Investigating integrin $\alpha M\beta 2$ outside-in signaling in human monocytes

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**INVESTIGATING INTEGRIN α M β 2 OUTSIDE-IN
SIGNALING IN HUMAN MONOCYTES**

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SCHOOL OF BIOLOGICAL SCIENCES

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INVESTIGATING INTEGRIN α M β 2 OUTSIDE-IN SIGNALING IN HUMAN MONOCYTES

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Abbreviations

Ab	antibody
APC	allophycocyanin
ATCC	American type culture collection
BCECF	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein
BCR	B cell receptor
βTD	β tail domain
BM	bone marrow
BSA	bovine serum albumin
CAMs	cell adhesion molecules
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CHO cells	Chinese hamster ovary cells
co-IP	co-immunoprecipitation
CR3	complement receptor 3
C-terminal	carboxy terminal
DAG	diacyl glycerol
DAPI	4',6-diamidino-2-phenylindole
ddH ₂ O	distilled deionized H ₂ O
DMSO	dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	deoxyribonucleotide triphosphate
DSP	dithiobis succinimidyl propionate
DTT	dithiothreitol
ECIS	electric cell-substrate impedance sensing
ECL	enhanced chemiluminescence
ECM	extracellular matrix
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGTA	ethylene-glycol-bis (β-aminoethylether)-tetraacetic acid
Erk	extracellular regulated protein kinase
FACS	fluorescence activated cell sorting
F-actin	filamentous-actin

FAK	focal adhesion kinase
FBS	fetal bovine serum
Fc	fragment crystallizable (of immunoglobulin)
FcR	Fc receptor
FERM	4.1-ezrin-radixin-moesin
FITC	fluorescein isothiocyanate
Fox	forkhead box
Foxp1	forkhead box P1
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GT	Glanzmann's Thrombasthenia
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMVE	human lung microvascular endothelial
HRP	horseradish peroxidase
HSA	human serum albumin
iC3b	inactive C3b
ICAM	intercellular adhesion molecule
I-EGF	integrin epidermal growth factor
IFN	interferon
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
I κ B	inhibitor of κ B
IKK β	inhibitor of nuclear factor κ B kinase subunit β
IL	interleukin
IP	Immunoprecipitation
IPs	Immunoprecipitates
IRAK1	IL-1 receptor-associated kinase-1
IRF3	interferon regulatory factor 3
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
JAM	junctional adhesion molecule
JEB	junctional epidermolysis bullosa
JNK	c-Jun N-terminal kinase
κ B	kappa-light-chain-enhancer of activated B cells
kD	kilodalton

KD	kinase dead
K562 con siRNA	K562 cells treated with non-targeting siRNA
K562 k3 siRNA	K562 cells treated with kindlin3 siRNA
K562/ α M β 2 (KM)	K562 cells stably expressing wild type integrin α M β 2
ctrl-KM	K562/ α M β 2 cells expressing non-targeting control siRNA
k3-KM	K562/ α M β 2 cells expressing kindlin3 siRNA
L	litre
LAD	leukocyte adhesion deficiency
LB	Lysogeny broth
LFA-1	leukocyte function-associated antigen-1
LIMBS	ligand-induced metal-binding site
LPS	lipopolysaccharide
LY	LY294002, PI3K inhibitor
mAb	monoclonal antibody
Mac-1	macrophage-1 antigen
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony stimulating factor
MEK	MAPK/Erk kinase
MIDAS	metal ion-dependent adhesion site
ADMIDAS	adjacent to MIDAS
min	minute(s)
mRNA	messenger ribonucleic acid
ms	millisecond(s)
NADPH	nicotinamide adenine dinucleotide phosphate
NIF	neutrophil inhibitory factor
NK cell	natural killer cell
NF κ B	nuclear factor κ B
NP-40	nonidet P-40
N-terminal	amino terminal
O.D.	optical density
PBS	phosphate buffered saline
PBS-T	PBS with 0.1% (v/v) tween-20
PCR	polymerase chain reaction
qPCR	quantitative PCR
PH domain	pleckstrin homology domain
PIC	picetannol, Syk inhibitor

PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PKD	protein kinase D
PMA	phorbol myristate acetate
PS	penicillin streptomycin
PSI	plexin-semaphorin-integrin
PTK	protein tyrosine kinases
PVDF	polyvinylidene fluoride membrane
PVP	polyvinylpyrrolidone 10000
RBC	red blood cell
rHck	recombinant Hck
RIP1	receptor-interacting protein-1
ROS	reactive oxygen species
rPKC δ	recombinant PKC δ
rpm	revolutions per minute
RPMI-1640	Roswell Park Memorial Institute formulation-1640
RT	reverse transcription
RT-PCR	reverse transcription-polymerase chain reaction
SBB	sodium bicarbonate buffer
SD	standard deviation
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFK	Src family kinase
SH2	Src homology 2
SH3	Src homology 3
SHIP-1	SH2-containing 5' inositol phosphatase
SHP-1	SH2-containing phosphatase-1
siRNA	small interfering RNA
Syk	spleen tyrosine kinase
TAE buffer	Tris-acetate-EDTA buffer
TAK1	transforming growth factor- β activated kinase-1
TBK1	TANK-binding kinase-1
TBS	Tris buffered saline
TBS-T	TBS with 0.1% (v/v) tween-20
TCR	T cell receptor

TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TM domain	transmembrane domain
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
TRIF	TIR domain-containing adapter-inducing interferon- β
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
v/v	volume per volume
w/v	weight per volume
WT	wild type

Single letter or triplet code is used to describe amino acid. Restriction enzymes are referred by their three letter names derived from that of the source microorganism. Other abbreviations are indicated in the text where first used.

Abstract

Integrins are type I transmembrane and α/β heterodimeric adhesion receptors that mediate the interaction of cells and the extracellular matrix (ECM) or adjacent cells. They are expressed on most eukaryotic cells and involved in diverse biological functions, such as immune defense, cell growth and differentiation, hemostasis, bone resorption, tissue development, and neural functions. Although integrins do not have catalytic activities, they are bidirectional signaling receptors since their cytoplasmic tails are docking sites for many cytoplasmic molecules.

