lesion growth. In the sub-endothelial space, interaction of CD40 on monocyte-derived macrophages with CD40L on infiltrating T lymphocytes induces the secretion of cytokines (IL-1β, IL-6, IL-12, and TNFα), and matrix degrading enzymes (MMP-1 and MMP-3) by macrophages, which favour SMCs proliferation and migration from the medial layer to the intima.[485, 571] Additionally, ligation of CD40 on migrating SMCs induces the secretion of MCP-1, IL-1β, IL-6, and IL-8, all of which contribute to the progression of the atherosclerotic lesion.[374, 571] Fibroblasts migrating from the adventitial layer into the atherosclerotic lesion also contribute to the progression of the atherosclerotic lesion by secreting chemoattractant cytokines, such as IL-6 and IL-8, following CD40 ligation.[580]

Irrigation of a developing atherosclerotic plaque is favoured by the formation of neovessels, which is greatly influenced by the CD40/CD40L dyad. Ligation of CD40 on endothelial cells and macrophages induces secretion of pro-angiogenic factors, namely growth factors (VEGF, FGF, and PAF) and MMPs (MMP-1, MMP-2, MMP-3, and MMP-9).[368-371] MMPs degrade the extracellular matrix, therefore allowing endothelial cells to infiltrate and to proliferate within the matrix, a process favoured by growth factors.

Accumulation of these events leads to the formation of a fibro-fatty plaque consisting of infiltrated leukocytes, foam cells, proliferating SMCs, extracellular matrix proteins, and lipid-rich particles.[581]

2.5.3.3 Plaque instability

A stable atherosclerotic plaque is characterized by a thick fibrous cap containing a considerable amount of intact fibrillar collagen overlying a small necrotic core. On the other hand, a vulnerable or unstable rupture-prone atherosclerotic plaque is characterized by a thin fibrous cap containing scarce amounts of collagen overlying a large necrotic core.[463] Rupture of a vulnerable atherosclerotic plaque exposes its thrombogenic necrotic core, as well as components of sub-endothelial matrix, to the circulation leading to thrombus formation and the subsequent partial or complete occlusion of the affected vessel. The CD40/CD40L dyad promotes plaque instability by favouring thinning of the fibrous cap and contributing to the thrombogenicity of the necrotic core.

Thinning of the fibrous cap of an atherosclerotic plaque is due to a disturbance in the balance between extra-cellular matrix production and MMP secretion.[582, 583] Activation of CD40

on SMCs, endothelial cells, and macrophages increases the production of MMP-1, MMP-2, MMP-3, and MMP-9.[350, 351, 368, 376, 378] In addition, MMP-1, MMP-8, MMP-13 have been shown to co-localize with CD40 within human and experimental atherosclerotic lesions.[584, 585] Therefore, MMP accumulation within the atherosclerotic plaque favours degradation of the extracellular matrix, specifically interstitial collagen, which leads to fibrous cap thinning and plaque instability.

The CD40/CD40L dyad enhances the thrombogenic property of the atherosclerotic plaque by increasing its TF content. Indeed, CD40 activation on SMCs, endothelial cells, and macrophages increases TF expression, which is a potent stimulator of the coagulation process and platelet activation.[350, 372, 377] Furthermore, CD40 ligation on endothelial cells decreases the expression of thrombomodulin, which is an anticoagulant factor that inactivates thrombin.[586] Finally, CD40L stimulates platelet activation and stabilizes thrombus formation through its interaction with the $\alpha_{IIb}\beta_3$ integrin, thereby enhancing thrombosis following plaque rupture.[4, 307] Figure 2.10 summarizes the role of the CD40L in atherothrombosis.

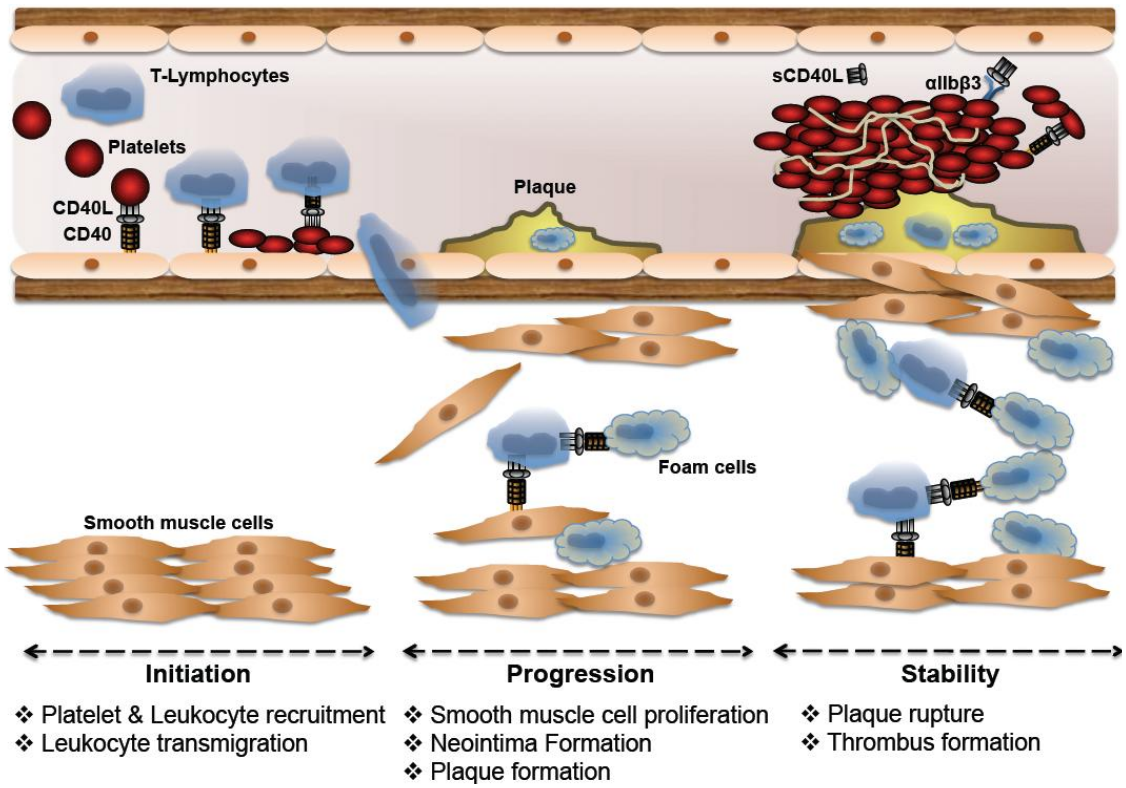

Figure 2.10: Role of CD40L in atherosclerotic plaque initiation, progression and stability. Gaxiola E. (2012). *Atherothrombosis*. INTECH. p. 84.

2.5.4 sCD40L as a marker of cardiovascular diseases

Given the significant contribution of the CD40/CD40L dyad to the initiation, development and instability of atherosclerotic lesions, in addition to the platelet origin of 95% of circulating sCD40L, the sCD40L has gained much attention in the scientific community as a probable predictor of cardiovascular diseases. Indeed, multiple clinical studies have assessed the correlation between the levels of circulating sCD40L and cardiovascular diseases, specifically in stable and acute coronary artery disease, stable and unstable angina and myocardial infarction (Table 2.4). Data from these studies showed increases in the level of circulating sCD40L in patients with acute myocardial infarction, unstable angina, or stable angina, as compared to healthy individuals.[8, 10, 587-593] In addition, the levels of circulating sCD40L were shown to correlate with the severity of ACS.[8, 591] Interestingly, the increase in the levels of sCD40L in these patients is independent of other important inflammatory markers, such as IL-6, soluble VCAM-1, soluble ICAM-1, C reactive protein, and troponin.[588, 594] Therefore, the levels sCD40L could be used as a better diagnostic marker of cardiovascular events.

Multiple clinical studies have assessed the correlation between the levels of plasma sCD40L and complications related to cardiovascular diseases. For instance, the CAPTURE (c7E3 Fab anti-platelet therapy in unstable refractory angina) trial showed a 3 fold higher risk for cardiovascular death and acute myocardial infarction in patients with higher levels of sCD40L.[595] Additionally, the MIRACL (myocardial ischemia reduction with aggressive cholesterol lowering) study showed that elevated levels of sCD40L are an independent risk factor for reoccurring cardiovascular events.[596] Moreover, individuals with a 3459A>G single nucleotide polymorphism within the CD40L gene have a higher risk of acute myocardial infarction incidence.[590] Finally, a small study demonstrated that high plasma levels of sCD40L could predict increased restenosis following coronary angioplasty.[597]

Whether the elevated levels of circulating sCD40L is a cause or a consequence of the underlying cardiovascular disease is still inconclusive. Elevated sCD40L levels could either be the consequence of a higher degree of platelet activation in these patients, or the cause of disease development and the associated complications related to the disease. Therefore, further investigations are required to clarify the role of the CD40/CD40L dyad in cardiovascular disease.

Table 2.4: CD40L in stable and unstable coronary syndrome. (UA: unstable angina; SA: stable angina; AMI: acute myocardial infarction; CAD: coronary artery disease)

Population	Syndromes	sCD40L levels vs. control	Reference
26 patients with UA 29 patients with SA 19 controls	UA SA	↑ -	Aukrust P. et *al.*[8]
20 patients with UA 10 patients with SA 10 controls	UA SA	↑ ↑	Wang Y. et *al.*[593]
20 patients with UA 24 patients with SA 12 patients with AMI 16 controls	UA SA AMI	↑ ↑ ↑	Yan J. et *al.*[589]
15 patients with AMI 12 patients with UA 23 patients with SA 30 controls	AMI UA SA	↑ ↑ -	Peng D. Q. et *al.*[591]
15 patients with AMI 25 patients with UA 15 patients with SA 12 controls	AMI UA SA	↑ ↑ -	Garlichs C. D. et *al.*[10]
109 patients with AMI 201 patients with stable CAD 286 controls	AMI Stable CAD	↑ ↑	Tousoulis D. et *al.*[588]
204 patients with stable CAD 189 controls	CAD	↑	Tayebjee M. H. et *al.*[592]
219 patients with AMI 389 controls	AMI	↑	Antoniades C. et *al.*[587]

Chapter 3

The CD40/CD40L Axis in Platelets

3.1 Differential expression of the CD40/CD40L dyad in platelets

As mentioned in the previous chapter, both members of the CD40/CD40L dyad are present in platelets. CD40 is constitutively expressed on the surface of resting or activated platelets, and its expression levels have been shown to slightly increase following platelet activation.[2, 330] On the other hand, as for other cell types expressing CD40, it remains unclear whether CD40 is arranged as monomers or multimers on the platelet surface (see section 2.1.3 of chapter 2).

Unlike CD40, CD40L is not expressed on resting platelets, but upon activation by thrombin, collagen, or ADP, CD40L is translocated to the platelet surface. The expression of CD40L on platelets seems to coincide with α-granule secretion, which is characterized by P-selectin expression and the release of granule contents (PF4, TGFβ, and PDGF), suggesting that CD40L is present within α-granules; however, further investigations are required to clarify this issue.[330] Once on the platelet surface, CD40L is cleaved into its soluble 18 kDa form within 1 to 2 hours of platelet activation, which accounts for approximately 95% of circulating plasmatic sCD40L levels. However, the mechanisms by which CD40L is cleaved on the platelet surface remain poorly characterized. MMP-9 has been shown to be involved in platelet CD40L cleavage, as its inhibition in Crohn disease patients significantly reduces CD40L shedding.[598] In addition, it has been demonstrated that shedding of sCD40L from the platelet surface requires engagement of CD40.[3] On the other hand, MMP-2 and its association with the $\alpha_{IIb}\beta_3$ integrin have been shown to be required for effective sCD40L release by activated platelets, given that the enzymatic activity of platelet MMP-2 depends on activation of the $\alpha_{IIb}\beta_3$ integrin.[599-601] Moreover, as for CD40L expressed and shed by T lymphocytes, CD40L is also expressed and shed as a trimer by platelets.[3]

Although the presence of the CD40L/CD40 dyad in platelets is now well established, the amount of information regarding its involvement in platelet function is scarce. Soluble CD40L has been shown to induce platelet activation, and secretion of ROS and the chemokine RANTES through binding of CD40.[310, 360, 361] In contrast, sCD40L has been shown to activate platelets and stabilize thrombus formation in an $\alpha_{IIb}\beta_3$-dependent manner.[4, 307] Moreover, platelet CD40L induces an inflammatory response in endothelial cells, which is characterized by chemokine and cytokine secretion and the expression of adhesion molecules.[2]

3.2 Platelet response following CD40 activation

In 2001, the first hint for a functional role of CD40 in platelets was put into evidence, where its ligation was shown to be required for platelet release of sCD40L.[3] In 2003, stimulation of resting platelets with sCD40L was shown to induce responses characteristic of platelet activation, such as P-selectin expression, platelet shape change, and formation of platelet/leukocyte aggregates.[310] These responses were shown dependent on CD40L/CD40 but not CD40L/$\alpha_{IIb}\beta_3$ interactions, as blockade of $\alpha_{IIb}\beta_3$ with epitifibatide does not affect them. Moreover, the formed platelet/leukocyte aggregates are due to the CD40L-induced expression of P-selectin on platelets and its interaction whit PSGL-1 on leukocytes, although CD40L expressed on T lymphocytes has been shown to bind platelet CD40.[361] Activation of CD40 on resting platelets by sCD40L was later shown to induce the secretion of the chemokine RANTES and the production of ROS.[360, 361] These studies also show activation of the p38 MAPK in platelets following sCD40L stimulation; however, the involvement of TRAFs and the NF-κB signalling pathway in sCD40L-induced platelet responses were not evaluated.

3.3 Platelet CD40L in thrombus formation

As mentioned in the previous chapter, CD40L harbours a KGD motif, which allows its recognition by the $\alpha_{IIb}\beta_3$ integrin on platelets.[4, 307] Mice deficient in CD40L ($CD40L^{-/-}$) are presented with an *in vivo* defect in arterial thrombus stability and a delay in arterial occlusion time, despite not having a defect in initial adhesion of single platelets or the time required for first thrombus growth.[4] Infusion of sCD40L into these mice corrects thrombus instability and reduces the arterial occlusion time, suggesting that CD40L is involved in the formation of stable and irreversible platelet aggregates. Furthermore, the effects of sCD40L on thrombus stability depend on its interaction with the $\alpha_{IIb}\beta_3$ integrin and not CD40; since injection of a mutated form of sCD40L that lacks $\alpha_{IIb}\beta_3$ binding dose not correct the haemostatic defects in $CD40L^{-/-}$ mice, as compared to its non-mutated form. Moreover, CD40 deficient mice do not exhibit a haemostatic defect, which further confirms the role of the CD40L/$\alpha_{IIb}\beta_3$ but not the CD40L/CD40 interactions in platelet aggregate stability. This CD40L/$\alpha_{IIb}\beta_3$ interaction was later shown to induce tyrosine phosphorylation within the cytoplasmic tail of the β_3 subunit of the integrin, which ultimately mediates "outside-in" signals by the latter.[307] In light of these studies, the CD40L/$\alpha_{IIb}\beta_3$ interaction probably favours thrombus stability by inducing "outside-in" signals through $\alpha_{IIb}\beta_3$

that mediate Bcl-3 synthesis in platelets, which is involved in fibrin clot retraction, given that platelets from Bcl-3 deficient mice exhibit a defect in fibrin retraction and stabilization.[602, 603]

3.4 Platelet CD40L in endothelial cell activation

In 1998, a functional role for platelet CD40L was put into evidence for the first time.[2] Ligation of CD40 on endothelial cells with CD40L on activated platelets was shown to induce inflammatory and pro-thrombotic responses in endothelial cells, which are characterized by the expression of adhesion molecules (E-selectin, VCAM-1, and ICAM-1) and TF, as well as the secretion of cytokines (MCP-1, IL-6, and IL-8) and MMP-9.[2, 3] These cellular response induced by platelet CD40L on endothelial cells are of great importance in the development of atherosclerotic lesions. Indeed, as mentioned in the first chapter, platelets are among the first (if not the first) cells to be recruited to the atherosclerotic-prone sites and their interaction with the activated endothelium is essential for plaque development. This highlights the importance of the platelet CD40L and endothelial CD40 interaction in inflammation and atherosclerosis.

Hypothesis and Objectives

Multiple lines of evidence now support the existence of a plethora of inflammatory mediators potentially involved in the pathogenesis of vascular disease. Among these, the CD40L/CD40 dyad has gained much attention and circulating levels of sCD40L are now considered as reliable predictors of cardiovascular events. Although the presence of the CD40L/CD40 dyad in platelets is well defined, its exact involvement in platelet function remains elusive. Soluble CD40L has been shown to activate platelets and stabilize thrombus formation in an $\alpha_{IIb}\beta_3$-dependent manner. In contrast, sCD40L has been shown to induce platelet activation, and secretion of ROS and the chemokine RANTES through binding of CD40. Nonetheless, the physiological impact, and the cellular and molecular mechanisms involved in CD40L-induced platelet activation are still undefined.

Hypothesis: Platelets play a central role in the development of cardiovascular diseases, and since platelets constitutively express CD40, elevated levels of circulating sCD40L, as seen in ACS patients, may activate quiescent platelets through CD40 and subsequently contribute to disease progression and complications.

Objectives:

1 - To evaluate the *in vitro* effects of sCD40L on platelet activation and aggregation
2 - To determine the receptor on platelets on which sCD40L mediates its effects
3 - To elucidate the intracellular signalling pathways induced by sCD40L
4 - To evaluate the *in vivo* effects of elevated levels of circulating sCD40L on thrombus formation in a mouse model

Expected results:

1 - sCD40L activates resting platelets and enhances platelet function
2 - sCD40L mediates its effects on platelet function via CD40 and the subsequent TRAF association
3 - Increased levels of circulating sCD40L exacerbate thrombus formation in response to vascular injury

Chapter 4
Scientific Contribution

Background for the first article

Since its discovery, the CD40/CD40L dyad has gained much attention within the scientific community. It has been shown to be involved in immune and inflammatory responses, and to have a broad expression pattern encompassing not only cells of the immune system, but also cells of the circulatory and vascular systems. Elevated levels of circulating sCD40L are tightly linked to incidence of cardiovascular diseases, which makes them potential diagnostic and preventative tools for future cardiovascular disease treatments.

Given that platelets express CD40 and account for 95% of circulating sCD40L, multiple studies have focused on the impact of the CD40/CD40L dyad on platelet function. However, the underlying cellular and molecular mechanisms by which this dyad affects platelet function, including platelet activation, aggregation, and *in vivo* thrombus formation remain poorly characterized; hence the objective of this study.

Authors contributions

Ahmed Hachem: Planning and execution of the flow cytometry (Figures 1, 4, and 5), TRAF association with CD40 (Figure 2), and intracellular and molecular signalling (Figures 4 and 5) experiments. Editing the article.

Daniel Yacoub: Planning and execution of the aggregation (Figures 1, 3, 4, and 5), and scanning electron and confocal microscopy (Figure 3) experiments. Planning of the mouse *in vivo* thrombosis model experiments (Figure 6). Writing and editing the article.

Jean-François Théorêt: Planning of the scanning electron microscopy experiments and editing the article.

Marc-Antoine Gillis: Execution of the mouse *in vivo* thrombosis model experiments (Figure 6).

Walid Mourad: General co-direction. Provided both the WT and mutant (R/Y) forms of CD40L. Participated in the interpretation of the results and helped in editing the article.

Yahye Merhi: General direction. Designed the study, provided intellectual input and helped in editing the article.

Enhanced Levels of Soluble CD40 Ligand Exacerbate Platelet Aggregation and Thrombus Formation through a CD40-Dependent Tumor Necrosis Factor Receptor-Associated Factor-2/Rac1/p38 Mitogen-Activated Protein Kinase Signaling Pathway

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Abstract

Objective—CD40 ligand (CD40L) is a thrombo-inflammatory molecule that predicts cardiovascular events. Platelets constitute the major source of soluble CD40L (sCD40L), which has been shown to influence platelet activation, although its exact functional impact on platelets and the underlying mechanisms remain undefined. We aimed to determine the impact and the signaling mechanisms of sCD40L on platelets.

Methods and Results—sCD40L strongly enhances platelet activation and aggregation. Human platelets treated with a mutated form of sCD40L that does not bind CD40, and $CD40^{-/-}$ mouse platelets failed to elicit such responses. Furthermore, sCD40L stimulation induces the association of the tumor necrosis factor receptor-associated factor-2 with platelet CD40. Noticeably, sCD40L primes platelets through activation of the small GTPase Rac1 and its downstream target p38 mitogen-activated protein kinase, which leads to platelet shape change and actin polymerization. Moreover, sCD40L exacerbates thrombus formation and leukocyte infiltration in wild type mice but not in $CD40^{-/-}$ mice.