Integrin $\alpha M\beta 2$ is mainly expressed on cells of the myeloid lineage, natural killer (NK) cells and $\gamma\delta$ T cells. It plays crucial roles in cell adhesion, degranulation and phagocytosis. Integrin $\alpha M\beta 2$ ligation-triggered intracellular signals are important for cell migration, cell growth and cell differentiation. This thesis describes the investigation of integrin $\alpha M\beta 2$ outside-in signaling pathway. The results of this thesis contain three sections.

In the first section (3.1), we provide data showing that protein kinase C (PKC) δ mediates integrin $\alpha M\beta 2$ outside-in signaling in monocytes. Integrin $\alpha M\beta 2$ clustering induces the translocation of PKC δ to the plasma membrane and PKC δ phosphorylation at Tyr311. PKC δ is activated by the src family kinases (SFKs), such as Hck and Lyn. We also show that the integrin $\beta 2$ sequence Asn727-Ser734 is important in integrin $\alpha M\beta 2$ -induced PKC δ Tyr311 phosphorylation. We found that clustering of integrin $\alpha M\beta 2$ induces down-regulation of forkhead box P1 (Foxp1), which is a transcription factor involved in monocyte differentiation. The expression of Foxp1 was reduced in monocytes following plating of cells on human lung microvascular endothelial (HMVE) cells. This was not observed in PKC δ -targeting small interfering RNA (siRNA)-treated monocytes. These results support a role for PKC δ in integrin $\alpha M\beta 2$ -induced Foxp1 regulation in monocytes.

In the second section (3.2), we examined the activation of signaling conduits downstream of PKC δ that may lead into Foxp1 down-regulation. We

found that mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinase (Erk) kinase (MEK)-Erk pathway is involved in integrin $\alpha\text{M}\beta 2$ -induced Foxp1 regulation. We also examined the molecules that are involved in the cross-talk between integrin $\alpha\text{M}\beta 2$ signaling and lipopolysaccharide (LPS)-induced Toll-like receptor (TLR) signaling. These molecules include interleukin-1 (IL-1) receptor-associated kinase-1 (IRAK1), PKC δ and MAPKs (such as Erk, c-Jun N-terminal kinase (JNK), and p38). We show that PKC δ interacts with IRAK1 and induces IRAK1T209 phosphorylation. These results suggest that the cross-talk between integrin $\alpha\text{M}\beta 2$ signaling and LPS-induced signaling is involved in the interaction between PKC δ and IRAK1.

In the third section (**3.3**), we show that the 4.1-Ezrin-Radixin-Moesin (FERM)-containing cytoplasmic protein kindlin3 is required for integrin $\alpha\text{M}\beta 2$ outside-in signaling that modulates cell spreading. Reduced kindlin3 expression in K562 cells expressing wild type (WT) integrin $\alpha\text{M}\beta 2$ or constitutively activated integrin $\alpha\text{M}\beta 2\text{N329S}$ abrogated the adhesion and spreading capacities of these cells on ligands inactive C3b (iC3b) and bovine serum albumin (BSA). We also show that kindlin3 is involved in the integrin $\alpha\text{M}\beta 2$ -spleen tyrosine kinase (Syk)-Vav1 signaling axis regulating the activities of Rac1 and Cdc42. These results provide further evidence that kindlin3 plays an important role in integrin outside-in signaling.

1. Introduction

1.1 Overview

Leukocytes derived from bone marrow (BM) are principal components of the immune system. During inflammation, the targeting of leukocytes to sites of infection involves a series of adhesion events mediated by cell adhesion molecules (CAMs), such as selectins, integrins, cadherins and immunoglobulin superfamily (IgSF) [1, 2].

Selectins and their ligands on both leukocytes and the endothelium allow leukocytes to attach loosely and roll on the endothelium. Leukocytes can therefore be activated by immobilized chemokines on the surface of the endothelium. This leads to the activation of integrins on the leukocytes that promote firm adhesion of the leukocytes on the endothelium [3]. Subsequently the leukocyte will polarize to have a leading edge and an elongated uropod, which are necessary for transmigration across the endothelium [4, 5].

Among these CAMs, integrins play an important role not only in leukocyte migration and trafficking, they are also crucial for leukocyte survival and differentiation [6-9].

1.2 Integrins

Integrins are type I transmembrane CAMs and they serve as cell signaling receptors despite the lack of intrinsic enzymatic function [10-12]. In addition of providing immunity, integrins are essential in other biological processes, including hemostasis, wound healing, angiogenesis, cell survival and differentiation [13].

The importance of integrins is highlighted by many debilitating diseases. Poor expression of $\beta 2$ integrins in leukocyte adhesion deficiency type I (LAD I) and dysfunctional $\beta 2$ integrins in LAD III affect leukocyte adhesion and migration into infected tissues, leading to persistent microbial infection in immune compromised patients [14, 15]. In the bleeding disorder Glanzmann's Thrombasthenia (GT), mutations in the integrin αIIb or $\beta 3$ subunit disrupt

normal α IIb β 3 expression and/or function, thus leading to poor platelet aggregation and blood clotting function [16, 17]. In the skin blistering disease junctional epidermolysis bullosa (JEB), hemidesmosome contacts between the epidermis and the basal lamina are disrupted because of mutations in the integrin β 4 subunit [18].

Each integrin contains an α and a β subunits that are joined non-covalently. Both subunits have a large extracellular domain, a transmembrane domain (TM domain) and a short cytoplasmic domain (Figure 1.1). In humans, there are 24 integrins formed by the specific pairing of 18 α and 8 β subunits (Figure 1.2). Based on their pairings, type of ligand or tissue expressions, integrins can be categorized into different subfamilies [10].

There can be more than one ligand for each integrin and many integrins bind to common ligands [5, 19-21]. Integrin-ligand binding is highly dependent on divalent cations in the extracellular micro-environment [22-26]. Ligand-binding is a tightly regulated process that involves bi-directional signaling and long-ranged conformational changes [10, 27, 28]. These processes require inter- and intra-subunit interactions of the integrin domains.

Figure 1.1 Integrin heterodimer

An integrin molecule includes an α and a β subunits joined by non-covalent bond. Both subunits contain a large extracellular domain, a TM domain and a cytoplasmic domain.

Figure 1.2 Human integrin α and β heterodimeric pairings

Integrins are formed by specific non-covalent association of different α and β subunits. A single subunit can pair with more than one partner subunit in some cases, for example, the $\beta 1$ subunit can pair with 12 different α subunits. Integrin α subunits that contain the Inserted (I)-domain are shaded in purple. Integrins expressed in immune cells are shown with green connecting lines [11].