Conclusion—sCD40L enhances agonist-induced platelet activation and aggregation through a CD40-dependant tumor necrosis factor receptor-associated factor-2/Rac1/p38 mitogen-activated protein kinase signaling pathway. Thus, sCD40L is an important platelet primer predisposing platelets to enhanced thrombus formation in response to vascular injury. This may explain the link between circulating levels of sCD40L and cardiovascular diseases. **(*Aretioscler Thromb Vasc Biol.* 2010; 30:2424-2433)**

Key Words: platelets ■ signal transduction ■ thrombosis ■ CD40L

Multiple lines of evidence now support a plethora of inflammatory mediators potentially involved in the pathogenesis of vascular disease. Among these, the CD40 ligand (CD40L)/CD40 dyad has been the focus of much attention and circulating levels of soluble CD40L (sCD40L) are now considered as reliable predictors of cardiovascular events.[1-4]

CD40L is a 48-kDa trimeric transmembrane protein belonging to the tumor necrosis factor superfamily originally identified on cells of the immune system.[5, 6] Interaction of CD40L with its respective receptor on B cells, CD40, a 39-kDa glycoprotein from the tumor necrosis factor receptor family, is of critical importance for immunoglobulin isotype switching during the immune response.[7] Today, we know that these two molecules are also present on cells of the vascular system, including endothelial cells, monocytes/macrophages, smooth muscle cells and platelets,[8, 9] and have important implications in inflammatory reactions, through up-regulation of cell adhesion molecules and production of pro-inflammatory cytokines, chemokines, growth factors, matrix metalloproteinases and pro-coagulants.[8, 10-12] The involvement of the CD40L/CD40 dyad in thrombo-inflammation has been highlighted in all pathogenic phases of atherosclerosis, including endothelial dysfunction, platelet activation, thrombosis, and neointima formation.[13-20]

Platelets are highly specialized blood cells of paramount importance in normal hemostasis and thrombo-inflammatory complications. The pioneering work of Heen et *al*. showed that both CD40L and its receptor CD40 are found in platelets.[8] Whereas CD40 is constitutively expressed on platelets, CD40L rapidly appears on the platelet surface following activation, on which it is subsequently cleaved, generating a soluble fragment of 18-kDa, termed sCD40L, accounting for >95% of its plasmatic concentration.[21] Circulating levels of sCD40L in patients have now emerged as strong indicators of cardiovascular risk, as there appears to be a significant correlation between levels of sCD40L and vascular complications such as atherosclerosis and acute coronary syndromes (ACS).[2-4]

Although the presence of the CD40L/CD40 dyad in platelets is well defined, its exact involvement in platelet function remains elusive. Andre and *al*. have shown that CD40L binds to $\alpha_{IIb}\beta_3$ and stabilizes arterial thrombi in mice,[13] whereas others have shown that CD40L can induce platelet activation and secretion of reactive oxygen species and the chemokine RANTES through binding of CD40.[14, 17, 22] Nevertheless, the physiological impact and the mechanisms involved in CD40L-induced platelet activation are poorly characterized. Here we show that

sCD40L primes and enhances agonist-induced activation and aggregation of human platelets through a CD40-mediated tumor necrosis factor receptor-associated factor (TRAF)-2/Rac1/p38 mitogen-activated protein kinase (MAPK)-dependant pathway, which ultimately leads to platelet shape change and actin polymerization. Moreover, we show that enhanced levels of sCD40L exacerbate thrombus formation and leukocyte infiltration in response to vascular injury, in a CD40-dependant manner.

Materials and Methods

Reagents and antibodies

Recombinant human soluble CD40L (sCD40L) was obtained from R&D systems (Minneapolis, MN), while recombinant mouse soluble CD40L (msCD40L) came from Alexis Biochemicals (San Diego, CA). Recombinant human mutant $sCD40L^{R/Y}$ (Arginin (R) 203 for Alanin and Tyrosin (Y) 145 for Alanin) and its wild type counterpart $sCD40L^{WT}$ were generated as previously described.[23] Antibodies against TRAF-1, -2, -3, and -6 were all purchased from Cell Signaling Technology (Beverly, MA). Anti-CD40 antibody used for immunoprecipitation of human CD40 was also from R&D systems, while anti-CD40 antibody used for detection of CD40 by immunoblotting came from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-VASP (Ser^{157}), anti-phospho-p38 (Thr^{180}/Tyr^{182}), anti-VASP (total) and anti-p38 (total), as well as the antibody against Rac1 were all procured from Cell Signaling Technology (Beverly, MA). Antibodies against P-selectin (AK4) and the active form of $\alpha_{IIb}\beta_3$ (PAC-1) were obtained from BD Biosciences (Mississauga, ON). The specific Rac1 NSC23766 inhibitor, the p38 SB203580 inhibitor and the actin polymerization Latrunculin B inhibitor were purchased from Calbiochem (San Diego, CA). Alexa Fluor 555-phalloidin came from Invitrogen (Carisbad, CA). Protein A agarose beads were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY) and p21-activated kinase-protein binding domain (PAK1-PBD) beads were from Cytoskeleton, Inc (Denver, CO). Native type I collagen and adenosine diphosphate (ADP) were from Chronolog Corp. (Havertown, PA), while human thrombin was purchased from Sigma-Aldrich (Oaskville, ON).

Animals

Age- and sex-matched wild type (WT) and $CD40^{-/-}$ mice, both on C57BLK/J6 background, were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed under pathogen free conditions. Handling and care of animals were in compliance with guidelines established by the animal care and ethical committee of the Montreal Heart Institute.

Isolation of human and mouse platelets

Venous blood was drawn from healthy volunteers, free from medication known to interfere with platelet function for at least 10 days before the experiment, in accordance with the guidelines of the human ethical committee of the Montreal Heart Institute. Platelet-rich plasma (PRP) was obtained by centrifugation of acid citrate dextrose (ratio of 1:5) anticoagulated blood at 200g for 15 minutes. Platelets were then pelleted from PRP, to which 1 μg/mL of PGE_1 was added, washed with HBSS-Hank's sodium citrate buffer (138 mM NaCl, 5 mM KCl, 0.34 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM Na_2HCO_3, 5.6 mM Glucose, 10 mM HEPES, 12.9 mM sodium citrate, pH 7.4), also containing PGE_1 (0.5 μg/mL), and finally resuspended in HBSS-Hank's buffer containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$.

Murine washed platelets were prepared from mice anesthetized with a mixture of 75 mg/kg of Ketamine (Vetalar, Belleville, QC) and 0.5 mg/kg of medetomidine (Domitor, Pfizer, Kirkland, QC). Blood was drawn by cardiac puncture in 1-cc syringes containing 50 μL of heparin (1000 iU/mL) and diluted (1:1) with modified Tyrode's buffer (150 mM NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mg/mL BSA, 1 mg/mL dextrose, pH 7.4), containing prostacyclin (0.2 μg/mL). PRP was obtained by centrifugation of blood at 164g for 8 minutes, to which prostacylin (0.1 μg/mL) was added, and platelets were pelleted by centrifugation at 1000g for 5 minutes. Platelets were finally resuspended in modified Tyrode's buffer.

Human and mouse platelets were adjusted to 250 x 10^6 platelets/mL, unless otherwise specified, and allowed to rest at 37ºC for 30 minutes before further manipulation.

Flow cytometry analysis of platelet activation

Translocation of platelet P-selectin and activation of $\alpha_{IIb}\beta_3$ were measured by flow cytometry, as previously described.[24] Platelets were preincubated with sCD40L prior to cell stimulation with

agonists, fixed with 1% paraformaldehyde, washed and stained with saturating concentrations of anti-P-selectin antibody (AK4-PE conjugated) for 30 minutes or its isotype-matched control IgG. For measurement of $\alpha_{IIb}\beta_3$ activation, PAC-1 antibody (FITC-conjugated) was incubated with platelet suspensions prior to activation with sCD40L and agonists. Samples were analyzed (20,000 events) on an Altra flow cytometer (Beckman Coulter, Mississauga, ON) and platelets were gated by their characteristic forward and side scatter properties.

Measurement of platelet aggregation

Aggregation of human and mouse washed platelets was monitored on a four-channel optical aggregometer (Chronolog Corp., Havertown, PA) under shear (1000 rpm) at 37ºC. sCD40L (human, mouse and the mutated R/Y and WT forms) was pre-incubated with platelet suspensions under static conditions at 37ºC, 30 minutes prior to addition of agonists, and traces were recorded until stabilization of platelet aggregation was reached.

Immunoprecipatation of CD40

Platelets (500 x 10^6/mL) were stimulated as indicated, pelleted, then lysed into ice-cold RIPA lysis buffer (1% NP-40, 0.25% deoxycholic acid, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 mM sodium-orthovanadate, 1 mM sodium fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 2 μg/mL benzamidin) for 1 hour at 4ºC. Lysates were sonicated on ice and pre-cleared with 100 μL of protein A agarose beads for 15 minutes at 4ºC. Beads were then pelleted and the supernatant was incubated with 5 μg/mL of anti-CD40 antibody overnight at 4ºC. Samples were treated with 100 μL of protein A agarose beads for 1 hour at 4ºC and precipitated by centrifugation, washed three time with ice-cold RIPA lysis buffer, resuspended in 2X Laemmli buffer, and boiled for 5 minutes. Supernatants were analyzed by immunoblot for the presence of TRAF-1, -2, -3, and -6, as well as CD40.

SDS-PAGE and immunoblotting

Proteins were resolved in 8% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk for 1 hour, washed three times with TBS/T (150 mM NaCl, 20 mM Tris, pH 7.4, 0.1 % Tween-20) and incubated with appropriate primary antibody overnight at

4ºC. Following washing steps, membranes were labeled with horseradish peroxidase-conjugated secondary antibody for 1 hour, washed and bound peroxidase activity was detected by enhanced chemiluminescence (PerkinElmer Life Sciences, Waltham, MA).

Rac1 activation assay

Platelets (1 x 10^9/mL) were treated with sCD40L and reactions were terminated by addition of an equi-volume of ice-cold 2X lysis buffer (50 mM HEPES, 300 mM NaCl, 2% Igepal, 20% glycerol, 20 mM $MgCl_2$, 50 mM sodium fluoride, 2 mM EDTA, 2 mM sodium orthovanadate, 20 μg/mL leupeptin, and 20 μg/mL aprotinin). Lysates were then clarified by centrifugation at 14,000g for 5 minutes at 4ºC and supernatants were treated with 10 μg of PAK1-PBD beads for 1 hour at 4ºC. Beads were centrifuged and washed three times with ice-cold lysis buffer. They were then resuspended in 2X Laemmli sample buffer, boiled for 5 minutes, and supernatants were analyzed by immunoblotting for the presence of Rac1.

GDP and GTPγS were used as negative and positive controls, respectively. Supernatants from lysates were treated with either GDP (1 mM) or GTPγS (100 μM) for 15 minutes at room temperature, and reactions were terminated by placing samples on ice and adding $MgCl_2$ (60 mM). Samples were then clarified by centrifugation, incubated with PAK1-PBD beads, and treated with the same procedure as described above.

Scanning electron microscopy

Platelets treated or not with sCD40L were allowed to immobilize on 2% bovine serum albumin (BSA)-treated glass cover slips for 30 minutes at 37ºC. Samples were then fixed in 2% paraformaldehyde overnight at 4ºC. Dehydration of surfaces was achieved by placing samples in ethanol/water followed by amyl acetate/ethanol baths for 15 minutes each, increasing the ethanol/water proportion from 30% to 100% and the amyl acetate/ethanol proportion from 25% to 100%. Slides were subsequently coated with gold palladium particles and analyzed on a Hitachi S-4700 Field Emission Gun Scanning Electron Microscope (FEG-SEM).

Actin polymerization assay

Platelets treated or not with sCD40L in the presence or absence of Latrunculin B were allowed to immobilize on 2% BSA-treated glass cover slips for 30 minutes at 37ºC. Samples were then

fixed in 2% paraformaldehyde for 20 minutes at room temperature. Platelets were permeabilized with 0.05% Triton X-100 in 2% BSA and stained with Alexa555-phalloidin in 1% BSA for F-actin detection. Series of fluorescent confocal images (Z stacks) were acquired with a LSM 510 confocal microscope (Zeiss. Oberkochen, Germany). Alexa555-phalloidin was visualized using a 543-nm helium-neon laser line and a 63×/1.4 plan-apochromat objective (Zeiss) was used for magnification (voxel size is 28 nm X 28 nm X 250 nm (X,Y,Z)). Final images were produced using the Zeiss LSM 510 software and saved as LSM files.

Thrombosis model

The effect of sCD40L on thrombus formation was determined in a $FeCl_3$ mouse carotid injury model, as previously described.[25, 26] Briefly, anesthetized C57BL6 WT and $CD40^{-/-}$ mice were injected with sCD40L (0.25 mg/Kg) through the jugular vein, 5 minutes prior to $FeCl_3$ (4%) injury of the right carotid artery, and blood flow and time to thrombotic occlusion (blood flow of 0 mL/minute) were measured with the aid of a miniature ultrasound flow probe (0.5 VB 552, Transonic Systems Ithaca, NY) interfaced with a flow meter (T206, Transonic Systems) and a computer-based data acquisition program (Iox 2.2.17.19, Emka, Falls Church, VA). Mouse plasmatic sCD40L was measured by ELISA (Bender MedSystems, San Diego, CA), according to the manufacturer's instructions.

Histology and Immunostaining

Following *in vivo* thrombosis measurements in mice, injured and contralateral non-injured carotid arteries were excised, fixed in 10% buffered formalin and analyzed by hematoxylin and eosin staining or CD45 immunostaining for leukocytes infiltration within the thrombus mass, as previously described.[25] Briefly, sections were embedded in paraffin, sectioned at 6 microns, and stained with hematoxylin and eosin, or an anti-CD45 antibody (Santa Cruz, Santa Cruz, CA). Samples were visualized using an Olympus BX60 microscope (Olympus imaging America Inc, Center Valley, PA) and images were captured with a Retiga 2000R camera (QImaging Corporation, Surrey, BC) and visualized through the Image Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD).

Measurement of intracellular calcium flux

Platelets in HBSS-Hank's sodium citrate buffer were incubated with 5 μg/ml Fluo-4 AM for 30 minutes at 37°C. Platelets were then removed from excess Fluo-4 AM by centrifugation and resuspended in final HBSS-Hank's buffer containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$. Platelets were then placed onto a FluoroDish and mounted on a LSM 510 confocal microscope (Zeiss. Oberkochen, Germany). sCD40L (1 μg/ml) or thrombin (0.1 U/ml) was added to the platelet suspension and series of fluorescent confocal images were acquired in real-time at a rate of 30 images/second (excitation wavelength selected was 488 nm).

Dense granule release

ATP release was measured by a Lumi-Aggregometer according to the manufacturer's instructions (Chrono-log Corp. Havertwon, PA). Briefly, 25 μl Luciferin-Luciferase (Chrono-Lume) reagent was added to a 475 μl platelet suspension 2 minutes before addition of sCD40L (1 μg/ml) or thrombin (0.1 U/ml).

Dense granule release was measured by mepacrine uptake into platelets.[27] Platelets in HBSS-Hank's sodium citrate buffer were incubated with 5 μM mepacrine (Quinacrine dihydrochloride, Sigma-Aldrich) for 30 minutes at 37°C. Platelets were then removed from excess mepacrine by centrifugation and resuspended in final HBSS-Hank's buffer containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$. Secretion of dense body constituents was evaluated by flow cytometry as the fluorescence remaining in platelets upon stimulation with sCD40L or thrombin; in comparison to resting platelets.

Statistical analysis

Results are presented as mean ± SEM of at least 3 independent experiments. Statistical comparisons were done using a one-way ANOVA, followed by a Dunnetts-*t*-test for comparison against a single group. Data with $P<0.05$ were considered statistically significant.

Results

sCD40L Enhances Platelet Activation and Aggregation Through Interaction With CD40

We first evaluated the functional effects of sCD40L on platelet activation and aggregation, as it remains poorly characterized. Incubation of platelets with sCD40L alone had no effect on platelet aggregation (Figure 1A, upper panel) but led to a significant and dose-dependent increase of platelet aggregation induced by a subthreshold or priming concentration of collagen, thrombin or ADP (Figure 1A), indicating that this is a broad platelet phenomena and not agonist-specific. As sCD40L showed a significant impact on platelet aggregation, we sought to determine its effect on platelet activation. As expected, sCD40L was unable to trigger activation of $\alpha_{IIb}\beta_3$ on resting platelets, whereas it caused a significant increase in P-selectin expression (Figure 1B). However, $\alpha_{IIb}\beta_3$ activation and P-selectin expression were both significantly enhanced in the presence of subthreshold concentrations of collagen, thrombin and ADP.

Because it has been shown that $\alpha_{IIb}\beta_3$, in addition to CD40, can constitute a CD40L receptor on the platelet surface,[13, 28] it was imperative to investigate through which receptor sCD40L acts. To address this issue, we first generated a mutant recombinant sCD40L ($sCD40L^{R/Y}$), that does not bind CD40 while retaining $\alpha_{IIb}\beta_3$ binding (Supplemental Figure I) and showed that this molecule failed to enhance platelet aggregation induced by a priming concentration of collagen compared to its WT counterpart ($sCD40L^{WT}$) (Figure 1C). Second, unlike platelets from WT mice, platelets from $CD40^{-/-}$ mice were insensitive to mouse sCD40L in response to a subthreshold concentration of collagen (Figure 1D). These data clearly show that sCD40L enhances platelet function through interaction with its counterreceptor CD40.

sCD40L Triggers TRAF-2 Association With CD40

It is well established that the TRAF family is tightly linked to CD40 signaling in immune cells. The TRAF family comprises six known members, among which TRAF-1, -2, -3 and -6 have been the most studied, although their expression in platelets is yet to be determined. We therefore investigated the expression of the major TRAF members and their association with CD40 on sCD40L stimulation. Interestingly, we found that in addition to CD40, platelets express TRAF-1, -2, and -6, and traces of TRAF-3 were detected (Figure 2A). Most importantly, only TRAF-2 associates with CD40 after stimulation of resting platelets with sCD40L, whereas none of these members were shown to be associated with CD40 at baseline conditions (Figure 2A and

2B). In addition, sCD40L^{WT}, but not sCD40L$^{R/Y}$, caused association of TRAF-2, further supporting the contribution of CD40 in response to sCD40L.

sCD40L Induces Platelet Shape Change and Actin Polymerization

In search of the underlying cellular and molecular events involved in the effects of sCD40L on platelet function, we first found that stimulation of resting platelets with sCD40L caused a significant morphological shape change characterized by an increase in lamellipodia and filopodia formation (Figure 3A). Second, sCD40L induced actin polymerization, as noted by the increase in F-actin staining in treated platelets (Figure 3B). To determine the implication of these cytoskeletal and morphological changes in sCD40L-induced potentiation of platelet aggregation, we pretreated platelets with latrunculin B, a specific inhibitor of actin polymerization, and found that it completely reversed sCD40L's capacity to increase platelet aggregation (Figure 3C and 3D).

The Small GTPase Rac1 and p38 MAPK Are Required for sCD40L Signaling

The Rho family GTPase member Rac1 and the VASP represent key signaling components required for cytoskeletal reorganization and shape change in platelets. Phosphorylation of VASP, particularly at Ser^{157}, regulates its anticapping activity and thereby promotes platelet filopodia formation.[29] As shown in Figure 4A, stimulation of resting platelets with sCD40L, but not with sCD40L$^{R/Y}$, induced phosphorylation of VASP on Ser^{157} and activation of Rac1. To assess the implication of the small GTPase Rac1, we used a specific Rac1 inhibitor, NSC23766. Pretreatment of platelets with NSC23766 significantly reversed sCD40L's ability to enhance platelet aggregation (Figure 4B) and P-selectin expression (Figure 4C) in resting platelets.

Activation of p38 MAPK is of significant importance in CD40 signaling, and in platelets it could therefore act as a downstream target of Rac1 in response to sCD40L. In fact, sCD40L induced a time-dependent activation of p38 MAPK and inhibition of Rac1 with NSC23766 significantly reduced its activation, indicating that p38 MAPK does indeed act as a downstream target of Rac1 (Figure 5A). Furthermore, specific blockade of p38 MAPK with SB203580, which prevented its phosphorylation (Supplemental Figure II), impaired the effects of sCD40L on platelet P-selectin expression (Figure 5B) and aggregation (Figure 5C), thus highlighting its

implication in these responses. These data establish Rac1 and its downstream effector p38 MAPK as key components involved in sCD40L signaling in platelets.

sCD40L Exacerbates Thrombus Formation and Leukocyte Infiltration

To date, no direct correlation between circulating levels of sCD40L and thrombosis has been established, and this could be of important clinical and physiopathological relevance. In order to explore this aspect, we injected sCD40L (0.25 mg/kg or approximately 5 μg/mouse) into WT and $CD40^{-/-}$ mice before vascular injury, thereby enhancing its plasma circulating levels to 47.5 ± 3.7 ng/mL (n=4), and assessed thrombus formation. Infusion of sCD40L into WT mice significantly exacerbated thrombus formation, in comparison to vehicle-treated mice, in which occlusion was only partial (Figure 6). Interestingly, $CD40^{-/-}$ mice were protected from increased levels of circulating sCD40L, as no significant difference in thrombosis between treated and non-treated groups was observed (Figure 6A and 6B), confirming the *in vivo* contribution of the CD40 receptor in sCD40L-induced thrombus formation. Because sCD40L induces surface expression of P-selectin on platelets (Figure 1B), we sought to determine the extent of leukocyte infiltration within the thrombus because of its involvement in platelet/leukocyte interactions, a well-established aspect of hemostasis known to potentiate thrombus formation.[30] Leukocyte infiltration, as measured by CD45 immunostaining (Figure 6C) and optical quantification of histological sections post-thrombosis (Figure 6D), was significantly increased in WT but not in $CD40^{-/-}$ mice that received sCD40L. These results establish a direct *in vivo* correlation between circulating levels of sCD40L and arterial thrombosis, while highlighting the requirement of the CD40 receptor in this process.