1.3 Overall structure of integrins

Both of α and β subunits consist of a large extracellular region, a single TM domain and a short cytoplasmic tail, except $\beta 4$ subunit which has a large cytoplasmic domain consisting of four fibronectin type III repeats [29]. Integrin α and β subunits non-covalently associate with each other to form a receptor with a large globular headpiece and two stalks. The globular headpiece is

involved in ligand-binding, and the two extracellular stalks are connected to the TM domain and cytoplasmic tail of the individual subunit, moving apart or coming together depending on inter- and intra-subunit interactions of individual domains of the integrin α and β subunit.

The general domain organization of integrin α and β subunits is shown in Figure 1.3.

Figure 1.3 Integrin domain organization

The domain organization within the primary structure of integrin is shown. The α subunits of α L, α M, α X, α D, α E, α 1, α 2, α 10 and α 11 contain an I domain, which is inserted between β -sheets 2 and 3 of the β -propeller domain, as denoted by the dotted line.

The extracellular region of the integrin α subunit is linearly organized from the N- to C-terminus into four distinct domains: β -propeller, thigh, calf-1 and calf-2 domains. A prominent feature in the integrin α subunit is a seven-bladed β -propeller domain similar to the β -propeller domain found in the β subunit of the G proteins [30]. The β -propeller domain contains 7 repeats of 60 amino acids that fold into 7 radially arranged blades [13, 31]. Depending on the α subunit, it may include an additional domain known as I domain. α subunits containing the I domain are the α L, α M, α X, α D, α E, α 1, α 2, α 10 and α 11 subunits. The I domain is inserted between β -sheets 2 and 3 of the β -propeller domain and adopts a Rossman fold with a metal ion-dependent adhesion site (MIDAS) [13], forming the major receptor binding site [32]. The thigh domain is

located after the β -propeller domain and adopts the C2-set immunoglobulin (Ig) fold. The 2 calf domains both have 2 anti-parallel β -sheets [31].

The integrin β subunit consists of eight extracellular domains: the plexin-semaphorin-integrin (PSI) domain, hybrid domain, I-like domain, four integrin epidermal growth factor (I-EGF) domains, and the β tail domain (β TD). At the amino terminal (N-terminal) of integrin β subunit is the PSI domain, which share sequence homology with the membrane proteins plexins and semaphorins [33]. The PSI domain is a two-stranded anti-parallel β -sheet flanked by two short helices and has a cysteine-rich region with eight cysteines paired by disulfide bonds. At the carboxy terminal (C-terminal) of the PSI lies the hybrid domain that is a β -sandwich domain formed from two segments flanking the I-like domain in the primary sequence [34]. The β I-like domain is inserted into the hybrid domain which is itself inserted in the PSI domain. The β I-like domain consists of a central 6-stranded β -sheet surrounded by 8 helices similar to the α -subunit I domain, and it has three metal ion binding sites, such as MIDAS, adjacent to MIDAS (ADMIDAS) and ligand-induced metal ion binding site (LIMBS). After the hybrid domain, there are four tandem cysteine-rich repeats, called I-EGF 1-4, which shares sequence homology with the epidermal growth factor (EGF) modules. The β TD consists of a four-strand β sheet.

The integrin TM domains are the transducers of activation signal between the integrin extracellular and intracellular domains. It is highly conserved between integrins, and has approximately 25-29 amino acids. It is predicted to assume a helical structure in the phospholipid bilayer.

The cytoplasmic tails of integrin α and β subunits are generally less than 70 residues, with the exception of the β 4 subunit [10, 29], and are generally less structured compared to the extracellular and TM domains. Despite the weak interaction of the α and β cytoplasmic tails, its disruption leads to integrin activation [35, 36]. In addition to the hydrophobic contacts, an electrostatic contact forming a salt-bridge between the α and β cytoplasmic tails was reported [37]. The salt-bridge is formed by an Arg found in the conserved α subunit membrane-proximal sequence GFFKR and an Asp found in the β

subunit membrane-proximal sequence LLv-iHDR (highly conserved residues are uppercase, less conserved residues are lowercase, and the dash represents nonconserved residue) [38, 39].

The integrin cytoplasmic tails are short, but they act as a signaling hub by interacting with a series of cytoplasmic proteins [40-42]. It is interesting to note that the β subunit cytoplasmic tails are highly conserved as compared to the α cytoplasmic tails. There are two highly conserved NPXY/F motifs in integrin β cytoplasmic tails (Figure 1.4) [7]. The membrane proximal NPXY/F motif is a docking site for integrin activator talin [43, 44], and the other NPXY/F motif has been reported to bind with the integrin co-activator kindlins [45-47]. Other cytoplasmic molecules that bind integrin β cytoplasmic tails have been reported [48].

	Membrane	Cytoplasmic
β1D 752	KLLMI I	HDRREFAKFEKEKMNAKWD TQENP IYKSPINNFKNPNYGRKAGL
β2 724	KALTHL	SDLREYRRFEKEKLKSQWNNDNPLFKSATTVMNPKFAES
β3 742	KLLITI	HDRKEFAKFEERARAKWD TANPLYKEATSTFTNITYRGT
β4 734	KYCACC	KACLALLPCCNRGH (another 1123 residues)
β5 743	KLLVTI	HDRREFAKFQSERSRARYEMASNPLFRKPISTHTVDFTFNKENKSYNGTVD
β6 731	KLLVSF	HDRKEVAKFEAERSKAKWQTGTNPLYRGSTSTFKNVTYKHREKQKVDLSTDC
β7 747	RLSVEI	YDRREYSRFEKEQQQLNWKQDSNPLYKSAITTTINPREQEADSPTL
β8 705	RQVILQ	WNSNLIKSSSDYRVSASKKDKLILQSVCTRAVTYRREKPEEIKMDISKLNHETFCNF

Figure 1.4 Sequences of the integrin β cytoplasmic tails

The membrane proximal NPXY/F motif in the integrin β cytoplasmic tails is highlighted in red, whereas the membrane distal NPXY/F motif is highlighted in green, with the exception of integrin β 4 and β 8 subunits.