Discussion

CD40L has gained much attention over the years for its involvement in the pathogenesis of atherosclerosis and today, numerous clinical studies show a tight association between levels of sCD40L and vascular diseases.[1-4] The majority of sCD40L found in plasma is believed to originate from activated platelets, and this in turn has been shown to influence platelet activation. Here, we provide novel insights into the regulation of platelet function by CD40L, as we show that sCD40L primes platelets and enhances aggregation through a CD40-mediated TRAF-2/Rac1/p38 MAPK-dependant pathway. This ultimately leads to shape change and actin

polymerization. Furthermore, we establish a direct correlation between circulating levels of sCD40L and thrombus formation.

To get insights into the cellular and molecular impact of sCD40L on platelet function, it was important that we first investigate its effect on platelet aggregation and activation. sCD40L was unable to trigger aggregation of resting platelets or to induce activation of the integrin $\alpha_{IIb}\beta_3$, whereas it significantly increased the expression of P-selectin, in accordance with previous studies.[14, 17, 22] This may attributed to the fact that sCD40L alone does not affect intraplatelet calcium influx (Supplemental Figure III) or dense granule secretion, as assessed by ATP release and mepacrine uptake (Supplemental Figure IV), which are necessary for integrin activation and platelet aggregation. However, we found that sCD40L strongly enhanced platelet aggregation, P-selectin expression and $\alpha_{IIb}\beta_3$ activation in response to subthreshold concentrations of platelet agonists, indicating that it rather acts as a broad and potent primer of platelets, such as matrix metalloproteinase-2, plasma protein growth arrest-specific 6 and stromal derived factor-1α.[31-33] Moreover, this priming phenomenon holds true for B cell proliferation, as sCD40L requires costimulation with interleukin-4 for immunoglobulin E secretion.[34, 35] Taken together, this would indicate that CD40L acts as an accessory, but important, element in platelet function.

One important aspect of controversy regards the identification of the platelet receptor for sCD40L. In our study, we were able to show by molecular and genetic approaches that sCD40L enhances platelet function by interacting with CD40. These results are in agreement with previously published data showing that sCD40L can induce platelet activation and secretion of reactive oxygen species and the chemokine RANTES through binding to CD40.[14, 17, 22] In addition to CD40, its constitutively expressed receptor on platelets, it has been reported that sCD40L binds to $\alpha_{IIb}\beta_3$.[13, 28] However, this occurs in the presence of 40 μg/mL of rsCD40L on preactivated platelets ($\alpha_{IIb}\beta_3$ already in its active form), which is 40 times higher than the concentration used in the present study. This may suggest that CD40 and $\alpha_{IIb}\beta_3$ constitute the high- and low-affinity receptors for sCD40L, respectively. However, additional studies are necessary to specifically address this issue. Thus, it appears that increased levels of circulating sCD40L, as seen in ACS patients, prime platelets via CD40, whereas its interaction with $\alpha_{IIb}\beta_3$ at the site of vascular injury, where higher levels of sCD40L may be generated, stabilizes platelet aggregates.

CD40 signaling in immune and endothelial cells requires its association to TRAF proteins. To our knowledge, the expression of TRAF members in platelets and their association with platelet CD40 following sCD40L stimulation are still unknown. Here, we found that only TRAF-2 associates with CD40 upon ligation, indicating that it may be responsible for sCD40L/CD40-induced signaling in platelets. Although present in platelets, neither TRAF-1 nor TRAF-6 associate with CD40, indicating that they may rather play a role in tumor necrosis factor signaling. Even though CD40 contains binding sites for both TRAF-2 and TRAF-6, binding of either one is sufficient to induce activation of nuclear factor-κB in B cells.[36] This would also hold true for platelets, as binding of TRAF-2 alone appears sufficient to trigger downstream signaling.

In platelets, Rac1 and VASP govern shape change, cytoskeletal reorganization and spreading, through lamellipodia and filopodia formation, respectively. VASP is typically phosphorylated on two main residues, Ser^{239} and Ser^{157}. Platelet inhibitors induce phosphorylation of both residues, whereas agonists that cause platelet activation trigger phosphorylation of VASP on Ser^{157}, thereby promoting anticapping activity and favoring actin polymerization and filopodia formation.[37, 38] Our finding that sCD40L promotes phosphorylation of VASP on Ser^{157} provides evidence for its role as an inducer of shape change in platelets. As further support for the role of sCD40L in these processes, we highlight its capacity to induce activation of the small GTPase Rac1. Rac1 activation in response to sCD40L was shown to be of physiological importance, given that its specific inhibition significantly reduced sCD40L's ability to potentiate platelet aggregation and P-selectin expression. Interestingly, in accordance with our results, it has recently been shown that in endothelial and WEHI 231 B cells, CD40 signaling-induced reactive oxygen species generation requires activation of Rac1.[19, 39] Moreover, in immune cells, CD40L is classically known to induce activation of MAPKs, such as p38. Here, we were able to confirm that sCD40L induces activation of p38 MAPK in resting platelets, [17, 22] and further highlight its involvement in platelet activation and aggregation as a downstream effector of Rac1 in response to sCD40L. Consistent with its role in sCD40L-induced shape change and platelet priming, p38 MAPK has been shown to be an important regulator of actin polymerization and platelet spreading.[40]

Whether enhanced levels of sCD40L seen in patients with ACS are a consequence of increased platelet activation or a predetermining cause of these complications (or perhaps both) is still unknown. Here, we provide novel evidence demonstrating a direct correlation between

enhanced levels of sCD40L and thrombosis. Mice that received sCD40L prior to vascular injury showed increased thrombus formation, indicating that they were predisposed to thrombotic stimulus. Our study adds new insights to a previous work showing that $CD40L^{-/-}$ mice develop unstable thrombi and that this deficiency can be overcome by infusion of 1.6 mg/kg sCD40L in a $\alpha_{IIb}\beta_3$-dependent manner.[13] Here, a different approach was employed, as we increased circulating levels of sCD40L in mice by injecting 0.25 mg/kg to reach approximately 50 ng/mL of plasma sCD40L, to mimic conditions similar to those seen in patients with ACS. In this experimental setting, sCD40L was shown to exacerbate thrombosis in WT mice but not in $CD40^{-/-}$ mice, indicating that enhanced levels of sCD40L prime resting platelets in a CD40-dependent manner, predisposing them to enhanced thrombus formation. Because $CD40^{-/-}$ mice do not show a defect in thrombus formation, it is likely that CD40 is not essential for platelet hemostasis in the absence of significant levels of sCD40L but rather has a pathological importance in atherothrombosis in the presence of elevated levels of circulating sCD40L as seen in patients with ACS. Given that sCD40L induces platelet P-selectin expression, which is involved in platelet/leukocyte interactions[14, 17, 20] and stabilization of thrombus mass,[30] we measured the extent of leukocyte infiltration within the thrombus. We found significantly more leukocytes within the thrombus of sCD40L-treated mice, presumably accounting for the increase in thrombus formation seen in these animals, concomitantly with enhanced platelet predisposition to activation and aggregation in response to vascular injury.

Elevated levels of sCD40L are associated with increased cardiovascular risk, as seen in patients with ACS, such as unstable angina[41] and acute myocardial infarction.[3] However, the relative importance of the soluble form versus the membrane-bound form in thrombus formation is still unknown. It is likely that both forms are involved in primary hemostasis, whereas under pathological conditions, thrombosis is exacerbated by increased levels of the soluble form. Nevertheless, the involvement of CD40L in atherogenesis, thrombus formation, platelet-mediated inflammation, and plaque destabilization makes it a potential therapeutic target in atherothrombosis. Accordingly, it would be pertinent for future clinical studies to evaluate the degree of platelet priming in ACS patients, specifically through sCD40L-induced TRAF-2 association with CD40, for instance. Hence, a direct clinical link between sCD40L and platelet function could be established for pharmacological targeting.

In summary, aside from this newly identified TRAF-2/Rac1/p38 MAPK pathway involved in platelet priming in response to sCD40L, we highlight the relevance of sCD40L in a physiopathological setting of platelet function and thrombus formation. Indeed, enhanced levels of sCD40L potentiate platelet aggregation and exacerbate thrombus formation and leukocyte infiltration in response to vascular injury, in a CD40-dependant manner. This study provides novel evidence for the regulation of platelet function by sCD40L and may partly explain the link between levels of circulating sCD40L and the occurrence of cardiovascular complications. The CD40L/CD40 axis may ultimately represent a therapeutic target in the treatment of thrombo-inflammatory diseases.

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Disclosures

None.

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Figure legends

Figure 1: sCD40L enhances platelet activation and aggregation through interaction with CD40. A, Dose-dependent effect of sCD40L on platelet aggregation. Platelets were preincubated with the indicated concentrations of sCD40L for 30 minutes at 37ºC, and aggregation was induced by a priming dose of collagen (0.25 μg/mL). Upper aggregation traces show the effect of sCD40L alone (1 μg/mL). Histogram represents the mean of data of platelet aggregation in response to sCD40L alone (no agonist) or in the presence of a priming (low) dose of collagen (0.25 μg/mL), thrombin (0.02 U/mL), or ADP (5 μM). High doses of agonists (5 μg/mL collagen, 0.5 U/mL thrombin, or 20 μmol/L ADP) were used as positive controls (n=5; **P*<0.05 versus low dose). B, Effect of sCD40L on P-selectin (CD62P) expression and $\alpha_{IIb}\beta_3$ activation, as assessed by flow cytometry. Left plots represent resting platelets (- collagen: gray for baseline, black for sCD40L). Right plots represent platelets in the presence of a priming concentration of collagen (+ collagen: gray for priming dose of collagen, black for priming dose + sCD40L, and white for high-dose collagen alone, as positive control). Histograms represent the mean of data of plots for CD62P expression and $\alpha_{IIb}\beta_3$ activation in response to sCD40L alone (no agonist) or in the presence of a priming (low) dose of collagen (1 μg/mL), thrombin (0.02 U/mL), or ADP (5 μM). High doses of agonists (collagen 5 μg/mL, thrombin 0.5 U/mL or ADP 20 μM) were employed as positive controls (n=5; †*P*<0.05 versus baseline and **P*<0.05 versus low dose). C, Effect of recombinant mutant $sCD40L^{R/Y}$ and $sCD40L^{WT}$ on human platelet aggregation. Platelets were incubated with 1 μg/mL of $sCD40L^{R/Y}$ or $sCD40L^{WT}$ for 30 minutes at 37ºC, and aggregation was induced by a priming dose of collagen (0.25 μg/mL). sCD40L was employed as a positive control. D, Effect of mouse (m) sCD40L on WT and $CD40^{-/-}$ mouse platelet aggregation. Platelets were incubated with msCD40L (1 μg/mL) for 30 minutes at 37ºC, and aggregation was induced by a priming concentration of collagen (1 μg/mL). Data in parentheses shown in C and D represent the mean ± SEM of n=4.

Figure 2: sCD40L induces TRAF-2 association with CD40. A, Representative blots of lysates from resting (baseline) or sCD40L (1 μg/mL)-stimulated platelets immunoprecipitated using an anti-CD40 monoclonal antibody and analyzed by SDS-PAGE for TRAF-1, -2, -3, and -6. B, Histogram represents the mean of data of blots in A, expressed as arbitrary units of optical density (n=5; **P*<0.05 versus baseline). C, CD40 was immunoprecipitated from lysates of

resting (baseline), or 1 μg/mL of sCD40L$^{R/Y}$ or sCD40L^{WT} stimulated platelets. Immunoprecipitates were then analyzed by SDS-PAGE for TRAF-2. Blot shown is representative of 4 independent experiments.

Figure 3: sCD40L induces platelet shape change and actin polymerization. A, Scanning electron micrographs of resting (baseline), sCD40L, sCD40L^{WT}, and sCD40L$^{R/Y}$ (all at 1 μg/mL)-stimulated platelets at low (top: ×2,500) and high (bottom: ×30,000) magnifications. Images are representative of 4 independent experiments. B, Actin polymerization in resting (baseline) and in sCD40L (1 μg/mL)-treated platelets, with or without 10 μmol/L latrunculin B (Lat), as assessed by confocal microscopy. Images are representative of 3 independent experiments. C, Platelets were preincubated with latrunculin B (10 μmol/L) or vehicle dimethyl sulfoxide for 15 minutes at 37ºC. Cells were then left unstimulated (control) or incubated with sCD40L (1 μg/mL) and aggregation was triggered by a priming dose of collagen (0.25 μg/mL). D, Represents the mean of data of traces in C (n=3; **P*<0.05).

Figure 4: The Rho-GTPase Rac1 is required for sCD40L signaling. A, VASP phosphorylation and Rac1 activation were detected from platelets left untreated (baseline) or incubated with sCD40L (1 μg/mL) for the indicated time. Control experiments were performed in parallel with 1 μg/mL of sCD40L$^{R/Y}$ and sCD40L^{WT} after 30 minutes stimulation. Blots are representative of 4 independent experiments. B, Effect of Rac1 inhibition on sCD40L-induced potentiation of platelet aggregation. Platelets were preincubated with the Rac1 inhibitor NSC23766 (50 μmol/L) or vehicle demethyl sulfoxide for 15 minutes at 37 ºC. Cells were then left unstimulated (control) or incubated with sCD40L (1 μg/mL). Aggregation was then monitor in the presence of a priming dose of collagen (0.25 μg/mL). Histogram represents the mean of data of aggregation traces (n=4; **P*<0.05 versus sCD40L). C, Platelets were left untreated (baseline) or incubated with sCD40L (1 μg/mL) with or without NSC23766 (50 μmol/L) and assessed by flow cytometry for CD62P expression. Histogram shows the mean data of overlay plot (n=3; **P*<0.05 versus sCD40L).

Figure 5: The p38 MAPK is an important Rac1 downstream target in response to sCD40L. A, Platelets were left untreated (time 0) or stimulated with sCD40L (1 μg/mL), with or without

NSC23766 (50 μmol/L), for the indicated time and assessed for p38 MAPK phosphorylation by SDS-PAGE. Results are expressed as fold increase in optical density (O.D.) over time 0 (n=4; *P<0.05). B, Platelets were left unstimulated (baseline) or incubated with sCD40L (1 μg/mL), with or without the p38 MAPK inhibitor SB203580 (5 μmol/L) and assessed for CD62P by flow cytometry. Histogram represents the mean of data of overlay plot (n=5; *P<0.05 versus sCD40L). C, Platelets were preincubated with SB203580 (5 μmol/L) or vehicle demethyl sulfoxide for 15 minutes at 37ºC. Cells were then left unstimulated (control) or incubated with sCD40L (1 μg/mL), and aggregation was then induced by a priming dose of collagen (0.25 μg/mL). Histogram represents the mean of data of aggregation traces (n=4; *P<0.05 versus sCD40L). p-p38 indicates phosphorylated p38

Figure 6: sCD40L exacerbates thrombus formation and leukocyte infiltration. A, WT and $CD40^{-/-}$ mice were injected with sCD40L (0.25 mg/kg) or vehicle (control) prior to $FeCl_3$-induced injury of the right carotid artery, and thrombus formation was monitored by residual blood flow measurements. Traces are representative of 6 mice/group (*P<0.05 versus control). B, Representative histological sections stained with hematoxylin–eosin and observed by optical microscopy (magnification, ×20). C, CD45-positive cells, observed by immunostaining of the injured carotid arteries, were mainly detectable in sCD40L-treated WT mice (arrows), indicative of leukocyte incorporation within the thrombus mass (magnification, ×40). D, Histogram shows quantitative measurements of leukocytes/thrombus section (n=6, *P<0.05 versus WT).

Figure 1

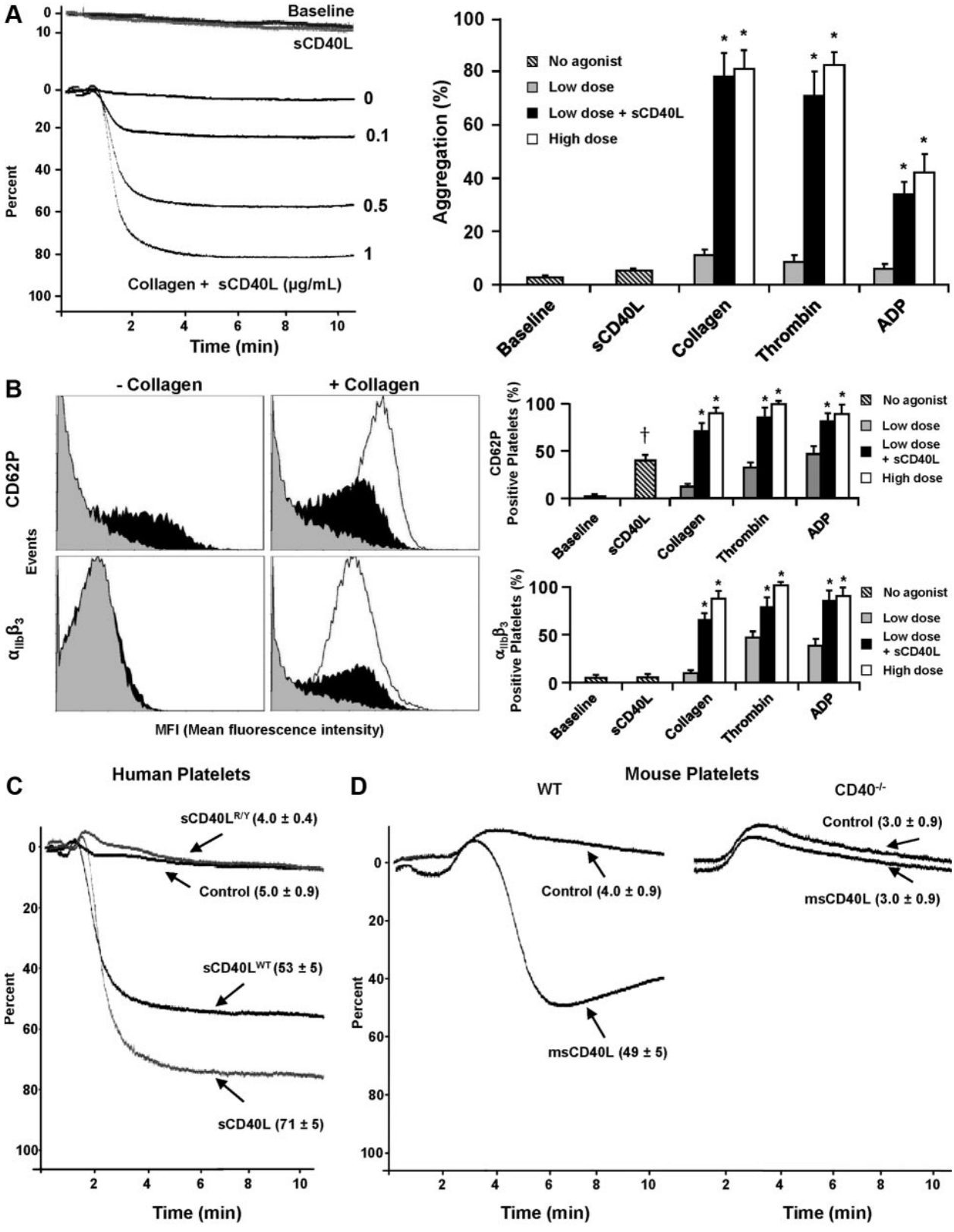
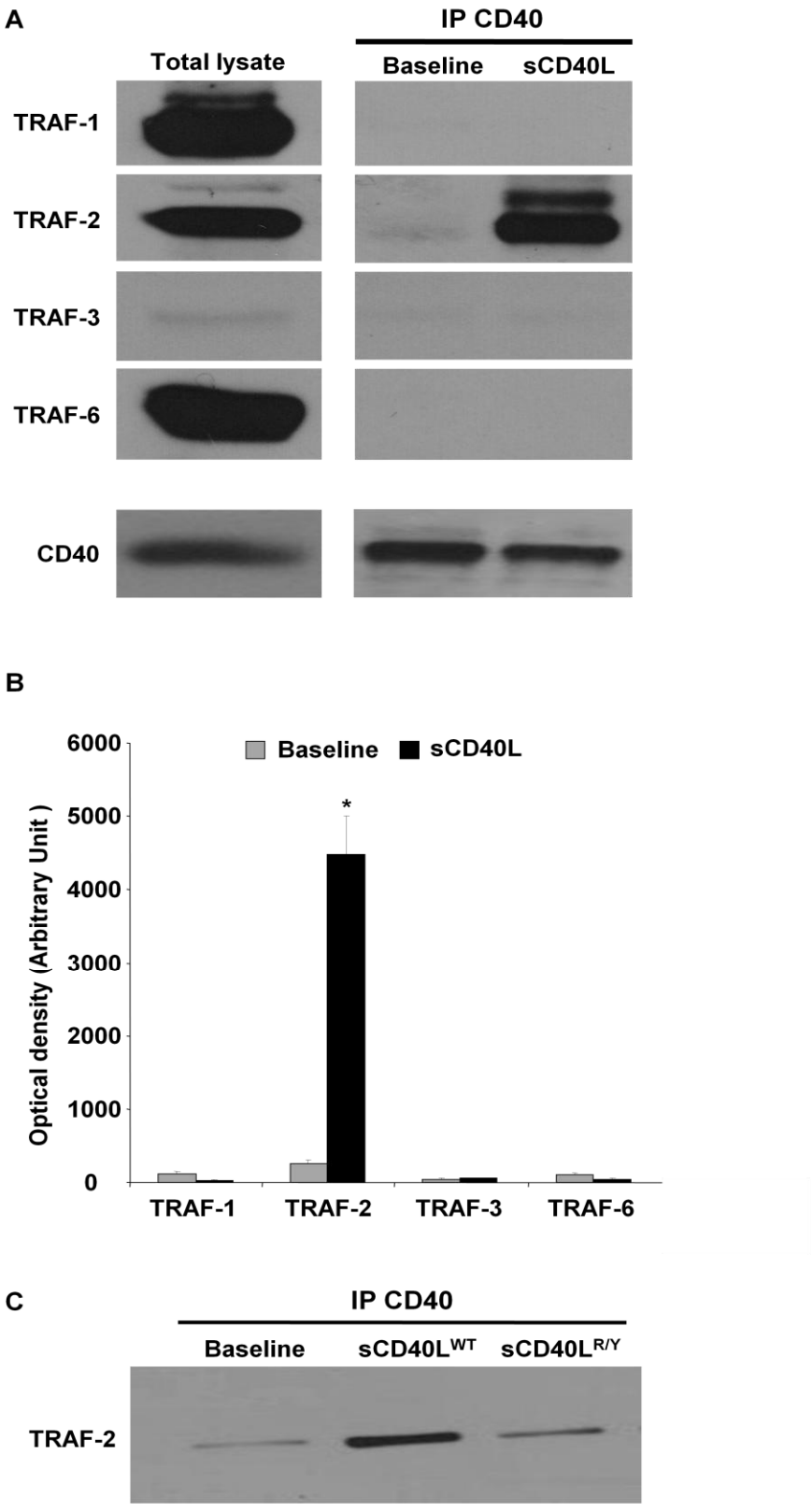