1.4 Conformational changes of integrin structure

Integrins are not just passive molecular glue; they also actively function in transmitting signals from the extracellular environment across the plasma membrane into the interior of the cell and vice versa. The bi-directional signaling is coordinated by integrin allostery through interactions of inter- and

intra-subunit domains, leading to integrin conformational changes and thus its affinity to different extracellular receptors [49]. Integrin conformational changes initiate many cellular processes, such as migration, polarization and aggregation [28]. It is well known that integrins can adopt three distinct conformations. These conformations are the 'bent, with closed headpiece and clasped cytoplasmic tails', the 'extended, with closed headpiece and separated cytoplasmic tails' and the 'extended, with open headpiece and separated cytoplasmic tails'. These three forms are suggested to be the low affinity, intermediate affinity and high affinity forms, respectively (Figure 1.5) [23]. However, this is only a generalized three-state model of integrin activation, and other forms of the integrin states are still being investigated [50, 51].

Figure 1.5 Structural transitions of the integrin molecule

The three generic conformations of the integrin molecule and their affinity states to the ligand. Integrin α -subunit is in green and integrin β -subunit is in blue.

Structural and cell-based studies on integrins have shown that integrin ligand-binding affinity is regulated by its conformational changes. The model of inside-out integrin activation indicates the conversion of a bent integrin to a highly extended integrin in a “switchblade” motion [52]. Upon activation through the integrin cytoplasmic regions, the interface between integrin headpiece and stalk opens in a switchblade-like manner, leading to the extension of ligand-binding integrin headpiece away from the plasma

membrane [52]. The headpiece remains in a closed conformation until it makes contact with its ligand.

1.5 α -less integrin and α l integrin

Depending on the presence and absence of the I domain, integrins are classified into α -less integrin and α l integrin. Nine of the α subunits (α L, α M, α X, α D, α E, α 1, α 2, α 10 and α 11) contain the I domain, which is inserted between β -sheets 2 and 3 of the β -propeller domain (Figure 1.3).

Three integrin conformational states of α -less and α l integrins are shown in Figure 1.6. The bent conformation (Figure 1.6 A and D) has a closed headpiece, with a low ligand-binding affinity. Extension of the α and β -knees releases the interface between integrin headpiece and lower legs, leading to extended-closed conformation with an intermediate ligand-binding affinity (Figure 1.6 B and E). The hybrid domain swings out with respect to β I-like domain, which results in the extended-open conformation with a high ligand-binding affinity (Figure 1.6 C and F).

The major difference between α -less and α l integrins is the ligand-binding site(s) [53]. In α -less integrins, the β -propeller and the I-like domain form the ligand-binding interface [54]. In α l integrins, the I domain is the primary binding site [55].

Figure 1.6 α I-less integrin and α I integrin

The integrin conformational states of α I-less integrin is shown in A, B and C, while α I integrin is shown in D, E and F.

1.6 Integrin functions

Integrins have two major functions. They mediate cell-cell, cell-ECM and cell-pathogen attachments, and transmit signals from the ECM into cells [6].

Cell-cell and cell-ECM attachments are required for the development of multi-cellular organisms. Apart from being an adhesion molecule, integrins transduce signals from the surrounding microenvironment into the cell. The signals from integrins and other receptors (such as EGF and vascular endothelial growth factor (VEGF)) enforce cells to decide on their biological action to take, attachment, movement, death, or differentiation [6, 56]. Thus integrins are involved in many cellular biological processes. Cell adhesion is a complex process that not only involves adhesion molecules such as integrins but also many cytoplasmic proteins, such as talin, kindlin, vinculin, α -actinin

and paxillin [6, 56]. They regulate focal adhesion kinase (FAK) and SFK members to phosphorylate their substrates, such as Cbl/phosphoinositide 3-kinase (PI3K) and the immunoreceptor tyrosine-based activation motif (ITAM)/Syk [49]. The adhesion complexes have been reported to connect to the actin cytoskeleton [57, 58], thus integrins link the two networks across cell plasma membrane: the intracellular actin filamentous system and the ECM.

Integrins also mediate cell-pathogen attachment during the immune response. As an example, integrin $\alpha M\beta 2$ has been reported to be involved in pathogen recognition through inducing lymphocyte adhesion to the fungus such as *Candida albicans* [59]. The human body is protected by the immune system against pathogens including fungus, bacteria, parasites and viruses. The complement system is a part of the immune system and plays an important role to clear the pathogens on an organism. The pathogens are recognized by leukocytes which can be divided into phagocytes and lymphocytes. Phagocytes form the first defense line by using various pathogen-uptake receptors such as fragment crystallizable (Fc) receptors (FcRs) and complement receptors (CRs). Integrin $\alpha M\beta 2$ (CR3) and $\alpha X\beta 2$ (CR4) bind to iC3b, which is an inactive form from the fragment C3b. After the activation of complement on monocytes, iC3b opsonizes apoptotic cells and pathogens [60].

Briefly, the complement system can be activated through three pathways such as classical, alternative and lectin pathways. Typically, the classical pathway needs immune complexes for activation, but the alternative and lectin pathways can be activated by C3 hydrolysis or antigens only. In all three activation pathways, the homologous variants of the protease C3-convertase can be generated. The component C3 can be activated and cleaved by C3-convertase into C3a and C3b, leading to a series of further activation and cleavage events. In this case, C3b can bind to a host cell or a pathogen surface, as it is free and abundant in the plasma. To prevent complement activation from proceeding on the host cell, C3b is cleaved into its inactive form iC3b, by a plasma protease (complement factor I) together with complement factor H, CR1, and membrane cofactor of proteolysis (MCP).

Integrins are important in cell signaling [6]. By connecting with ECM molecules, integrin causes a signal transmitted into cells through protein kinases. These protein kinases directly or indirectly, stably or transiently associate with the cytoplasmic tail of integrin, leading to integrin conformational changes mediated by the binding of ECM. The signals that the cell receives via integrins influence cell survival, cell growth, cell division, cell differentiation, and cell apoptosis [6].