A
Baseline
sCD40L
0
0.1
0.5
1
Collagen + sCD40L (µg/mL)
Percent
Time (min)
Aggregation (%)
No agonist
Low dose
Low dose + sCD40L
High dose
Baseline
sCD40L
Collagen
Thrombin
ADP
B
- Collagen
+ Collagen
CD62P
Events
αIIbβ3
MFI (Mean fluorescence intensity)
CD62P Positive Platelets (%)
αIIbβ3 Positive Platelets (%)
C
Human Platelets
sCD40LR/Y (4.0 ± 0.4)
Control (5.0 ± 0.9)
sCD40LWT (53 ± 5)
sCD40L (71 ± 5)
D
Mouse Platelets
WT
CD40-/-
Control (4.0 ± 0.9)
msCD40L (49 ± 5)
Control (3.0 ± 0.9)
msCD40L (3.0 ± 0.9)

Figure 2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IP CD40
Total lysate
Baseline
sCD40L
TRAF-1
TRAF-2
TRAF-3
TRAF-6
CD40
B
Baseline
sCD40L
Optical density (Arbitrary Unit)
6000
5000
4000
3000
2000
1000
0
*
TRAF-1
TRAF-2
TRAF-3
TRAF-6
C
IP CD40
Baseline
sCD40L^WT
sCD40L^R/Y
TRAF-2

Figure 3

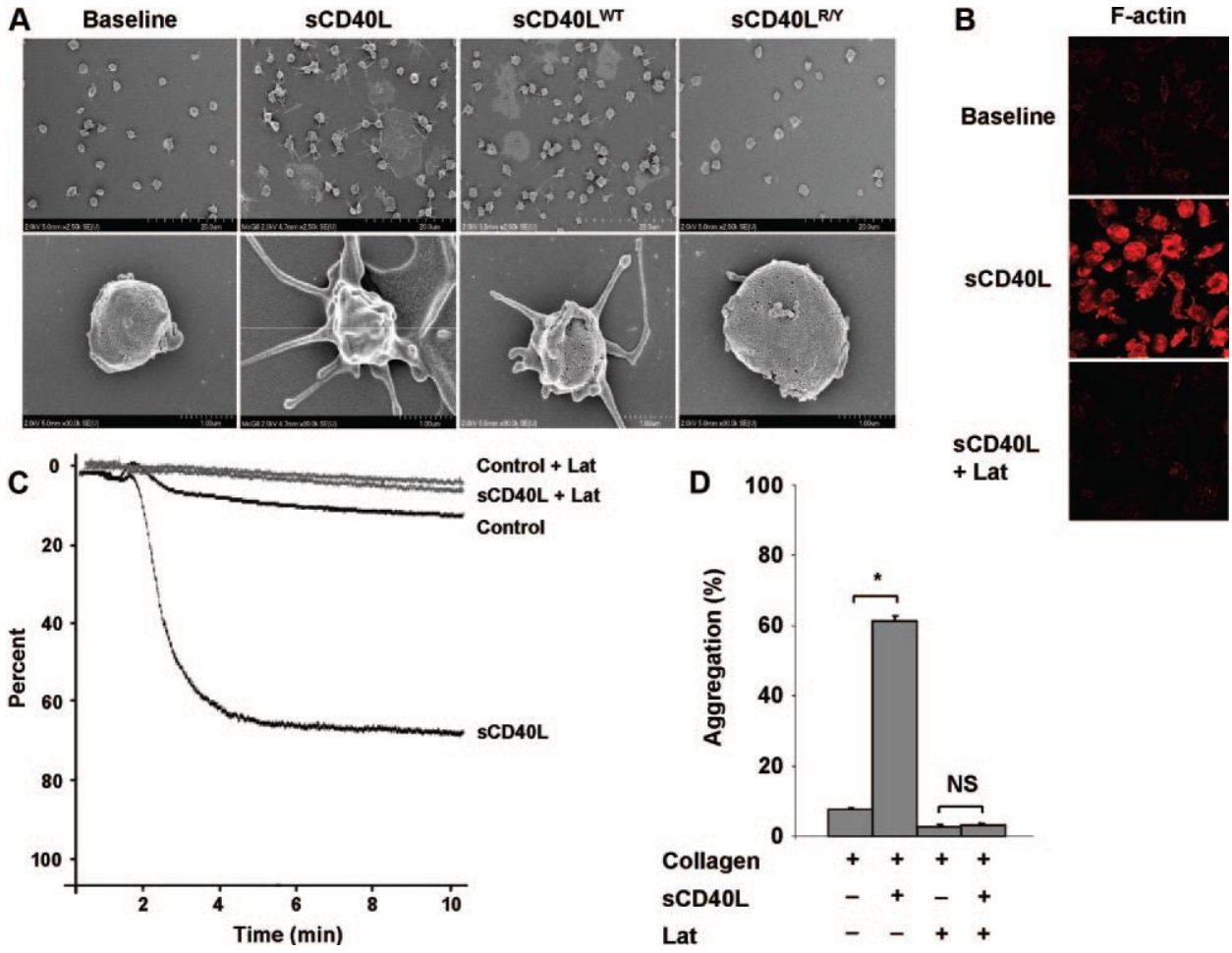

A
Baseline
sCD40L
sCD40L^WT
sCD40L^R/Y
B
F-actin
Baseline
sCD40L
sCD40L + Lat
C
Control + Lat
sCD40L + Lat
Control
sCD40L
Percent
Time (min)
D
Aggregation (%)
NS
Collagen
sCD40L
Lat

Figure 4

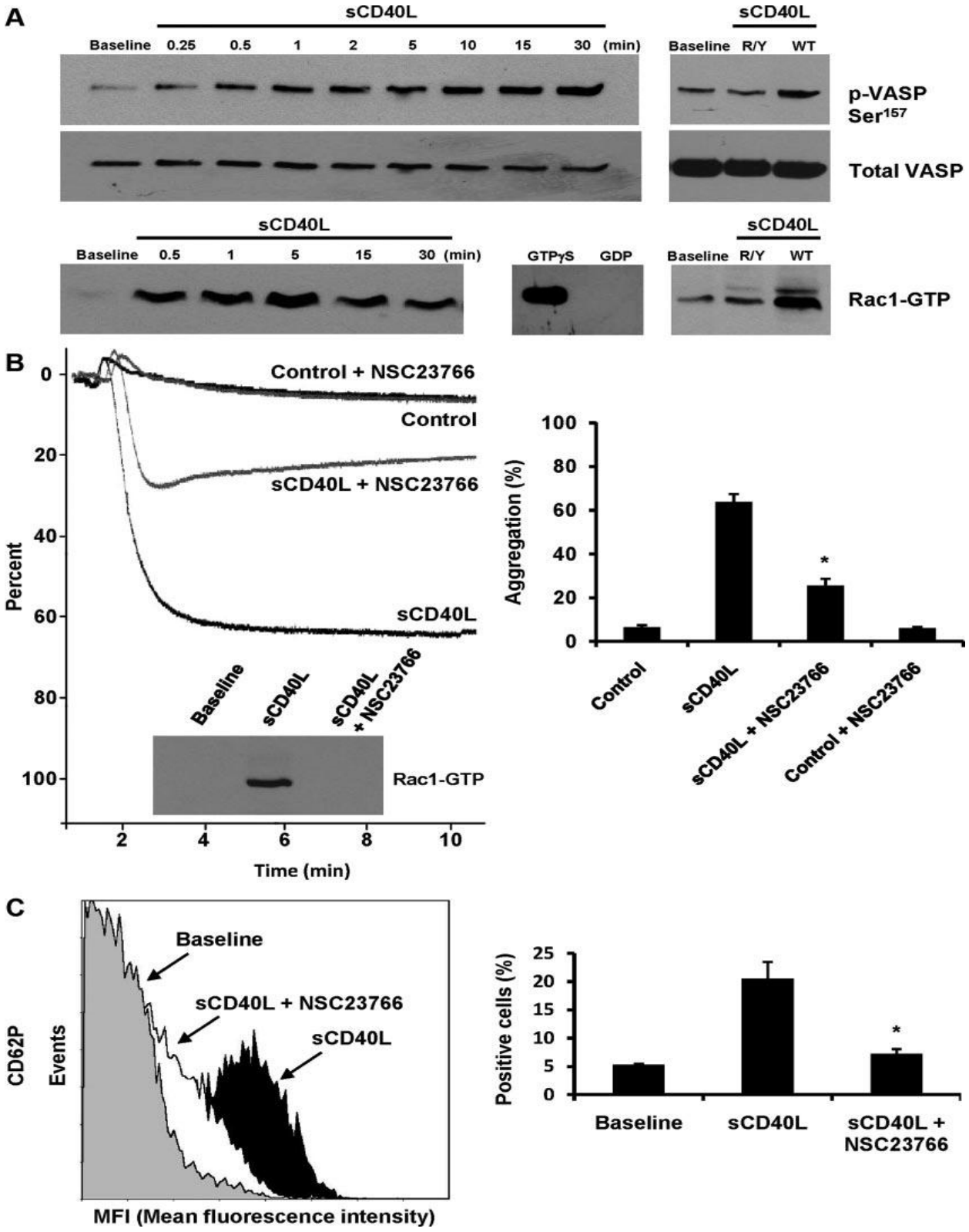

A
sCD40L
Baseline 0.25 0.5 1 2 5 10 15 30 (min)
sCD40L
Baseline R/Y WT
p-VASP Ser157
Total VASP
sCD40L
Baseline 0.5 1 5 15 30 (min)
GTPγS GDP
sCD40L
Baseline R/Y WT
Rac1-GTP
B
Control + NSC23766
Control
sCD40L + NSC23766
sCD40L
Baseline
sCD40L
sCD40L + NSC23766
Rac1-GTP
Percent
Time (min)
Aggregation (%)
Control
sCD40L
sCD40L + NSC23766
Control + NSC23766
C
Baseline
sCD40L + NSC23766
sCD40L
CD62P
Events
MFI (Mean fluorescence intensity)
Positive cells (%)
Baseline
sCD40L
sCD40L + NSC23766

Figure 5

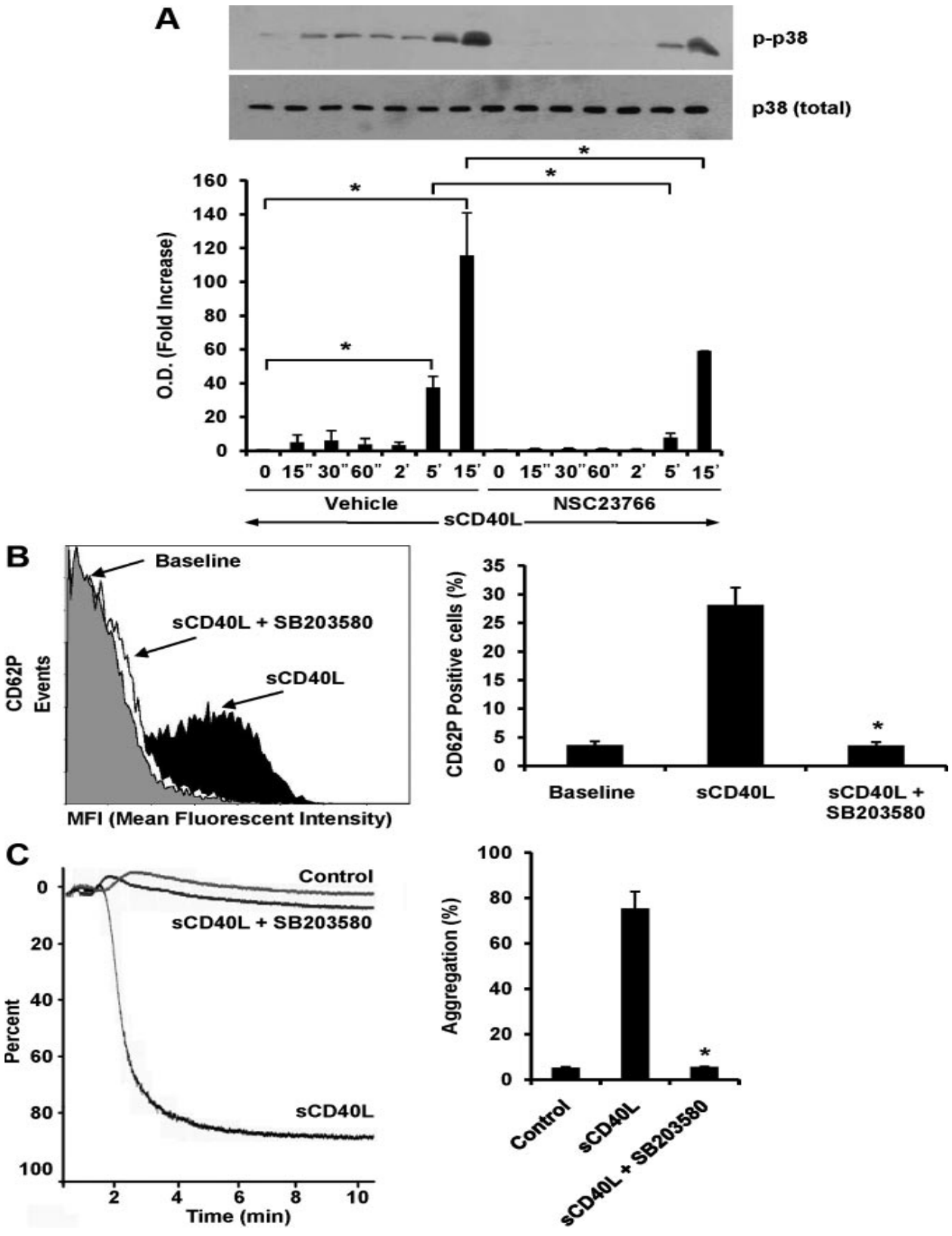
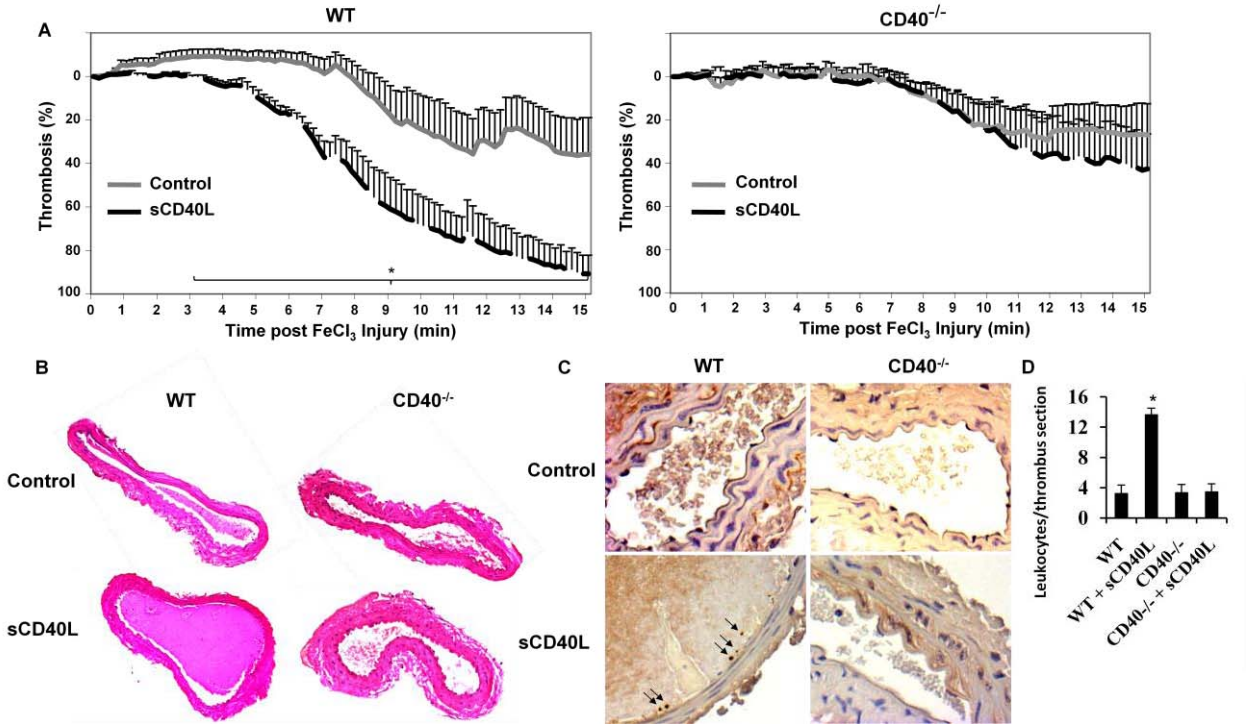
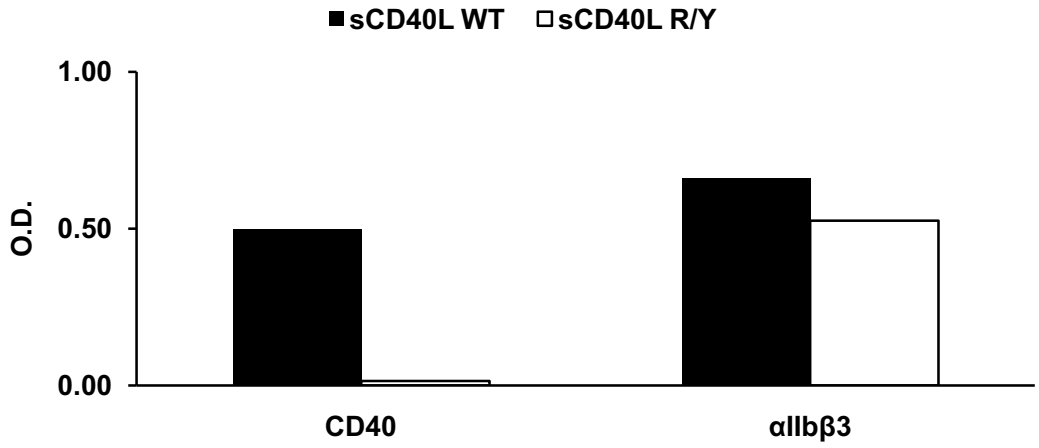

A
p-p38
p38 (total)
O.D. (Fold Increase)
0 15" 30" 60" 2' 5' 15' 0 15" 30" 60" 2' 5' 15'
Vehicle
NSC23766
sCD40L
B
Baseline
sCD40L + SB203580
sCD40L
CD62P Events
MFI (Mean Fluorescent Intensity)
CD62P Positive cells (%)
Baseline
sCD40L
sCD40L + SB203580
C
Control
sCD40L + SB203580
sCD40L
Percent
Time (min)
Aggregation (%)
Control
sCD40L
sCD40L + SB203580

Figure 6

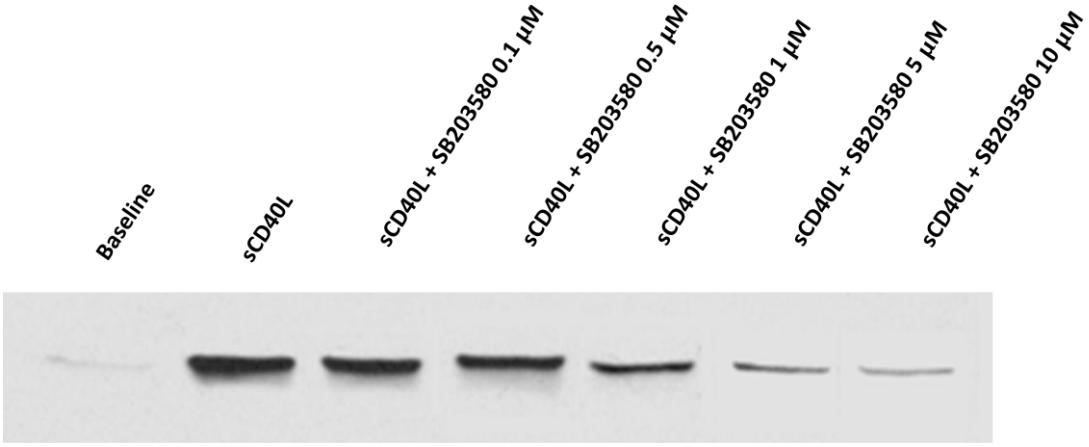

A
WT
CD40-/-
Thrombosis (%)
Control
sCD40L
Time post FeCl3 Injury (min)
B
WT
CD40-/-
Control
sCD40L
C
WT
CD40-/-
Control
sCD40L
D
Leukocytes/thrombus section
WT
WT + sCD40L
CD40-/-
CD40-/- + sCD40L

Supplemental Figure I



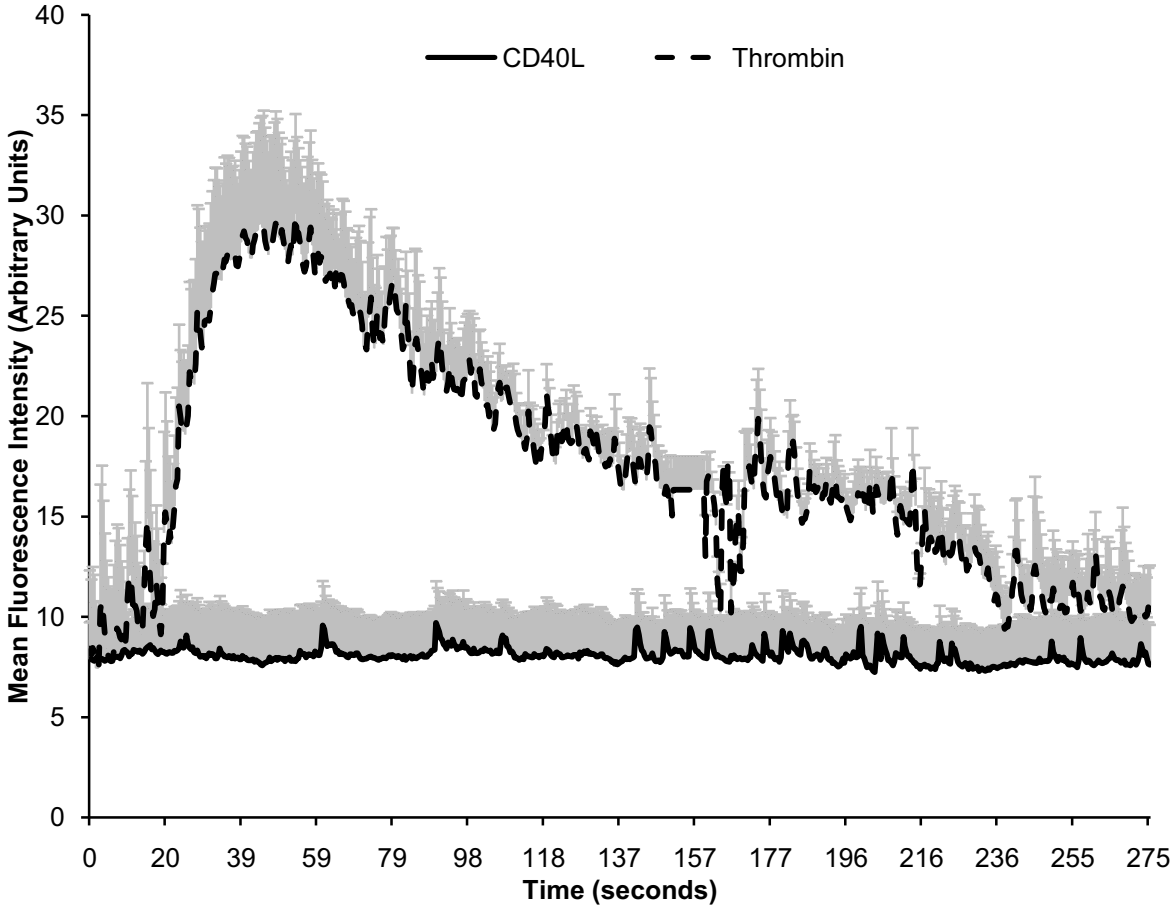
Supplemental Figure I: Specific binding of CD40 ligand to CD40- or αIIbβ3-coated well plates. Plates were coated with 4 μg of soluble hCD40 or $\alpha_{IIb}\beta_3$ overnight at room temperature. After extensive washing, plates were blocked with 1% BSA, then recombinant soluble hsCD40L wild type (WT) or hsCD40L R/Y was added overnight at a concentration of 100 ng/mL. A polyclonal anti-hCD40L-biotinylated antibody was used followed by addition of striptavidin-HRP. The signal was detected by addition of TMB substrate. The reaction was halted by the addition of 1N H_2SO_4. Optical density (O.D.) was then measured by spectrophotometry.

Supplemental Figure II



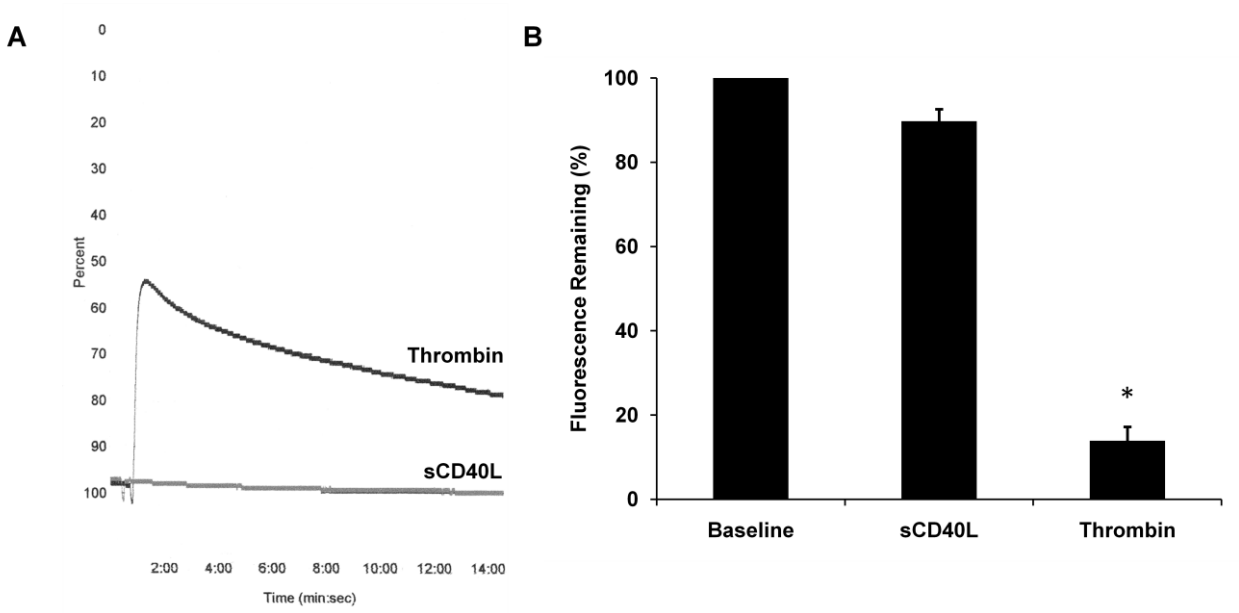
Supplemental Figure II: Effect of SB203580 (0.1 µM -10 µM) on sCD40L-induced p38 MAPK phosphorylation. Resting platelets were preincubated with the indicated concentration of SB203580 for 15 minutes at 37ºC or left untreated, and then stimulated with sCD40L (1 µg/mL). Total platelet lysates were analyzed by SDS-PAGE for phospho-p38 MAPK. Blot shown is representative of 3 independent experiments.

Supplemental Figure III



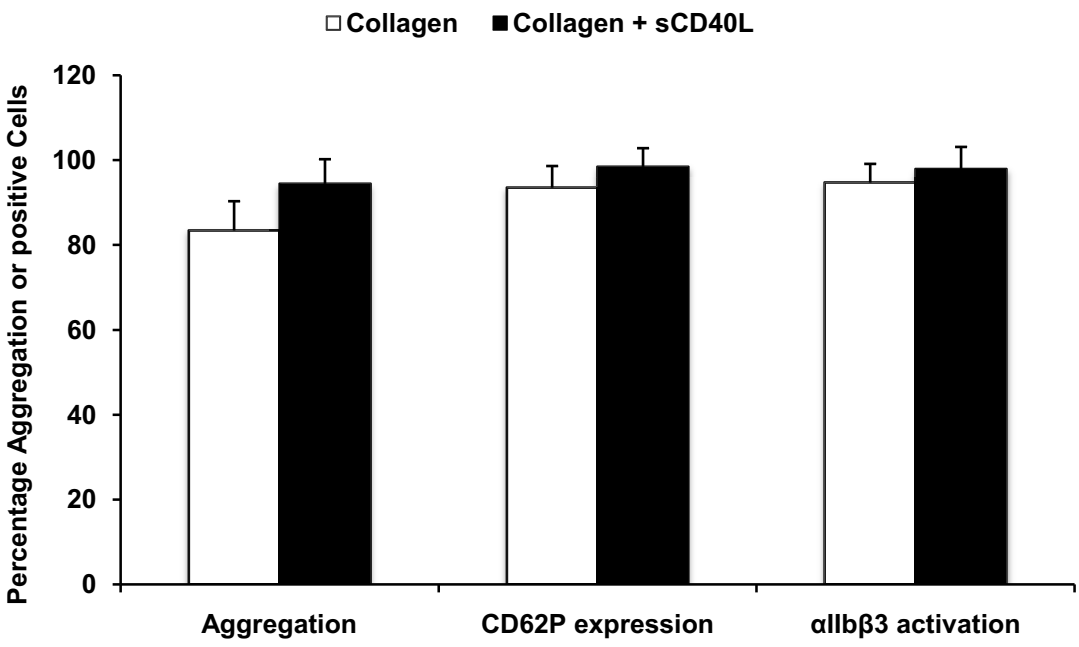
Supplemental Figure III: Effect of sCD40L on intracellular calcium flux. Intracellular calcium was measured by Fluo-4 AM fluorescence by real-time confocal microscopy following stimulation of platelets with either sCD40L (1 μg/mL) or thrombin (0.5 U/mL), as described in materials and methods. Tracings are representative of 3 experiments.

Supplemental Figure IV



Supplemental Figure IV: Effect of sCD40L on platelet dense granule secretion. A, ATP secretion as measured by Luciferase assay (Chrono-Lume, Chrono-log). Results are expressed as a measure of increase in luminescence. Blot is representative of 3 independent experiments. B, Dense granule secretion was evaluated by measuring the loss of mepacrine fluorescence following activation by sCD40L (1 μg/mL) or thrombin (0.5 U/mL); in comparison to untreated cells which was set as 100% fluorescence (n=3; **P*< 0.05 vs. baseline).

Supplemental Figure V



Supplemental Figure V: Effect of sCD40L on high dose collagen. Washed platelets were incubated with sCD40L (1 μg/mL) or left untreated for 30 minutes at 37°C, then stimulated with collagen (aggregation: 2 μg/mL; flow cytometry: 5 μg/mL), and assessed by optical aggregometry and flow cytometry for CD62P expression and $\alpha_{IIb}\beta_3$ activation, as described in materials and methods.

Background for the second article

In our first study we demonstrated that sCD40L enhances platelet activation and aggregation in response to subthreshold concentrations of platelet agonists, including thrombin, collagen and ADP, through a CD40-dependent TRAF2/Rac1/p38 MAPK signalling pathway. We also showed that increased levels of circulating sCD40L, as seen in ACS patients, exacerbates thrombus formation and leukocyte infiltration within the thrombus mass in a CD40-dependent manner.

In B lymphocytes, it is well documented that CD40 induces activation of the NF-κB signalling pathway through either TRAF2 or TRAF6. In platelets, the members of the NF-κB family have recently been identified and shown to be involved in platelet activation and aggregation. However, their role in platelet CD40 signalling remains unknown; hence the objective of this study.

Authors contributions

Ahmed Hachem: Planning and execution of all the experiments in this article except (Figure 4B). Writing and editing the article.

Daniel Yacoub: Planning and execution of the aggregation experiments (Figure 4B). Intellectual input and editing the article.

Younes Zaid: Platelet isolation for aggregation experiments and editing the article.

Walid Mourad: General co-direction. Participated in the interpretation of the results, helped in editing the article.

Yahye Merhi: General direction. Designed the study, analysed the data, provided intellectual input and helped in editing the article.

Involvement of nuclear factor κB in platelet CD40 signaling

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Abbreviations: TNF, tumor necrosis factor; sCD40L, soluble CD40 ligand; TRAF, tumor necrosis factor receptor-associated factor; NF-κB, nuclear factor κB; MAPK, mitogen-activated protein kinase; JNK, c-jun amino terminal kinase; ERK, extracellular signal-regulated protein kinase; SNAP-23, synaptosomal-associated protein 23.

Abstract

CD40 ligand (CD40L) is a thrombo-inflammatory molecule that predicts cardiovascular events. Platelets constitute the major source of soluble CD40L (sCD40L), which has been shown to potentiate platelet activation and aggregation, in a CD40-dependent manner, via p38 mitogen-activated protein kinase (MAPK) and Rac1 signaling. In many cells, the CD40L/CD40 dyad also induces activation of nuclear factor kappa B (NF-κB). Given that platelets contain NF-κB, we hypothesized that it may be involved in platelet CD40 signaling and function. In human platelets, sCD40L induces association of CD40 with its adaptor protein the tumor necrosis factor receptor associated factor 2 and triggers phosphorylation of IκBα, which are abolished by CD40L blockade. Inhibition of IκBα phosphorylation reverses sCD40L-induced IκBα phosphorylation without affecting p38 MAPK phosphorylation. On the other hand, inhibition of p38 MAPK phosphorylation has no effect on IκBα phosphorylation, indicating a divergence in the signaling pathway originating from CD40 upon its ligation. In functional studies, inhibition of IκBα phosphorylation reverses sCD40L-induced platelet activation and potentiation of platelet aggregation in response to a sub-threshold concentration of collagen. This study demonstrates that the sCD40L/CD40 axis triggers NF-κB activation in platelets. This signaling pathway plays a critical role in platelet activation and aggregation upon sCD40L stimulation and may represent an important target against thrombo-inflammatory disorders. **(*Biochem Biophys Res Commun*. 2012; 425:58-63)**

Keywords: Platelet, CD40L, TRAF, NF-κB, p38 MAPK

1. Introduction

CD40 is a 48 kDa membrane glycoprotein belonging to the tumor necrosis factor (TNF) receptor family. It was first discovered on human bladder carcinoma cells [1], but is now known to be present on a plethora of cell lines including B lymphocytes, endothelial cells, monocytes, dendritic cells and platelets [2,3,4,5]. Interaction of CD40 with its CD40 ligand (CD40L), a member of the TNF superfamily, plays a pivotal role in the immune response. The cytoplasmic domain of CD40 lacks direct kinase activity and therefore utilizes members of the TNF receptor-associated factors (TRAFs) as adapter proteins to mediate signaling events. TRAF1, 2, 3, 5 and 6 have been shown to interact with the cytoplasmic domain of CD40 and regulate downstream signaling pathways upon its ligation [6,7,8]. TRAF6 and TRAF2 mediate the activation of the canonical and non-canonical NF-κB pathways in response to CD40 engagement.

Nuclear factor κB (NF-κB) proteins are formed by hetero- or homo-dimerization of the five Rel/NF-κB DNA-binding subunits, which include RelA (p65), RelB (p68), c-Rel, p50 (NF-κB1) and p52 (NF-κB2). These NF-κB complexes are maintained in the cytoplasm in an inactive state through the inhibitor κB (IκBα or IκBβ). In response to stimuli, the NF-κB dimers in association with the inhibitory IκB subunit are regulated by the IκB kinase (IKK), which consists of two kinase subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO. Upon phosphorylation by IKK, the IκB subunit is targeted for proteosomal degradation, thereby releasing an active form of NF-κB that translocates into the nucleus. Nuclear translocation of p50/RelA and p52/RelB is responsible for the canonical and non-canonical NF-κB pathways, respectively [9]. Liu et *al*. [10] has demonstrated the presence of NF-κB and IκBα in platelets, as well as the induction of the NF-κB signaling pathway following platelet activation. Thereafter, Malaver et *al.* [11] has shown that IκBα is phosphorylated in thrombin-activated platelets and pharmacological inhibition of this factor leads to impairment of platelet function, thereby attributing non-genomic functions to NF-κB in anucleated platelets. In contrast, Gambaryan et *al*. [12] showed that NF-κB negatively regulates platelet activation by thrombin and collagen via PKAc activation.

Platelets are pivotal contributors to thrombosis and homeostasis, but also participate in inflammation and immunity [13]. The work by Henn et *al.* [5,14] demonstrated the presence of the CD40L/CD40 dyad in platelets; they showed that CD40 is constitutively expressed on the platelet surface, while CD40L rapidly appears on the platelet surface following activation.

Surface expressed CD40L is subsequently cleaved into an 18 kDa fragment, which accounts for >95% of plasmatic soluble CD40L (sCD40L) concentrations [15]. Circulating levels of sCD40L in patients have now emerged as strong indicators of cardiovascular risk, as there appears to be a significant correlation between elevated levels of sCD40L and vascular complications such as atherosclerosis and acute coronary syndromes [16,17,18].

We have previously shown that sCD40L enhances agonist-induced platelet activation and aggregation through a CD40-dependent TRAF2/Rac1/p38 mitogen-activated protein kinase signaling (MAPK) pathway [19]. Although the presence of both the CD40L/CD40 dyad and NF-κB/IκBα in platelets is recognized, the involvement of NF-κB/IκBα in platelet CD40 signaling and function remains unknown. This study was therefore designed to test the hypothesis that NF-κB is involved in platelet CD40 signaling and function.

2. Materials and methods

2.1 Reagents and Antibodies

Recombinant human sCD40L was obtained from R&D systems. Antibodies against TRAF2 (rabbit polyclonal), phospho-IκBα (mouse monoclonal, $Ser^{32/36}$), phospho-p38 MAPK (rabbit polyclonal, Thr^{180}/Tyr^{182}) and β-actin (rabbit polyclonal) were purchased from Cell Signaling Technology. The mouse monoclonal anti-CD40 antibody used for immunoprecipitation of human CD40 was also from R&D systems, while the rabbit polyclonal anti-CD40 antibody used for detection of CD40 by immunoblotting came from Santa Cruz Biotechnology. Antibody against CD62P (mouse monoclonal, AK4-PE conjugated) was obtained from BD Biosciences. The specific IKK inhibitor VII and the p38 MAPK inhibitor SB203580 were purchased from Calbiochem, while the IκBα phosphorylation inhibitor BAY 11-7082 was purchased from Sigma-Aldrich. Protein A agarose beads were obtained from Upstate Biotechnology, Inc. Native type I collagen was from Chronolog Corp.

2.2 Platelet isolation

Venous blood was drawn from healthy volunteers, free from medication known to interfere with platelet function for at least 10 days before the experiment. The protocol was approved by the human ethical committee of the Montreal Heart in accordance with the declaration of Helsinki for experiments involving humans. Washed platelets were prepared as

previously described [19], adjusted to the indicated concentrations and allowed to rest at 37 ºC for 30 min before further manipulation.