In order to determine the exact function of each integrin subunit, knock out and chimeric animals were generated. Knockout of integrin $\beta 1$ subunit ablates 12 integrins and it is embryonic lethal [61]. Integrin $\beta 2$ subunit knockout mice are viable but with LAD symptoms [62], while integrin $\beta 3$ subunit knockout mice show prolonged bleeding and placental defects [61]. Chimeric methods have been used to understand the diverse functions of integrins in development and hemostasis [63, 64]. Mutational studies have also been useful in dissecting the relationship between integrin sequence and its functions [49]. For example site-directed mutagenesis (SDM) at the $\alpha 4$ integrin cytoplasmic tail has been reported to modulate integrin activation [64].

1.7 Integrin affinity and avidity

Adhesion avidity is needed for cells to attach firmly to the substrate. Avidity is dependent on the affinity and valency of the receptors (Figure 1.7) [65, 66]. Clustering increases the valency of receptors in a specific area. The overall strength or avidity of integrin-mediated cell adhesion therefore includes regulation of integrin affinity and clustering. Whereas integrin affinity is regulated by conformational changes, integrin clustering can be induced by binding of integrins to multi-valent or multiple ligands and the lateral movement of integrins in the membrane via cytoskeletal remodelling [65, 66].

Figure 1.7 Valency and affinity modulations of integrins

An intermediate affinity may be achieved by unbending of the integrin, but conversion to the high affinity conformation needs full opening of the receptor. Clustering of integrins in their high-affinity (unbent) conformation enhances avidity required for firm adhesion.

1.8 Integrin bidirectional signaling

1.8.1 Inside-out signaling

Inside-out signaling is defined as the intracellular event that induces integrin structural changes, leading to an increase in its affinity for ligand binding (Figure 1.8) [49]. Circulating leukocytes normally maintain their integrins in a resting state. Under this state, the ectodomains of integrin are presented in a bent conformation, and the cytoplasmic tails are held in close proximity of each other through a salt bridge formed by residues in the membrane proximal regions of the tails. When leukocytes encounter chemokines or T cell receptor (TCR)/B cell receptor (BCR) agonists, a series of signaling events induce the activation of GTPase Rap1. It has been reported that the Rap GTPases act as major regulators in lymphocytes inside-out signaling pathway [67]. Rap1 and Rap2 are activated upon chemokines or TCR/BCR signaling, resulting in α L and α 4 integrin-dependent adhesion [68].

Figure 1.8 Inside-out signaling pathways of integrin

Key signaling events that occur downstream of chemokines and TCR/BCR agonists lead to integrin activation. In resting state, integrin is in a bent conformation, and the two tails are held in close proximity. Upon integrin activation by a series of signaling pathways recruiting of activated talin and GTP-bound Rap1, the two tails are separated. The conformational change of the cytoplasmic tails is transmitted through the TM domain and induces the structural changes in the extracellular region, which result in an open conformation of integrin required for ligand-binding.

A series of guanine nucleotide exchange factors (GEFs) have been reported to be activated by various upstream stimuli, such as PKC or protein kinase D (PKD) activation, Ca^{2+} and cAMP, and the activated GEFs trigger the activation of Rap GTPases through catalyzing exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP), suggesting that multiple pathways may converge on Rap GTPases [69].

Several effectors of GTP-bound Rap1 have been identified. These are RIAM, RapL, and Mstl (Figure 1.8). The final trigger point for integrin activation converges on the large cytoskeletal FERM-containing protein talin [67, 70]. In vertebrates, there are two talins known as talin 1 and talin 2, and talin 1 (referred herein as talin) is the most studied with regards to integrin activation [48]. Talin contains a head domain and a long rod domain [71]. The head domain of talin binds to the cytoplasmic tail of integrin β subunit, leading to the

disruption of the associated integrin α/β cytoplasmic tails and the activation [70], whereas its rod domain connects to the actin cytoskeleton [72]. The separation of the integrin cytoplasmic tails followed by that of the TM domains leads to conformational changes in the integrin extracellular region that promote ligand-binding.

1.8.2 Outside-in signaling

Outside-in signaling pathway includes events following extracellular ligand-driven clustering of integrins (Figure 1.9) [49]. Ligand-bound integrins transmit intracellular signals that modulate various cellular processes, such as cell migration, cell adhesion, cell survival and differentiation [10, 73]. SFKs play a key role in integrin outside-in signaling because activated SFKs phosphorylate many cytoplasmic molecules, including the ITAM, allowing the recruitment and activation of ZAP-70 or Syk kinases [74, 75]. These kinases then phosphorylate their substrates, such as Vav and SLP76. Rho GTPases activation by Vav leads to the cytoskeletal reorganization of actin [76-78]. Pyk2 and FAK kinases are also activated by SFKs, resulting in recruitment and activation of PI3K and Cbl phosphorylation [79, 80].

Figure 1.9 Outside-in signaling pathways of integrin

An illustration of integrin-induced signaling events following ligand binding. Upon ligand-binding, integrin becomes activated and transmits intracellular signals to modulate a series of cellular processes. SFKs are recruited by integrin and phosphorylate many cytoplasmic molecules, such as ITAM, which activates Syk or ZAP-70 kinases. These kinases then phosphorylate their substrates SLP76 and Vav. Vav activates Rho GTPases which leads to actin cytoskeletal reorganization.

Cellular responses triggered by integrin outside-in signaling include firm cell adhesion, spreading and migration. In T cells, integrin $\alpha\text{L}\beta 2$ outside-in signaling for example is important for immune synapse formation, secretion of IL-2, and proliferation [81]. In neutrophils, $\beta 2$ integrin outside-in signaling mediates the degranulation and activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, resulting in reactive oxygen species (ROS) production [82]. In macrophages, $\beta 2$ integrin outside-in signaling has a role in the stabilization of cytokine messenger ribonucleic acids (mRNAs) and cell differentiation [82]. In platelets, integrin $\alpha\text{IIb}\beta 3$ outside-in signaling responses are defined by lamellipodial and filopodial extensions producing firm adhesion and full spreading, that are required for thrombus formation [83].

As mentioned before, the activation of tyrosine kinases (e.g. Src and Syk families) is one of the earliest events of integrin outside-in signaling. It has

been reported that these enzymes are rapidly activated by the engagement of $\beta 1$, $\beta 2$ and $\beta 3$ integrins in myeloid cells, lymphocytes and platelets [74]. The importance of these kinases in inducing outside-in signaling has been unanimously demonstrated in knockout mouse models. Deficiency of the SFKs (e.g. Hck, Fgr and Lyn) completely abolishes ROS production and neutrophil degranulation upon the engagement of $\beta 2$ and $\beta 3$ integrins [74]. In macrophages, the lack of SFKs blocks cell adhesion and spreading and affects the phosphorylation of specific downstream substrates (e.g. Cbl, Vav and other molecules) [83]. Platelets without SFKs expression have shown defects in cell spreading upon integrin-binding to the fibrinogen-coated surfaces [83].