2.3 Flow cytometry

Platelet P-selectin (CD62P) expression, as a marker of α granule secretion and platelet activation, was measured by flow cytometry as previously described [19]. Briefly, platelets (250 × 10^6/mL) were pre-incubated with or without the indicated inhibitor for 10 min at 37 ºC. Platelets were then stimulated with sCD40L for 30 min at 37 ºC, fixed with 1% paraformaldehyde, washed and stained with saturating concentrations of anti-CD62P antibody for 30 min or its isotype-matched control IgG. Platelets were analyzed (20,000 events) on an Altra flow cytometer (Beckman Coulter) after gating their characteristic forward and side scatter properties.

2.4 Platelet aggregation

Platelets were adjusted to 250 x 10^6 /mL and aggregation was monitored on a four-channel optical aggregometer (Chronolog Corp.) under shear (1000 rpm) at 37 ºC. Platelets were pre-incubated with or without the indicated inhibitors for 10 min at 37 ºC prior to incubation with sCD40L for an additional 30 min under static conditions at 37 ºC. Platelet aggregation was then monitored following the addition of collagen (0.5 μg/mL) and recorded until stabilization of platelet aggregation.

2.5 Immunoprecipitation of CD40

Platelets were stimulated as indicated and lysed into ice-cold modified RIPA lysis buffer (1% NP-40, 0.25% deoxycholic acid, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 mM sodium-orthovanadate, 1 mM sodium fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 2 μg/mL benzamidin) for 1 h at 4 ºC. Lysates were sonicated on ice and pre-cleared with 100 μL of protein A agarose beads for 15 min at 4 ºC. Beads were then pelleted and the supernatant was incubated with an anti-CD40 antibody overnight at 4 ºC. Samples were treated with 100 μL of protein A agarose beads for 1 h at 4 ºC. Beads were then precipitated by centrifugation, washed three times with ice-cold modified RIPA lysis buffer, resuspended in 2×

Laemmli buffer and boiled for 5 min. Supernatants were analyzed by immunoblotting for the presence of TRAF2 and CD40 proteins.

2.6 SDS-PAGE and immunoblotting

Proteins were resolved in 8% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk for 1 h, washed three times with TBS/T (150 mM NaCl, 20 mM Tris, pH 7.4, 0.1 % Tween-20) and incubated with the appropriate primary antibody overnight at 4 ºC. Following washing steps, membranes were labeled with horseradish peroxidase-conjugated secondary antibody for 1 h, washed and bound peroxidase activity was detected by enhanced chemiluminescence (PerkinElmer Life Sciences). To assess equal protein loading, membranes were stripped, blocked with 5% milk and blotted for β-actin.

2.7 Statistical analysis

Results are presented as mean ± SEM of at least three independent experiments. Statistical comparisons were done using a one-way ANOVA, followed by a Dunnetts-*t*-test for comparison against a single group. Data with $P < 0.05$ were considered statistically significant.

3. **Results**

3.1 sCD40L induces TRAF2 association to CD40 and IκBα phosphorylation in platelets

We first confirmed our previous finding that sCD40L induces the association of TRAF2 to platelet CD40 [19] and showed that pretreatment with a blocking anti-CD40L antibody entirely prevents TRAF2 binding to CD40 in response to sCD40L (Fig. 1A). We then evaluated the activation of the NF-κB signaling pathway downstream of platelet CD40 by assessing IκBα phosphorylation on residues $Ser^{32/36}$, which leads to degradation and release of the active form of NF-κB. Our results show for the first time that sCD40L induces a time-dependent phosphorylation of IκBα in human platelets (Fig. 1B). Since the phosphorylation of IκBα at 5 min was close to maximum, we chose this time point for subsequent experiments. Pretreatment with a blocking anti-CD40L antibody prevents platelet IκBα phosphorylation in response to sCD40L (Fig. 1C).

3.2 IκBα phosphorylation downstream of CD40 is independent of p38 MAPK

Signaling pathways downstream of CD40 have been shown to involve both NF-κB and p38 MAPK [20,21]. Indeed, we have previously shown that sCD40L enhances platelet function through activation of p38 MAPK [19], albeit the link between this MAPK and NF-κB activation remains uncharacterized. In order to delineate the cross-talk between these signaling cascades downstream of platelet CD40, we employed specific inhibitors of IκBα and p38 MAPK phosphorylation, BAY 11-7082 and SB203580 respectively. In platelets, inhibition of IκBα phosphorylation does not influence p38 MAPK phosphorylation following sCD40L treatment, and vice versa (Fig. 2A and B). These results indicate that platelet CD40 signals via two distinct pathways, one involving the NF-κB pathway and another involving p38 MAPK.

3.3 IκBα is required for sCD40L-induced platelet activation and potentiation of aggregation

We have previously shown that sCD40L induces platelet CD62P expression and potentiates platelet aggregation in response to sub-threshold doses of platelet agonists [19]. In order to show the importance of IκBα in this process, the IKK inhibitor VII, which targets the kinase responsible for IκBα phosphorylation, was employed. Pretreatment of platelets with the IKK inhibitor VII not only decreases IκBα phosphorylation in a dose-dependent manner (Fig. 3A), but also abolishes platelet activation in response to sCD40L stimulation, as assessed by CD62P expression (Fig. 3B). In functional studies, pretreatment of platelets with either BAY 11-7082 or IKK inhibitor VII reverses the pro-aggregating effects of sCD40L (Fig. 4A and B). These results indicate that the NF-κB signaling pathway, which involves both IκBα and IKK, plays an important role in platelet CD40 signaling, activation and aggregation in response to sCD40L.

4. **Discussion**

The presence of CD40L in platelets, which is cleaved to generate most of the sCD40L within the circulation, and its ability to induce an inflammatory response in the vascular system is well documented [5,14]. However, its modulation of platelet function remains a matter of debate. Andre and *al.* have shown that sCD40L binds to $\alpha_{IIb}\beta_3$ and stabilizes arterial thrombi in mice [22]. In contrast, we and others have shown that sCD40L induces platelet activation through CD40 [19,23,24,25]. Indeed, we have previously demonstrated that sCD40L exacerbates

platelet activation and aggregation through a CD40-dependent TRAF2/Rac1/p38 MAPK signaling pathway. We have also shown that elevated levels of sCD40L predisposed platelets to enhanced thrombus formation in response to vascular injury [19]. In this study, we further investigated the signaling pathways downstream of platelet CD40 in response to sCD40L.

In B lymphocytes, it is well documented that CD40 induces activation of the NF-κB signaling pathway through either TRAF2 or TRAF6 [26]. Having shown that sCD40L induces the association of TRAF2/CD40, but not TRAF6/CD40 [19] in platelets, we sought to evaluate whether CD40 ligation could trigger activation of the NF-κB signaling pathway in platelets. We showed for the first time that treatment of platelets with sCD40L activates the NF-κB signaling pathway, which was revealed by the phosphorylation of IκBα on $Ser^{32/36}$. These results add insights to previous work [11], showing that NF-κB may be a novel mediator of platelet responses. Although previous work has focused on the involvement of p38 MAPK in platelet CD40 signaling [19,23], the interrelation between this pathway and the NF-kB cascade remains undetermined. Craxton et *al.* [27] have shown that p38 MAPK regulates NF-κB activation in B cells, whereas p38 MAPK inhibition does not affect CD40-mediated NF-κB DNA binding, suggesting that NF-κB is not a direct target of the p38 MAPK pathway. In the present study, we show a divergence between the NF-κB and p38 MAPK signaling pathways downstream of platelet CD40. This branching phenomenon suggests that these pathways regulate different aspects of CD40-mediated platelet responses. For instance, the p38 MAPK pathway may be involved in actin polymerization, cytoskeleton reorganization and platelet spreading [28], while the NF-κB pathway may regulate de novo protein synthesis through its interaction with microRNAs [29], which have been shown to be present in platelets [30]. However, further investigations are needed to specifically address this issue.

Malaver et *al*. [11] have demonstrated the implication of NF-κB in platelet function and showed that blockade of the NF-κB pathway by BAY 11-7082 reverses platelet activation as well as aggregation triggered by thrombin, collagen and ADP. Since we have previously demonstrated that sCD40L induces platelet activation as assessed by α-granule secretion [19]; here we show that pretreatment of platelets with the IKK inhibitor VII, which inhibited IκBα phosphorylation, reversed sCD40L-induced platelet α-granule secretion, as assessed by CD62P translocation to the platelet membrane. Therefore, aside from activating NF-κB, IKK could regulate the activation of critical elements involved in the degranulation process, such as the

synaptosomal-associated protein 23 (SNAP-23). In fact, IKK2 regulates mast cell degranulation by phosphorylating SNAP-23 in a NF-κB-independent manner [31]. In functional studies of platelet aggregation, sCD40L stimulation of platelets potentiates aggregation by a mechanism involving the NF-κB signaling pathway, since inhibition of this pathway by either BAY 11-7082 or the IKK inhibitor VII abolished sCD40L-induced potentiation of platelet aggregation. These results indicate that sCD40L via NF-κB, as well as p38 MAPK as previously demonstrated [19], primes platelets and predisposes them to enhanced aggregation responses in the presence of thrombotic stimulus.

In summary, this study shows for the first time that sCD40L is an important inducer of the NF-κB signaling pathway activation in platelets independently of the p38 MAPK pathway. This translates into platelet priming and enhancement of platelet activation and aggregation. Thus, the CD40L/CD40/NF-κB axis may ultimately represent a therapeutic target in the treatment of thrombo-inflammatory diseases.

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Figure legends

Figure 1: sCD40L induces TRAF2 association to CD40 and IκBα phosphorylation in platelets. (A) Platelets (500 × 10^6/mL) were left untreated or incubated with 1 μg/mL sCD40L for 15 min at 37 ºC in the presence or absence of a blocking anti-CD40L antibody (5 μg/mL). Total cell lysates were then immunoprecipated using an anti-CD40 antibody and immunoblotted for TRAF2 and CD40 expression. Blots are representative of three independent experiments. Histogram represents the mean of data of overlay blots, expressed as arbitrary units of optical density (n = 3; **P* < 0.05 vs. baseline or sCD40L). (B) Time-dependent course of IκBα phosphorylation on $Ser^{32/36}$ (p-IκBα $Ser^{32/36}$) following sCD40L treatment. Lysates form 1000 × 10^6 platelets/mL from untreated (Baseline) or sCD40L-treated (1 μg/mL) were resolved in 12% SDS-PAGE and assessed for p-IκBα $Ser^{32/36}$. Blots are representative of three independent experiments. Histogram represents the mean of data of overlay blots, expressed as fold increase in optical density, as compared to baseline (n = 3, **P* < 0.05 vs. baseline). (C) Dose-dependent effect of anti-CD40L treatment on IκBα phosphorylation. Platelets were left untreated or pretreated with the indicated dose of anti-CD40L for 5 min at 37 ºC prior to stimulation with sCD40L. Platelet lysates were then analyzed for p-IκBα $Ser^{32/36}$. β-actin blots shown are from stripped p-IκBα $Ser^{32/36}$ membranes. Blots are representative of five independent experiments. Histogram represent the mean of data of overlay blots, expressed as arbitrary units of optical density (n = 5, **P* < 0.05 vs. baseline and †*P* < 0.05 vs. sCD40L alone).

Figure 2: IκBα phosphorylation downstream of CD40 is independent of p38 MAPK phosphorylation. (A) Dose-dependent effect of BAY 11-7082 on IκBα and p38 MAPK phosphorylation. Platelets (1000 × 10^6/mL) were incubated with vehicle DMSO or the indicated concentrations of BAY 11-7082 for 5 min at 37 ºC and stimulated with sCD40L (1 μg/mL) for 5 min. Platelet lysates where resolved in 12% SDS-PAGE and assessed for p-IκBα $Ser^{32/36}$ (n = 4) and phospho-p38 MAPK (p-p38 MAPK) (n = 3). (B) Dose- dependent effect of SB203580 on IκBα and p38 MAPK phosphorylation. Platelets were incubated with vehicle DMSO or the indicated concentration of SB203580 for 10 min at 37 ºC prior to sCD40L stimulation (1 μg/mL) for 5 min. Platelet lysates where assessed for p-IκBα $Ser^{32/36}$ (n = 4) and p-p38 MAPK (n = 4). β-actin blots are from stripped membranes of either p-IκBα or p-p38 MAPK blots. Blots are representative of the indicated number of independent experiments. Histograms represent the

mean of data of overlay blots, expressed as arbitrary units of optical density (*P < 0.05 vs. baseline and †P < 0.05 vs. sCD40L alone).

Figure 3: IκBα is required for sCD40L-induced platelet activation. (A) Platelets (1000 × 10^6/mL) were incubated with vehicle DMSO or the indicated concentration of IKK inhibitor VII for 5 min at 37 ºC and stimulated with sCD40L for 5 min. Platelet lysates where resolved in 12% SDS-PAGE and assessed for p-IκBα $Ser^{32/36}$. β-actin blot is from stripped membranes of p-IκBα blot. Blots are representative of four independent experiments. Histogram represent the mean of data of overlay blots, expressed as arbitrary units of optical density (n = 4; *P < 0.05 vs. baseline and †P < 0.05 vs. sCD40L alone). (B) Effect of IκBα inhibition on platelet activation in response to sCD40L. Platelets (250 × 10^6/mL) were left untreated or stimulated with sCD40L (1 μg/mL) in the absence or presence of the indicated concentration of IKK inhibitor VII. Platelet activation was assessed by flow cytometry for the expression of CD62P. Histogram represents the mean of data expressed as percent of CD62P positive platelets (n = 3; *P < 0.05 vs. baseline and †P < 0.05 vs. sCD40L alone).

Figure 4: IκBα is required for sCD40L-induced potentiation of platelet aggregation. (A and B) Effect of IκBα inhibition on the potentiation of platelet aggregation induced by sCD40L. Platelets were preincubated with the IκBα phosphorylation inhibitors BAY 11-7082 (10 μM) or IKK inhibitor VII (2.5 μM), or vehicle DMSO for 5 min at 37 ºC. Cells were then left unstimulated (control) or treated with sCD40L (1 μg/mL). Aggregation was then monitor in the presence of a priming dose of collagen (0.5 μg/mL). Histograms represent the mean of data of aggregation traces (n = 3; *P < 0.05 vs. Control).

Figure 1

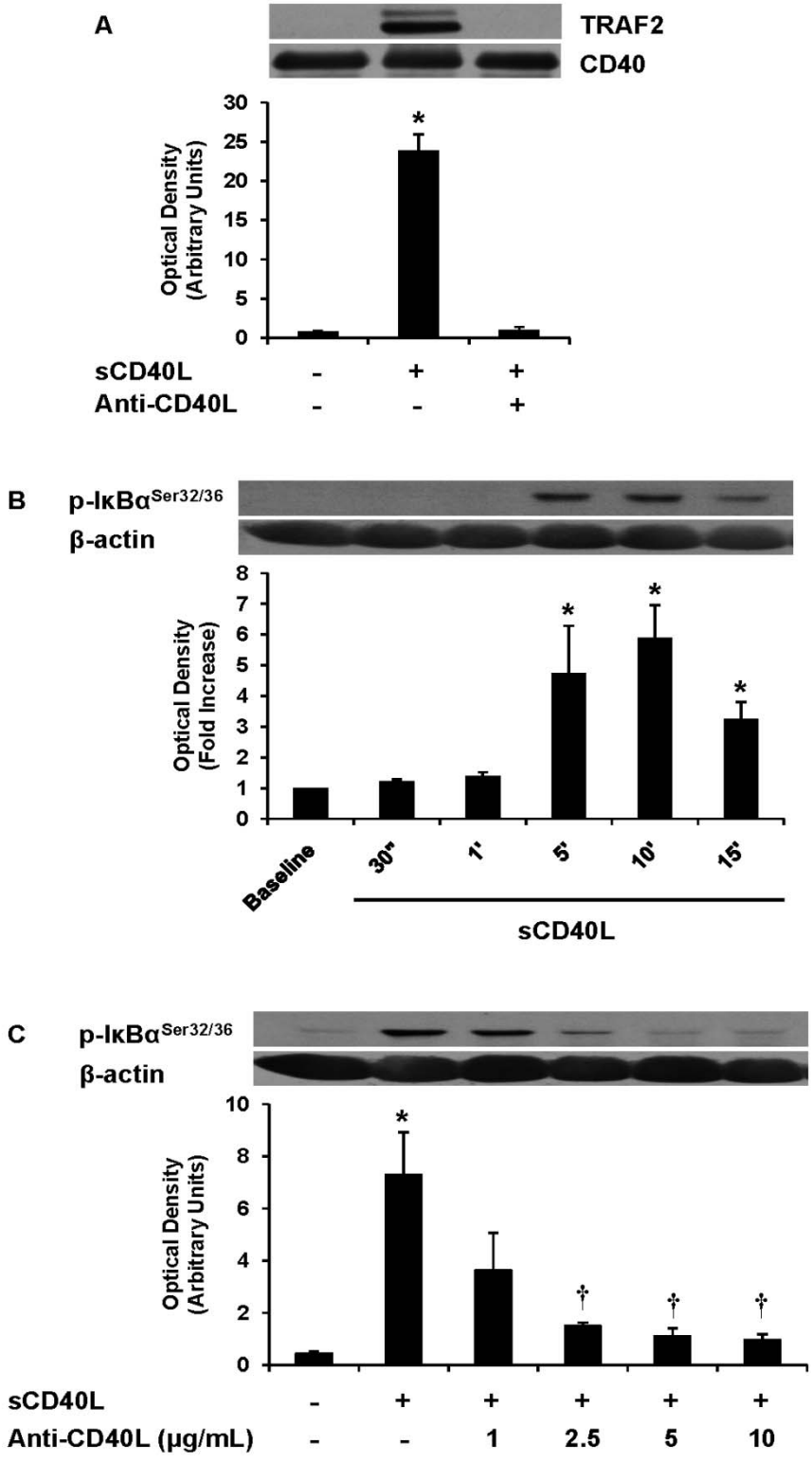
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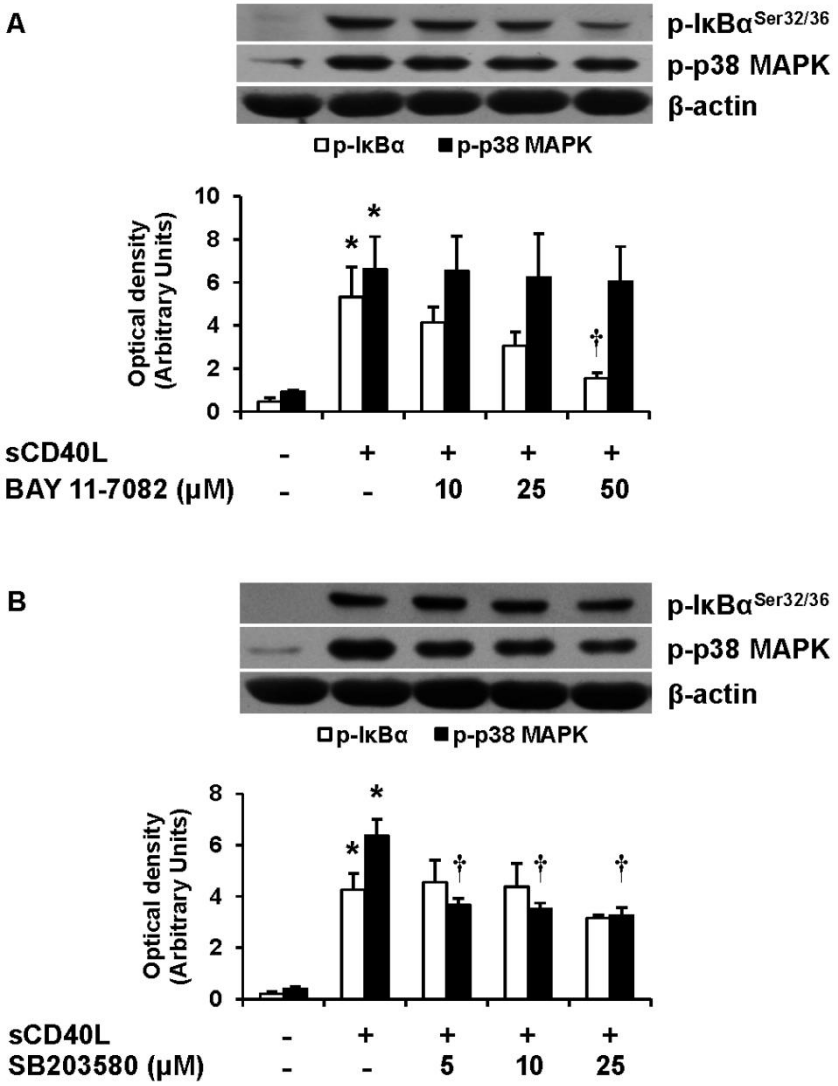
Figure 2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sCD40L
SB203580 (μM)

Figure 3

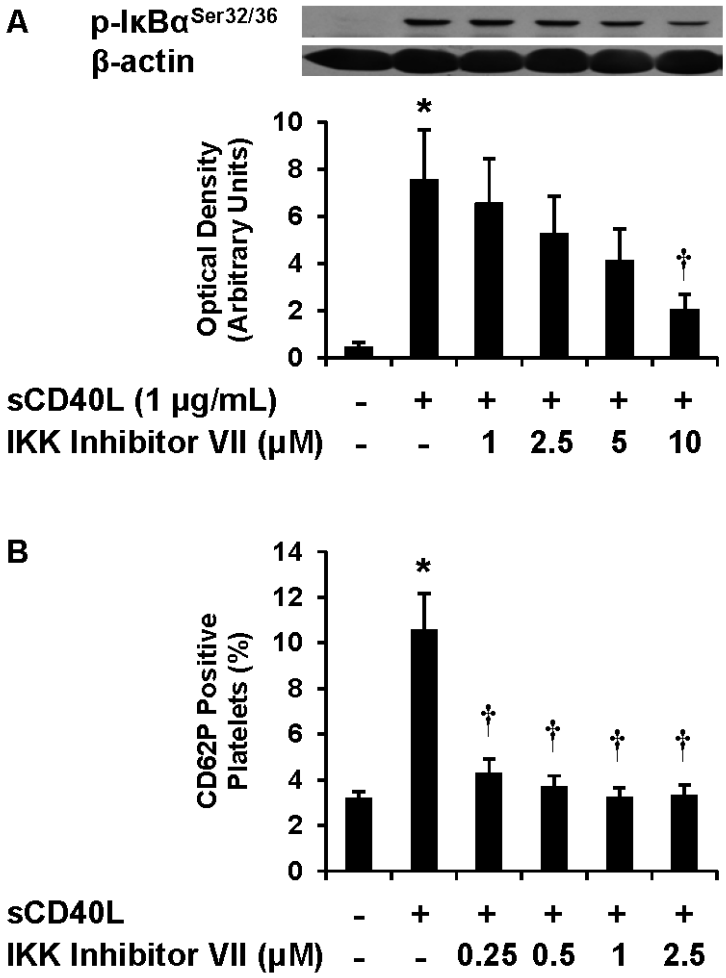
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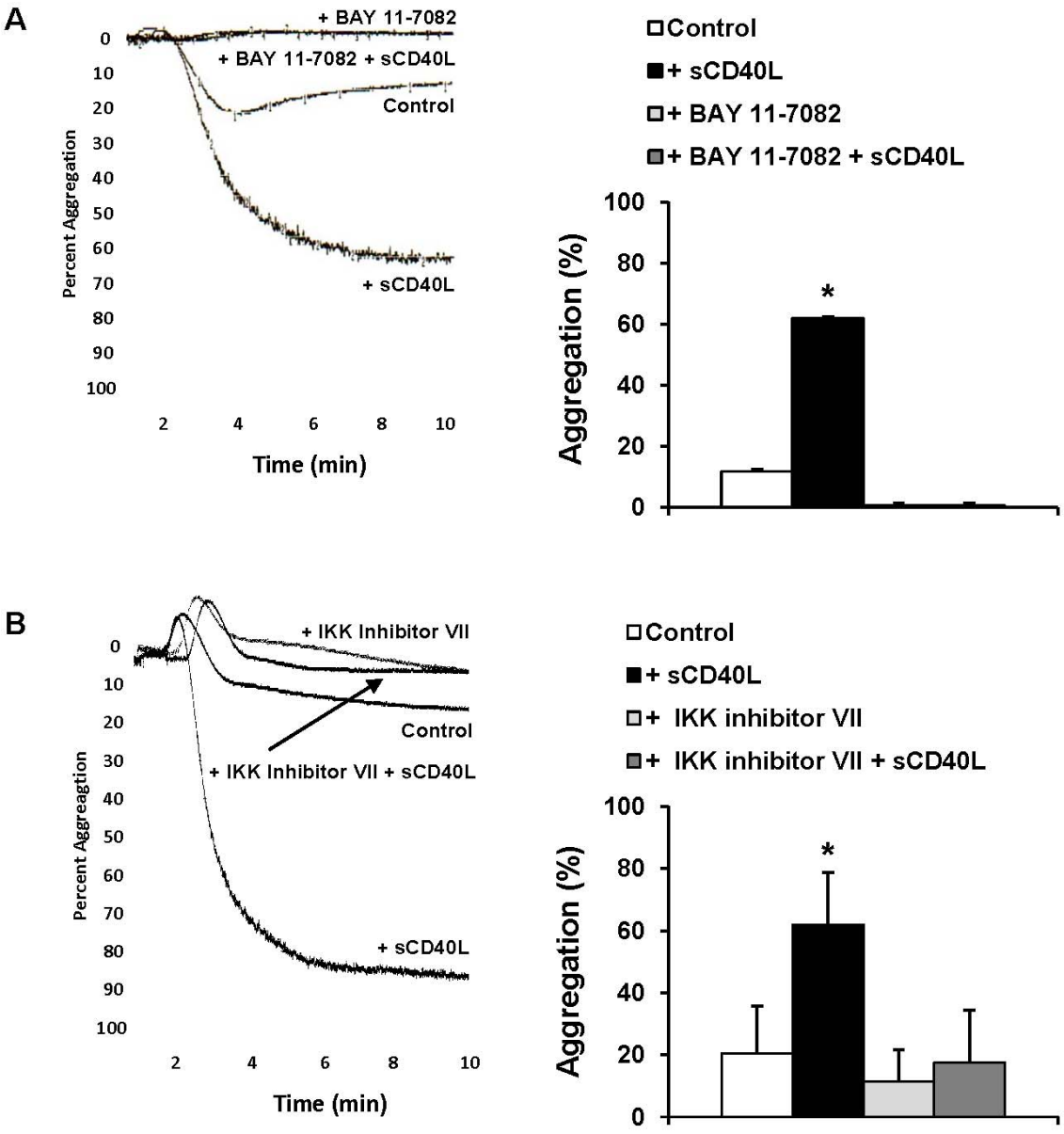
Figure 4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Time (min)
Control
+ sCD40L
+ BAY 11-7082
+ BAY 11-7082 + sCD40L
Aggregation (%)
B
+ IKK Inhibitor VII
Control
+ IKK Inhibitor VII + sCD40L
+ sCD40L
Percent Aggreagtion
Time (min)
Control
+ sCD40L
+ IKK inhibitor VII
+ IKK inhibitor VII + sCD40L
Aggregation (%)

Discussion

The CD40/CD40L dyad was initially thought to be restricted to cells of the immune system and to occupy a crucial role in regulating adaptive immunity. Now, it is well known that these two molecules have a much broader expression pattern, encompassing cells of the circulatory and vascular systems. The broad expression pattern of this dyad shed light into its contribution not only to immune responses, but also to inflammatory reactions. Indeed, the implication of the CD40/CD40L in all the facets of atherosclerosis, which is considered to be a chronic inflammatory disease, as well as the tight correlation between circulating levels of sCD40L and ACS incidence support its role as an inflammatory modulator. As platelets express members of this dyad and occupy a significant role in the development and progression of cardiovascular diseases, multiple studies attempted to determine the impact of sCD40L on platelet function. However, the precise effect of sCD40L on the *in vitro* platelet function and *in vivo* thrombus formation, as well as the underlying signalling mechanisms remain unclear; hence the objective of this project.

The main results of these studies demonstrate that sCD40L potentiates platelet activation and aggregation in a CD40-dependent manner through two signalling pathways. The first signalling pathway involves TRAF2/Rac1/p38 MAPK, while the second involves TRAF2/NF-κB. In addition, these results show that an increase in circulating sCD40L levels exacerbates *in vivo* thrombus formation in response to arterial injury, and leukocyte infiltration within the thrombus mass via CD40.

As aggregation is the main physiological function of platelets, we initially evaluated the effect of sCD40L on this important aspect of platelet function. Our results indicate that treatment of resting platelets with sCD40L alone induces a slight membrane translocation of P-selectin; however, it is unable to induce neither platelet aggregation nor activation of the $\alpha_{IIb}\beta_3$ integrin. This effect of sCD40L on platelets differs from those induced by other platelet agonists, such as collagen and thrombin, which trigger $\alpha_{IIb}\beta_3$ activation and platelet aggregation. In fact, sCD40L alone was unable to induce intra-platelet calcium influx and dense granule secretion, which are essential for $\alpha_{IIb}\beta_3$ activation and platelet aggregation. In contrast, sCD40L potentiates P-selectin expression, $\alpha_{IIb}\beta_3$ activation, and platelet aggregation in response to sub-threshold concentrations of platelet agonists, including collagen, thrombin and ADP. Therefore, these results indicate that sCD40L probably acts as a broad and potent primer that predisposes platelets to an enhanced response to physiological agonists. Aside from sCD40L, platelet priming has been shown to be

induced by other molecules, namely MMP-2, MDC, plasma protein growth arrest-specific 6 (Gas-6), and stromal cell-derived factor-1α (SDF-1α).[230, 604, 605] Similarly to sCD40L, these molecules potentiate platelet responses to activating stimuli without occupying a main role in primary haemostasis. These molecules play a significant role in pathological thrombus formation, which makes them attractive potential targets for novel antiplatelet therapies.[606] Moreover, the priming effect of sCD40L is seen in other cell types, such as proliferating B lymphocytes, in which sCD40L requires IL-4 co-stimulation for IgE production. Taken together, these findings indicate that sCD40L acts as important accessory element in platelet function.

To this date, the identity of the sCD40L receptor on platelets remains a matter of controversy, given that platelets express three receptors for CD40L (CD40, $\alpha_{IIb}\beta_3$, and $\alpha_5\beta_1$).[4, 308, 310, 311] The results of our study using molecular and genetic approaches show that CD40 is the main receptor through which sCD40L mediates its potentiating effect on platelet responses. These results are consistent with previous studies demonstrating that sCD40L induces platelet activation, and secretion of ROS and RANTES through its interaction with CD40, the constitutively expressed CD40L receptor on the platelet surface.[310, 360, 361] In addition, sCD40L has been shown to interact with the $\alpha_{IIb}\beta_3$ integrin on platelets.[4, 307] However, this interaction was demonstrated by treating preactivated platelets ($\alpha_{IIb}\beta_3$ already in its active conformation) with 40 μg/mL of sCD40L, which is 40 times higher than the concentration used in our studies. This suggests that CD40 is probably the high-affinity receptor for sCD40L, whereas $\alpha_{IIb}\beta_3$ is the low-affinity one. Therefore, additional investigations are required to clarify this issue. Nonetheless, we could hypothesize that the increased levels of circulating sCD40L, as seen in ACS patients, prime quiescent platelets through CD40, as the $\alpha_{IIb}\beta_3$ integrin is in its inactive conformation. However, at the site of vascular injury, higher levels of sCD40L generated within the thrombus by activated platelets stabilize platelet aggregates via $\alpha_{IIb}\beta_3$ in its active conformation. However, this does not exclude the possibility that within a growing thrombus mass, CD40L expressed on activated platelets could interact with CD40 on adjacent platelets, thereby mediating CD40-dependent signals. Indeed, our unpublished results indicated that this is most probably the case (discussed in more detail below).

CD40 signalling in immune and non-immune cells requires the recruitment and association of TRAF molecules with its cytoplasmic tail. To our knowledge, the expression of TRAF members in platelets and their association with platelet CD40 following sCD40L

stimulation have not been reported. Our results demonstrate for the first time that platelets express TRAF1, 2, and 6, and that only TRAF2 associates with CD40 following sCD40L stimulation, indicating that it may ultimately be responsible for the downstream signalling events arising from platelet CD40 activation. Despite their presence in platelets, neither TRAF1 nor TRAF6 associates with CD40, suggesting that they might instead have a role in TNF receptor and TLR signalling. However, TRAF1 might play a role in enhancing TRAF2-mediated CD40 signals in platelets by promoting membrane localization of TRAF2.[387, 388] On the other hand, the cytoplasmic domain of CD40 contains binding sites for both TRAF2 and TRAF6, and binding of either one is sufficient to induce activation of NF-κB in B lymphocytes.[391] This is probably the case for platelets, as binding of only TRAF2 is sufficient to induce downstream signalling. However, unlike TRAF6, TRAF2 can activate both the canonical and non-canonical NF-κB signalling pathways, suggesting that in platelets TRAF2 binding potentially leads to activation of these two pathways. In addition, the almost complete lack of TRAF3 in platelets probably enhances TRAF2-mediated CD40 signals, since TRAF3 negatively regulates CD40 signalling by sequestering TRAF2 in a complex with cIAP1/2.[402, 408]

In B lymphocytes, it is well documented that CD40 induces activation of the NF-κB signalling pathway through either TRAF2 or TRAF6.[444] In platelets, the members of the NF-κB signalling pathway have recently been characterized and shown to be involved in platelet activation; however, their role in platelet CD40 signalling is still unknown.[607-609] As we have shown that sCD40L induces the association of TRAF2 but not TRAF6 with CD40 in platelets, we sought to assess whether CD40 ligation could trigger activation of the NF-κB signalling pathway in platelets. Our results demonstrate for the first time that treatment of platelets with sCD40L activates the NF-κB signalling pathway, specifically the canonical pathway, which was revealed by the phosphorylation of IκBα on $Ser^{32/36}$. These results add new insights to previous work showing that NF-κB may be a novel mediator of platelet responses.[608] Moreover, as mentioned earlier, it is probable that within a growing thrombus mass, CD40L expressed on activated platelets could interact with CD40 on adjacent platelets and induce signalling. Our unpublished results (Annex I, figure 1) demonstrate that platelet treatment with thrombin, which is also generated during thrombus formation, induces rapid expression of CD40L. This is in agreement with previous published studies demonstrating that CD40L is expressed on platelets upon activation; however, these studies failed to demonstrate whether the newly expressed

CD40L is capable of inducing CD40 signalling in platelets.[2] Our results add new insights to these previous works by documenting that upon thrombin activation, TRAF2 associates with CD40 and IκBα is phosphorylated on $Ser^{32/36}$ in a CD40L-dependent manner. This suggests that the newly expressed CD40L on platelets is capable of interacting with CD40 and subsequently trigger signalling, which contributes to platelet function upon thrombin stimulation. Whether the membrane or soluble form of CD40L generated by thrombin-stimulated platelets induces CD40 signalling remains to be determined. However, it is unlikely to be the soluble form that interacts with CD40 in thrombin-stimulated platelets, given that IκBα phosphorylation and TRAF2 association are detectable as early as 5 and 15 minutes, respectively, whereas platelets shed sCD40L within 1 to 2 hours of platelet activation and shedding also requires CD40 ligation.[3]

In search of the underlying molecular mechanisms by which sCD40L mediates its pro-activating and pro-aggregating effects on platelets, we initially hypothesized that sCD40L might induce the secretion of weak platelet agonists, such as TxA_2 and ADP, which could be responsible for the increase in platelet activation and aggregation in response to sub-threshold concentrations of agonists. Given that platelets secrete ADP from dense granule stores, we assessed dense granule release following sCD40L stimulation of resting platelets. Our results demonstrate that sCD40L alone does not affect dense granule secretion, as assessed by ATP release and mepacrine uptake. Additionally, we did not detect any changes in TxA_2 release (unpublished results; Annex I, figure 2), as measured by enzyme-linked immunosorbent assay (ELISA), from resting platelets following sCD40L stimulation, suggesting that sCD40L does not influence COX-1 activation in platelets. At light of these results, we speculate that patients under aspirin regiment would not be protected from the sCD40L effects on platelet function. Moreover, Chakrabarti et *al*.[360] have shown that platelets generate ROS following sCD40L stimulation; however, whether sCD40L potentiates platelet activation and aggregation via ROS production remains to be determined, since ROS have already been shown to contribute to platelet activation.[359] On the other hand, the morphological changes observed in platelets by scanning electron microscopy reveal that sCD40L rather acts directly on platelets by inducing intracellular signals that trigger platelet shape change characterized by the formation of filopodia and lamellipodia, which predispose platelets to enhanced responses in thrombogenic environments.

In platelets, Rac1 and VASP regulate shape change, cytoskeletal reorganization, and spreading, via lamellipodia and filopodia formation, respectively.[54, 610] VASP is mainly

phosphorylated on two principal residues, Ser^{239} and Ser^{157}. Platelet inhibitors, such as NO and PGI_2 induce phosphorylation of VASP on both residues, whereas platelet agonists, such as thrombin and collagen, trigger phosphorylation of VASP on Ser^{157}, thereby promoting the anticapping activity of VASP and the subsequent actin polymerization and filopodia formation.[54, 611, 612] Our results demonstrate that sCD40L induces phosphorylation of VASP on Ser^{157}, thereby providing evidence for its role as an inducer of shape change in platelets. The role of sCD40L in this process is further highlighted by its capacity to activate the small GTPase Rac1, which is involved in lamellipodia formation. Soluble CD40L-induced activation of Rac1 is physiologically significant, as specific inhibition of Rac1 considerably reduces sCD40L's ability to potentiate platelet aggregation and P-selectin expression. These results are in agreement with previous published findings demonstrating that CD40 signalling induces Rac1-dependent ROS production in WEHI 231 cells and endothelial cells.[613, 614] Interestingly, inhibition of Rac1 activation does not completely reverse sCD40L's ability to potentiate platelet aggregation and P-selectin expression, probably due to VASP and NF-κB activation, which are possibly not related to Rac1 activation and may therefore be responsible for the residual effects of sCD40L on platelets.

In immune cells, CD40L is typically known to induce activation of MAPKs, such as p38.[400] This seems to hold true for platelets. Indeed, our results confirm that stimulation of resting platelets with sCD40L induces the activation of p38 MAPK, and further highlight its involvement in platelet activation and aggregation, given that its inhibition abolishes the pro-activating and pro-aggregating effects of sCD40L. Furthermore, our results demonstrate that p38 MAPK is a downstream effector of Rac1, since its inhibition affects p38 MAPK activation. However, inhibition of Rac1 activation delays but does not completely inhibit p38 MAPK activation, which might explain in part the lack of complete reversal of sCD40L-induced potentiation of platelet aggregation and P-selectin expression following Rac1 inhibition. Nonetheless, the role of p38 MAPK in platelets seems to compliment that of Rac1, because it appears to be an important modulator of actin polymerization and platelet spreading, hence further confirming that sCD40L has an important role in inducing platelet shape change.[615] Moreover, extensive work has focused on the involvement of p38 MAPK in CD40 signalling; however, the association between this pathway and the NF-κB cascade remains a matter of debate.[360, 616, 617] Craxton et *al*.[618] have shown that p38 MAPK regulates NF-κB activation in B

cells, whereas p38 MAPK inhibition does not affect CD40-mediated binding of NF-κB to DNA, suggesting that NF-κB mediates its effects via both p38 MAPK-dependent and -independent pathways. Our results demonstrate a divergence between the canonical NF-κB and p38 MAPK signalling pathways downstream of platelet CD40, suggesting that these two pathways regulate different aspects of CD40-mediated platelet responses. For instance, the p38 MAPK pathway may be involved in cytoskeleton reorganization, as it has already been demonstrated to play an important role in actin polymerization and platelet spreading, whereas the NF-κB pathway may regulate *de novo* protein synthesis by modulating mRNA translation through its interaction with microRNAs (miRNAs), which have already been shown to be present in platelets.[615, 619, 620] Nonetheless, further investigations are required to clarify this issue.

Malaver et *al*.[608] have implicated the canonical NF-κB pathway in platelet function and showed that blocking IκBα phosphorylation, which is responsible for its activation, reverses platelet activation and aggregation triggered by a wide array of platelet agonists, including thrombin, collagen and ADP. In the present study, we show that sCD40L induces platelet activation as assessed by P-selectin translocation to the membrane, which is indicative of α-granule secretion, as well as activation of the canonical NF-κB signalling pathway. We then hypothesized that this pathway may be involved in platelet α-granule secretion. In fact, our results demonstrate that blockade of IKK, which phosphorylates IκBα, reverses sCD40L-induced α-granule secretion as assessed by P-selectin translocation. Therefore, apart from its role in activating the canonical NF-κB pathway, IKK could regulate key elements involved in the degranulation process, such as SNAP-23, which is involved in membrane and vesicle fusion. In fact, in mast cells, IKK2 regulates degranulation by phosphorylating SNAP-23 in a NF-κB-independent manner.[621] Whether this is the case in platelets merits further investigation, given that SNAP-23 is involved in platelet α-granule secretion.[188] Furthermore, we evaluated the involvement of the canonical NF-κB signalling pathway in the effects of sCD40L on platelet function. Our results show that inhibition of the canonical NF-κB signalling pathway by preventing either IKK activation or IκBα phosphorylation abolishes sCD40L-induced potentiation of platelet aggregation. These results indicate that sCD40L, through both NF-κB and p38 MAPK, primes platelets and predisposes them to enhanced aggregation responses in the presence of thrombotic stimuli. In addition, the NF-κB signalling pathway does not influence activation of the p38 MAPK pathway, and vice versa. However, this does not remove the

possibility of a crosstalk between NF-κB and p38 MAPK, and that inhibition of either abolishes the sCD40L-induced platelet responses.