Syk is widely expressed in myeloid cells and platelets, while ZAP-70 is mainly expressed in T cells and NK cells [84, 85]. The deficiency of Syk kinase leads to the abrogation of $\beta 1$, $\beta 2$ and $\beta 3$ integrin signaling in macrophages and neutrophils [75]. The deficiency of Syk in platelets causes a failure of firm adhesion and full spreading on fibrinogen [84]. ZAP-70-deficient T cells have also been shown to be defective in integrin $\beta 1$ signaling [86]. FAK and its homolog Pyk2 are also involved in leukocyte integrin signaling. These kinases are normally considered to act downstream of SFK members [86, 87].

1.9 The leukocyte $\beta 2$ integrins

The $\beta 2$ integrins are expressed exclusively on leukocytes which have four members, $\alpha L\beta 2$ (leukocyte function-associated antigen-1 (LFA-1, CD11aCD18), $\alpha M\beta 2$ (CR3, macrophage-1 antigen (Mac-1), CD11bCD18), $\alpha X\beta 2$ (CR4, p150,95, CD11cCD18,) and $\alpha D\beta 2$ (CD11dCD18) [88]. The four members of $\beta 2$ integrin family and their cellular localization are listed in Table 1.1 [7]. A series of distinct but often overlapping ligands of $\beta 2$ integrins are identified (Table 1.2) [7]. $\beta 2$ integrins are αI integrins and are involved in leukocyte transmigration, monocyte activation, neutrophil apoptosis and phagocytosis of foreign materials [89-102]. The importance of the $\beta 2$ integrins is exemplified by the poor capacity of leukocytes to migrate into sites of inflammation in LAD I patients. These individuals suffer from recurrent bacterial and fungal infections [103, 104]. In these patients, the expression of

$\beta 2$ integrins on the leukocytes is defective as a result of mutations in the $\beta 2$ subunit.

Table 1.1 $\beta 2$ integrins and their cellular localization

Integrin	Cellular localization
$\alpha L\beta 2$	All leucocytes
$\alpha M\beta 2$	Monocytes, macrophages, NK cells, neutrophils and $\gamma\delta$ T-cells
$\alpha X\beta 2$	Monocytes, macrophages, dendritic cells, NK cells
$\alpha D\beta 2$	Macrophages and eosinophiles

Table 1.2 $\beta 2$ integrins and their ligands

Integrin	Ligands/recognition sequences
$\alpha L\beta 2$	Intercellular adhesion molecule (ICAM)-1, -2, -3, -4, -5 [105-109], junctional adhesion molecule (JAM)-1 [110]
$\alpha M\beta 2$	ICAM-1, -2, -4 [109, 111], iC3b [112], JAM-3 [113], BSA, fibrinogen [114], factor X [115], heparin [116], myelin basic protein [117], neutrophil inhibitory factor (NIF) [118], connective denatured proteins [119], microbial saccharides e.g. LPS [120], high-molecular-mass kininogen [121], plasminogen [122], fibronectin [123], laminin [123], collagen I, II, VI [124, 125], tissue growth factor [126], RAGE [127], cysteine-rich 61 [126], uPAR [128]
$\alpha X\beta 2$	ICAM-1,-4 [129, 130], iC3b [131], fibrinogen [132], collagen I [94], heparin [133], Osteopontin [134], LPS [135], denatured proteins [119]
$\alpha D\beta 2$	ICAM-3 [136], vascular cell adhesion molecule (VCAM)-1 [137]

Integrin $\alpha M\beta 2$ is mainly expressed on leukocytes of the myeloid lineage, NK lymphocytes and $\gamma\delta$ T cells [89, 138]. It is a promiscuous adhesion receptor, binding both endogenous ligands as well as an array of microbial ligands. It

has a wide range of ligands, including ICAM-1, JAM-3, complement protein iC3b, fibrinogen, microbial saccharides and denatured proteins [21, 113, 139, 140]. The complement proteolysis product iC3b is the most important ligand for integrin $\alpha M\beta 2$. The efficiency of $\alpha M\beta 2$ -mediated phagocytosis is enhanced significantly when the foreign body is opsonized by iC3b [139].

In myeloid cells, integrin $\alpha M\beta 2$ mediates cell adhesion, degranulation and phagocytosis [141]. It can recognize many pathogens directly and mediate the phagocytosis of microbes. In dendritic cells, a role of $\alpha M\beta 2$ in maintaining tolerance has been reported [142]. Integrin $\alpha M\beta 2$ is also involved in modulating the life span of neutrophils [143]. Fibrinogen-induced clustering of integrin $\alpha M\beta 2$ has been shown to regulate monocyte differentiation into macrophages by the down-regulation of Foxp1 expression [144]. Foxp1 is a transcriptional repressor of the *c-fms* gene encoding the macrophage colony stimulating factor (M-CSF) receptor [145]. Shi et al. [144] has also reported a reduction in Foxp1 expression in monocytes isolated from WT mice, but not $\alpha M\beta 2$ deficient mice, treated with thioglycollate to induce peritonitis. Clustering of integrin $\alpha M\beta 2$ on monocytic THP1 cells has been shown to trigger Toll/IL-1 pathway that affects nuclear factor κB (NF κB) activity [146].

1.10 The cytoplasmic molecules relating to integrin $\alpha M\beta 2$ signaling studied

Many molecules are involved in integrin $\alpha M\beta 2$ signaling pathway. In this study, we have investigated PKC δ , SFKs, Syk, Foxp1 during integrin $\alpha M\beta 2$ signaling by anti- αM antibody (Ab) ligation or clustering by ligands. We also examined the cross talk between integrin $\alpha M\beta 2$ signaling and the TLR activation. In addition, kindlin3 was also studied for its role in integrin $\alpha M\beta 2$ -mediated cell adhesion and cell spreading.