To date, it is still unclear whether the elevated levels of sCD40L seen in patients with ACS are a consequence of increased platelet activation or a predetermining cause of disease development (or possibly both). Our results demonstrate for the first time a direct correlation between enhanced levels of sCD40L and thrombosis. Infusion of sCD40L into mice prior to vascular injury exacerbates thrombus formation as compared to control mice, indicating that these mice were predisposed to enhanced thrombotic stimuli. These results add new insight to the work by Andre et *al*.[4] that shows correction of thrombus instability, in a $\alpha_{IIb}\beta_3$-dependent manner, in CD40L deficient mice that were treated with 1.6 mg/kg of sCD40L. In our study, a different approach was used, as we aimed to mimic conditions in ACS patients where sCD40L levels are elevated. Under these conditions, injection of 0.25 mg/kg of sCD40L into mice results in plasma concentrations of approximately 50 ng/ml. These concentrations probably reflect the rapid clearance of a significant portion of the molecule by the liver or another metabolic organ. Nonetheless, circulating concentrations result in exacerbated thrombus formation in WT but not $CD40^{-/-}$ mice, indicating that elevated circulating sCD40L concentrations influence platelets through CD40 but not $\alpha_{IIb}\beta_3$, given that the integrin is in its inactive conformation. Moreover, this circulating concentration of 50 ng/mL of sCD40L that was achieved in our experimental model is noteworthy, since it is similar to the concentrations noted in multiple clinical studies, in patients with coronary and peripheral artery diseases.[594, 595] In addition, sCD40L levels are further increased in individuals with high cardiovascular risk factors, such as diabetes, obesity, metabolic syndrome, hypertension, and smoking.[622, 623] Furthermore, $CD40^{-/-}$ mice do not show defect in thrombus formation, therefore it is likely that CD40 is not required for platelet primary haemostasis, but rather has a pathological importance in atherothrombosis in the presence of elevated levels of sCD40L as seen in ACS patients. Nonetheless, the possibility that the $\alpha_{IIb}\beta_3$ integrin may be in an active conformation in circulating platelets of ACS patients could not be excluded, which suggests that sCD40L may interact with $\alpha_{IIb}\beta_3$ on circulating platelets and induce "outside-in" signals through the integrin. However, the effects of sCD40L on platelets through its interaction with $\alpha_{IIb}\beta_3$ are seen at concentrations of 1.6 mg/kg, which are many folds greater than the concentrations seen in these patients and our study. In this regard, the CD40L/CD40 interactions in ACS patients and individuals with high cardiovascular risk factors

would prime and predispose platelets to an exaggerated response to thrombotic stimuli, whereas the CD40L/$\alpha_{IIb}\beta_3$ interaction would be implicated in thrombus stability at the lesion site where higher concentration of sCD40L are generated by activated platelets.

Expression of P-selectin on the platelet surface plays an important role in thrombus stabilization, as well as in platelet/leukocyte interactions and the recruitment of leukocytes to the site of vascular injury.[310, 312, 360, 624] As sCD40L induces the expression of P-selectin on the platelet surface, we evaluated the extent of leukocyte infiltration within the thrombus mass. Although sCD40L has already been shown to promote platelet/leukocyte aggregate formation, we were the first to demonstrate in an *in vivo* setting that the increase in leukocyte infiltration within the thrombus of sCD40L-treated mice is CD40-dependent.[310] The synergy between the increase in leukocyte (probably TF expressing leukocytes) infiltration and the enhanced platelet predisposition to activation and aggregation in response to vascular injury may explain the increased thrombus formation in the animals' in response to elevated levels of sCD40L.

The relative contribution of the soluble versus the membrane-bound form of CD40L in thrombus formation is still unknown. It is most likely that both forms are involved in the pathophysiological functions of the CD40/CD40L axis. In fact, our preliminary results (unpublished results) suggest that thrombin, which induces platelet membrane expression of CD40L, results in TRAF2 association with CD40 and activation of the canonical NF-κB signalling pathway in a CD40L-dependent manner. On the other hand, elevated levels of sCD40L, as seen under pathological conditions, predispose platelets to enhanced activation and aggregation in response to thrombotic stimuli. Nonetheless, the involvement of CD40L in atherogenesis, thrombus formation, platelet-mediated inflammation, and plaque instability makes it a potential target for the treatment of thrombo-inflammatory complications. In this regard, it be would relevant for future clinical studies to evaluate the level of platelet priming in ACS patients, who present elevated levels of sCD40L. This could be done by assessing the levels of TRAF2 association with CD40 or the activation of the NF-κB signalling pathway. Thus, a direct clinical association between the levels of circulating sCD40L and platelet function could be established for pharmacological targeting.

In summary, our studies show for the first time the *in vitro* and *in vivo* pathophysiological implication of sCD40L in different aspects of platelet function. We demonstrated that sCD40L potentiates platelet activation and aggregation in a CD40-dependent manner through two

divergent signalling pathways (Annex I, figure 3). The first signalling pathway involves TRAF2/Rac1/p38 MAPK, while the second involves TRAF2/NF-κB. In addition, our results show that an increase in circulating sCD40L levels exacerbates *in vivo* thrombus formation in response to arterial injury, and leukocyte infiltration within the thrombus mass in a CD40-dependent manner. Therefore, these studies provide novel evidences for the regulation of platelet function by sCD40L and may explain in part the link between enhanced levels of sCD40L and the incidence of cardiovascular complications. The CD40/CD40L axis may eventually represent a therapeutic target for the treatment of thrombo-inflammatory diseases.

Conclusion and Future Directions

The results of our studies reveal a novel role for sCD40L in regulating platelet function and thrombus formation. In fact, the underlying cellular and molecular mechanisms induced by sCD40L, which affect platelet activation, aggregation, and thrombus formation, were unexplored. Interestingly, the most relevant results of our studies stem from the correlation between increased levels of circulating sCD40L and exacerbated *in vivo* thrombosis, which may explain in part the link between enhanced levels of sCD40L seen in ACS patients and disease complications. Moreover, the identification of the intracellular signalling pathways by which sCD40L affects platelet function provides important clues for the development of specific therapeutic targets for the treatment of thrombotic diseases.

However, as for any other pharmacological target, care should be taken in developing specific antagonists against the CD40/CD40L dyad in order to avoid unwanted side effects. For instance, blockade of either CD40 or CD40L could result in immune defects, such as the one seen in HIGM patients who lack effective development of an adaptive immune system in addition to increased cancer incidence due to a lack of an immune response against tumour cells. Therefore, a better understanding of the mechanisms by which this dyad affects platelet function would be necessary in order to develop effective pharmacological tools against specific signalling pathways involved in platelet CD40 signalling. A suggestive approach would be to target CD40-induced TRAF2 signalling specifically in platelets through either the use of peptides that interfere with TRAF2/CD40 interactions, or the use of vectors or nanoparticles that block CD40 or TRAF2.

Although our studies add significant novel insights into the cellular and molecular mechanisms by which sCD40L affects platelet function, they do not answer all the questions and issues related to CD40/CD40L interactions in platelets. For instance, future studies addressing the following issues could provide more insights into the role and the mechanisms of action of the CD40L/CD40 axis in platelets:

1 - What is the exact role of TRAF2 in platelet CD40 signalling, specifically in response to thrombin?

2 - What is the exact role of the canonical NF-κB signalling pathway in platelet CD40 signalling? Is it involved in regulating *de novo* protein synthesis by modulating mRNA translation through its interaction with miRNAs or degranulation?

3 - Is the non-canonical NF-κB signalling pathway activated by sCD40L, given that TRAF2 is involved in the activation of both NF-κB pathways? If yes, what is its role in platelet function in response to sCD40L?

4 - What is the relative contribution of the p38 MAPK and NF-κB signalling pathways downstream of platelet CD40?

5 - Does sCD40L induce its effects on platelets through ROS generation?

6 - What is the relative contribution of CD40 and $\alpha_{IIb}\beta_3$ in platelet function in response to the membrane-bound or soluble forms of CD40L?

7 - Can elevated levels of sCD40L be used as a cardiovascular disease diagnostic marker, such as troponin?

Answers to these questions will undoubtedly bring important contributions to our knowledge regarding the precise role of the CD40/CD40L axis in platelets, and consequently in thrombotic cardiovascular disease development.

In conclusion, our studies confirm the tight link between the CD40/CD40L axis, inflammation and thrombotic cardiovascular disease pathogenesis, and suggest that this dyad may ultimately represent a potential therapeutic target in the treatment of these diseases. Nonetheless, further investigations are required to better understand the role of this dyad and its molecular partners in disease development and progression.

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Publications

List of publications 2010 - 2012

1 - **Hachem A**, Yacoub D, Théorêt JF, Gillis MA, Mourad W, Merhi Y. Enhanced levels of soluble CD40 ligand exacerbate platelet aggregation and thrombus formation through a CD40-dependent tumor necrosis factor receptor associated factor-2/Rac1/p38 mitogen-activated protein kinase signaling pathway. *Arterioscler Thromb Vasc Biol* 2010; 30:2424-2433.

2 - Théorêt JF, Yacoub D**, Hachem A**, Gillis MA, Merhi Y. P-selectin ligation induces platelet activation and enhances microaggregate and thrombus formation. *Thrombosis Research* 2011; 128:243-250.

3 - **Hachem A**, Yacoub D, Zaid Y, Mourad W, Merhi Y. Involvement of Nuclear Factor κB in Platelet CD40 Signaling. *Biochemical and Biophysical Research Communications*. 2012; 425:58-63.

Annex I

Unpublished results

This section includes data that were not published.

Figure 1

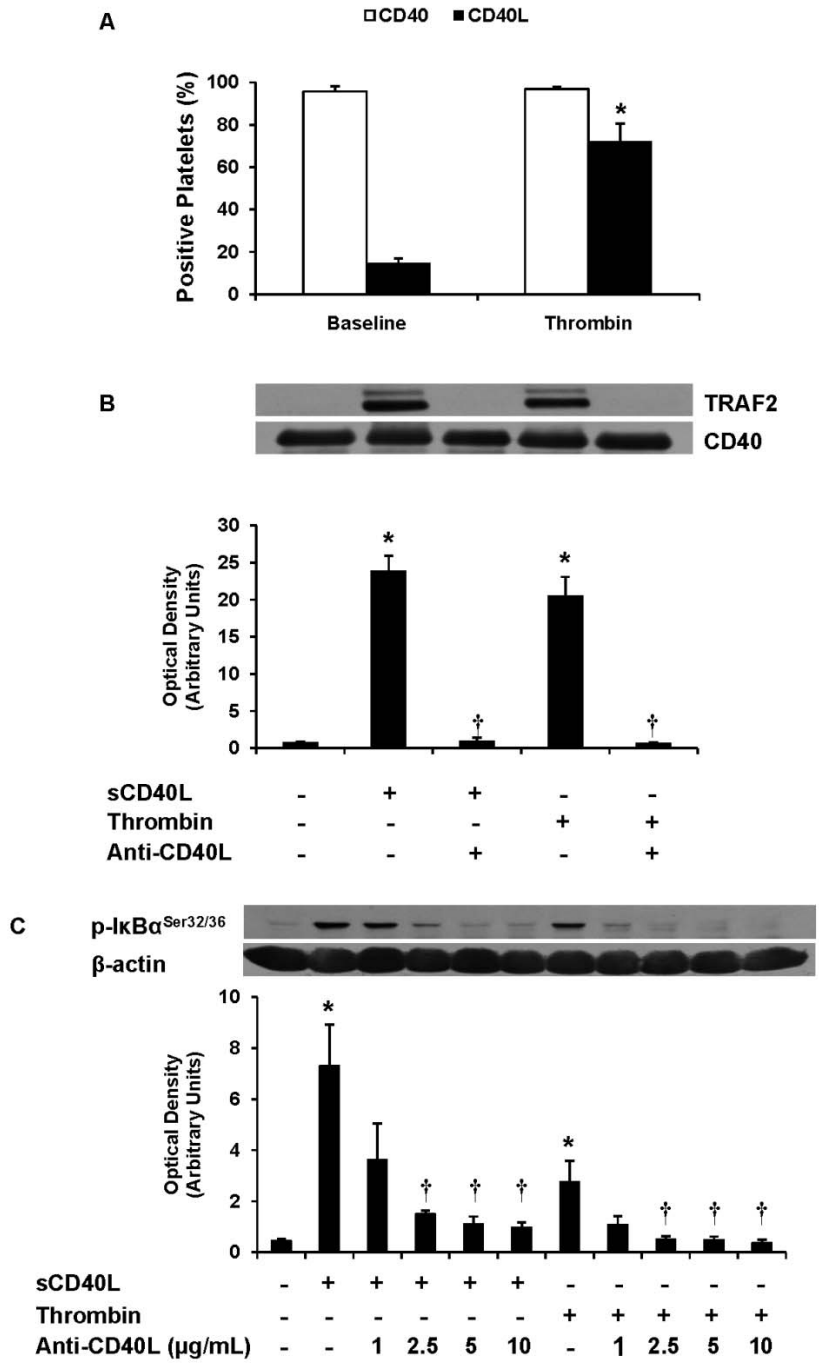

Figure 1: Thrombin and sCD40L induce TRAF2 association with CD40 and IκBα phosphorylation in platelets. A. CD40 and CD40L expression on platelets following thrombin stimulation. Platelets (250 X 10^6/mL) were left untreated or stimulated with thrombin (0.1 U/mL) and assessed for CD40 and CD40L expression by flow cytometry. Histogram represents the mean of data expressed as percent of CD40 and CD40L positive platelets (n=3; **P*<0.05 vs. baseline). B. Platelets (500 X 10^6/mL) were left untreated or incubated with 1 μg/mL sCD40L or 0.1 U/mL thrombin for 15 minutes at 37ºC in the presence or absence of a blocking anti-CD40L antibody (5 μg/mL). Total cell lysates were then immunoprecipated using an anti-CD40 antibody and immunoblotted for TRAF2 and CD40 expression. Blots are representative of 3 independent experiments. Histogram represents the mean of data of overlay blots, expressed as arbitrary units of optical density (n=3; **P*<0.05 vs. baseline and †*P*<0.05 vs. sCD40L or thrombin alone). C. Dose-dependent effect of anti-CD40L treatment on IκBα phosphorylation. Platelets (1000 X 10^6/mL) were left untreated or pretreated with the indicated dose of anti-CD40L for 5 minutes at 37ºC prior to stimulation with sCD40L (n=5) or thrombin (n=3) for 5 minutes. Platelet lysates were then analyzed for p-IκBα $Ser^{32/36}$. β-actin blots shown are from stripped p-IκBα $Ser^{32/36}$ membranes. Blots are representative of the indicated number of independent experiments. Histogram represent the mean of data of overlay blots, expressed as arbitrary units of optical density (**P*<0.05 vs. baseline and †*P*<0.05 vs. sCD40L or thrombin alone).

Figure 2

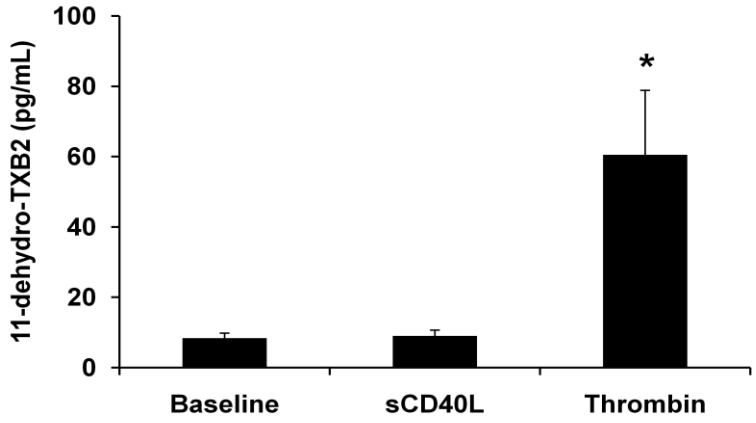

Figure 2: Effects of sCD40L and thrombin on TxA_2 secretion. Platelets (250 X 10^6/mL) were left untreated or treated with sCD40L (1 μg/mL) or thrombin (0.5 U/mL) and supernatants were assessed for 11-dehydro-TxB_2 (the stable metabolite of TxA_2) by ELISA. Histogram represents the mean of data expressed as pg/mL of 11-dehydro-TxB_2 (n=4; **P*<0.05 vs. baseline).

Schematic representation

This section includes a schematic representation of the signalling pathways downstream of platelet CD40 that were discovered in our studies.

Figure 3

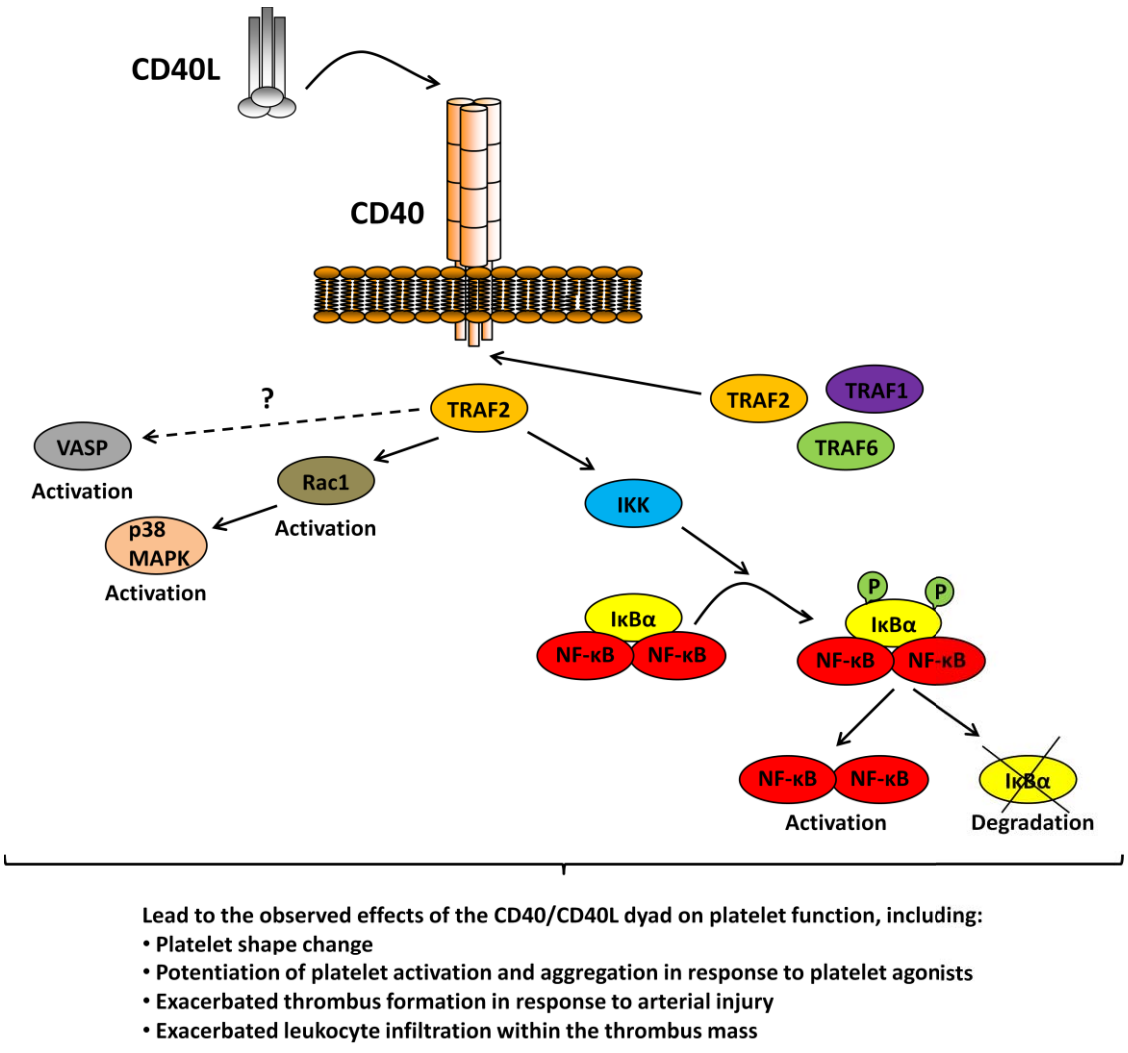

Figure 3: Schematic representation of the two divergent signalling pathways downstream of platelet CD40. Upon sCD40L/CD40 interaction, TRAF2 associates with the cytoplasmic tail of CD40. TRAF2 then induces activation of IKK, which in turn is responsible for IκBα phosphorylation and its subsequent degradation and release of the active form of NF-κB. On the other hand, TRAF2 activates Rac1, which in turn activates p38 MAPK. It is still unknown how TRAF2 induces activation of VASP. Culmination of these signalling pathways leads to the observed effects of the CD40/CD40L dyad on platelet function. Image is produced by Hachem A.

Annex II

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[redacted]

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