1.10.1 Protein kinase Cs (PKCs)

PKCs belong to a large multigene family of serine/threonine kinases involved in cell growth and apoptosis. PKCs are grouped into three different

classes: conventional (α , β I, β II and γ), novel (δ , ϵ , η and θ) and atypical (ζ and ι/λ) (Table 1.3) [147].

Table 1.3 PKC subfamilies and their second messengers for activation

Subfamily	Second messenger	Members
Conventional	diacyl glycerol (DAG), Ca^{2+} and phospholipid	PKC α , PKC β I, PKC β II, PKC γ
Novel	DAG, but not Ca^{2+}	PKC δ , PKC ϵ , PKC η , PKC θ
Atypical	neither Ca^{2+} nor DAG	PKC ζ , PKC ι/λ , PKN1, PKN2

PKCs are a family of key enzymes that are involved in cell growth and cell signal transduction. They also play an important role in the regulation of integrin-dependent cellular functions, such as cell motility, cell adhesion and spreading [148]. Phorbol myristate acetate (PMA), an analog of the second messenger 1, 2-DAG, is a well reported inducer of monocyte differentiation by activating the conventional and novel family members of the PKC [147]. It has been used in many studies to investigate the relationship between PKC activation and modulation of integrin-dependent functions. These studies have shown that cell adhesion, spreading and metastasis are partially regulated by the action of PKCs. PKCs have also been shown to activate the adhesive properties of cells by increasing the affinity of their cell surface integrins [147]. In addition, PKCs mediate signaling events leading to integrin-dependent cell spreading and motility [148].

PKC δ , a member of the novel PKCs, comprises a regulatory domain and a catalytic domain. There is a hinge region between the two domains, which includes two tyrosines 311 and 332 (Figure 1.10). *(The numbering of tyrosine sites 311 and 332 is based on the amino acid no. of mouse PKC δ protein.)* PKC δ is important in many processes such as cell apoptosis and cell differentiation [149, 150]. Over-expression of PKC δ induces myeloid cell differentiation and the catalytic domain of PKC δ is implicated in this process [149, 150].

Figure 1.10 The linear domain organization of PKC δ

The linear domain organization of PKC δ includes regulatory and catalytic domains. There is a hinge region between regulatory and catalytic domains. The phosphorylation sites of tyrosine (Y), threonine (T) and serine (S) residues are schematically shown.

1.10.2 Src family kinases (SFKs)

SFKs are a family of non-receptor tyrosine kinases including nine members: the SrcA subfamily (Src, Fyn, Fgr and Yes), the SrcB subfamily (Hck, Lck, Lyn and Blk) and Frk. Src homologs exist from unicellular organisms, such as choanoflagellates, to vertebrates. Frk homologs are specific to invertebrates (e.g. flies and worms), while the SrcA and SrcB subfamilies have homologs in vertebrates [151].

SFKs are involved in regulating signal transduction through a series of cell surface receptors under multiple cellular environments. SFKs interact with a diverse set of cellular membrane, cytosolic and nuclear proteins, and phosphorylate these proteins on their tyrosine residues [151-153]. A few of substrates have been discovered for these kinases.

In immune cells, SFKs play key roles in regulating a variety of intracellular signaling pathways [153]. In a large number of signaling transduction cascades such as those emanating from the FcRs, TCRs/BCRs, cytokine receptors, growth factor receptors and integrins, SFKs localize in a membrane proximal position. Other than their positive regulatory roles, SFKs have also been reported to be involved in negative regulation of cell signaling. They

phosphorylate immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on inhibitory receptors and ITIMs subsequently recruit and activate Src homology 2 (SH2) domain-containing phosphatases, such as SH2-containing 5'-inositol phosphatase-1 (SHIP-1) and SH2-containing phosphatase 1 (SHP-1) [152].

The members of SFK family have a highly conserved domain organization. N-terminal is a myristoylated segment, which is a membrane localization site. The Src homology 3 (SH3) and SH2 are located in the middle of SFK. The tyrosine kinase domain is found on the C-terminal region (Figure 1.11) [154]. The SFKs (Hck, Lyn and c-Yes) have been shown to interact with the cytoplasmic tail of $\beta 2$ integrin in pull-down assays using recombinant integrin β tails [155].

Figure 1.11 General linear domain organization of SFKs

The linear domain organization of SFKs includes four domains: a unique region, SH3, SH2 and tyrosine kinase domains. The activating (Y416) and autoinhibitory (Y527) phosphorylation sites are shown. SH2 domain recognizes phosphotyrosine by its conserved residue R175. The residue W260 in the kinase domain is important for autoinhibition. By convention, the numbering of amino-acid residues is based on chicken Src.

1.10.3 Spleen tyrosine kinase (Syk)

Syk is a non-receptor type protein tyrosine kinase (PTK), which was isolated from porcine spleen complementary deoxyribonucleic acid (cDNA) library [156]. Syk comprises tandem SH2 domains and a kinase domain on its C-terminal. The three domains are interrupted by interdomain A and interdomain B (Figure 1.12). After binding of tandem SH2 domains to ITAM, Syk is activated and plays a key role in activation of immune cells and

lymphocyte development [157]. Tyrosine residues of interdomain B undergo autophosphorylation upon increase in Syk catalytic activity, resulting in the formation of docking sites for interaction with Syk substrates/downstream molecules [158]. Syk is widely expressed in hematopoietic cells and essential in mediating a variety of cellular responses, such as cell differentiation, proliferation and phagocytosis [159]. Syk has also been reported to modulate epithelial cell growth and act as a potential tumor suppressor in human breast carcinomas [160].

Figure 1.12 The linear domain organization of human Syk

Syk has two tandem SH2 domains on its N-terminal and a kinase domain on its C-terminal. The three domains are interrupted by interdomain A and interdomain B. The tandem SH2 domains bind to ITAM, leading to Syk activation. A series of tyrosine (Y) phosphorylation sites are shown. The numbers refer to the amino acid sequence of human Syk.

Syk acts as a central component in immunoreceptor signaling and is activated following the engagement of $\beta 2$ or $\beta 3$ integrin in many cell types, such as macrophages, neutrophils, platelets and osteoclasts [161-165].

1.10.4 Forkhead box P1 (Foxp1)

The forkhead box (Fox) gene family consists of a very large number of transcription factors [166]. They all share a 'winged helix' DNA binding domain, which was first defined in 1990 [166]. Fox transcription factors comprise three

α -helices flanked by two 'wings' of β strands and loops. More than 100 proteins with forkhead domains have been found and classified into 17 subclasses, from FoxA through FoxQ. They play important roles in many biological processes such as metabolism, development, cancer and aging [166]. Both during development and in adulthood, Fox transcription factors are critical in the regulation of cell type- and tissue-specific gene transcriptions [167].

Foxp1 is a member of subfamily P in Fox family of transcription factors. In mammals, Foxp1 is needed for the proper development of the lung and brain. Foxp1 protein has both protein-protein binding domain and DNA binding domain. Foxp1 gene is lost in several tumor types and maps to a chromosomal region (3p14.1) containing a tumor suppressor gene(s), which suggests that Foxp1 may act as a tumor suppressor [168].

Foxp1 has been reported to regulate monocyte differentiation [144]. Treatment of the promyelocyte HL60 cells with PMA induces down-regulation of Foxp1 expression [144]. In integrin α M β 2-crosslinked monocytes, a reduction in Foxp1 expression level has also been observed [169]. Fibrinogen-induced clustering of α M β 2 has been shown to regulate monocyte differentiation into macrophages by down-regulating the expression of Foxp1 [144, 145].

The target gene of Foxp1 is c-fms, which encodes for M-CSF receptor. Clustering of integrin α M β 2 induces Foxp1 down-regulation, which leading to up-regulation of c-fms expression and the induction of monocyte differentiation. Foxp1 acts as a transcription repressor of c-fms gene by binding to the promoter of c-fms gene. The transcriptional regulation of c-fms is a key point during monocyte differentiation into macrophage. Expression of c-fms is regulated by the coordinated effects of a series of transcription factors, including PU.1, AML1 and C/EBP α [170]. PU.1, as a member of the ets transcription family, binds to the PU box on the promoter of c-fms gene [171]. C/EBP α is a member of the CCAAT enhancer-binding proteins and its binding site is on upstream of the PU box [170, 171]. AML1, as a member of core-

binding factor family, binds to the promoter of c-fms gene and and physically interacts with C/EBP α , which results in synergistic activation of promoter activity [170].

1.10.5 Kindlins

Kindlins are evolutionarily conserved focal adhesion proteins, including kindlin1 (also known as FERMT1 and kindlerin), kindlin2 (also known as MIG-2) and kindlin3 (also known as URP2 and FERMT3) [172]. These three mammalian kindlins exhibit high sequence similarities and identical domain architecture, comprising of a FERM domain at the carboxyl terminus and a F0 domain at the amino terminus [173]. Absence of catalytic domain in kindlins possibly suggests a possible function that they mediate interactions between proteins. Kindlin FERM domain, which shares high sequence homology to that of talin, is also composed of 3 sub-domains: F1, F2 and F3. The F0 domain and F1 sub-domain have been demonstrated to interact with migfilin and integrin-linked kinases [173]. The F2 sub-domain is split by a pleckstrin homology (PH) domain. The F3 sub-domain interacts with the β -integrin cytoplasmic tail. The linear domain organization for kindlin and talin is shown in Figure 1.13.

Figure 1.13 General linear domain organizations of kindlins and talins

All three kindlins share a similar domain architecture as Talins. They all have a FERM domain which includes three sub-domains: F1, F2 and F3. F3 sub-domain binds to the cytoplasmic domain of integrin β subunit. In kindlins, FERM domain is located on the C-terminal and F2 sub-domain is split by a PH domain. In Talins, a

rod domain is located on the C-terminal.

Kindlin3 is a cytoplasmic protein widely expressed in all hematopoietic cells, platelets and endothelial cells. As for kindlin1 and kindlin2, they are present at low levels in these cells. Kindlin3 shares high sequence similarity of 49% and 53% with kindlin1 and kindlin2, respectively [174]. Additionally, there are two spliced forms of kindlin3 consisting of a longer and shorter variant. The longer variant has an additional 4 amino-acid insertion in its PH domain, resulting in a possible SH3 binding site at the region. The well-established function of kindlin3 is to activate integrin together with talin. The F3 sub-domain of kindlin3 is involved in interacting with membrane-distal NPXY/F motif in the cytoplasmic tail of integrin β subunit [172].

Recent evidence has shown that kindlin3 deficiency in humans is a causative factor of an autosomal recessive disease-LAD III [175]. In LAD III patients, the leukocytes are incapable of firm attachment to vessel walls and transmigrate to tissues during inflammation. Due to mutations in the kindlin3 gene, weak bones, abnormalities in erythrocyte shape and affected hemostasis have been observed in these patients [176]. Kindlin3-deficient mice suffer from severe osteopetrosis and bleeding tendency [172].

1.10.6 Rho GTPases

GTPases belong to a large family of enzymes binding and hydrolyzing GTP. The GTP binding and hydrolysis by GTPases occur in the highly-conserved G domain common in all GTPases [177].

Ras GTPases are a superfamily of GTPase family. All of the members are small monomeric proteins that are homologous to Ras. Ras GTPases are also known as small GTPases or “Ras-like” proteins. Their molecular weight is about 21 kilodalton (kD). As molecular switches, Ras GTPases play key roles in a diverse of cellular signaling events [178-180]. The Ras GTPases superfamily is further classified into 5 subfamilies—Ras, Rho, Rab, Arf and Ran—according to their biochemical properties and original amino acid sequences [181, 182].

The Rho GTPases are one subfamily of the Ras superfamily. They are small signaling G proteins and include 20 members in mammals. Three most studied members of the Rho family are Cdc42, Rac and RhoA (Table 1.4) [183].

Table 1.4 Rho family members and their roles in cellular architecture

Rho family member	Function in the formation of
Cdc42	Filopodia
Rac	Lamellipodia
RhoA	Stress fibres

Cdc42, Rac and RhoA share a common G-domain fold, which comprises a six-stranded β -sheet surrounded by a few α -helices [184]. In addition to regulating cytoskeletal re-organization, the Rho GTPases are involved in a large number of cellular functions, such as gene expression, cell cycle progression, cell proliferation and apoptosis, cell adhesion and migration, membrane trafficking and cell polarity. In these processes, the Rho GTPases play key roles and function as "molecular switches" [185, 186].

Upon the binding of regulatory and cytoskeletal molecules with the regions of integrin cytoplasmic tail, integrin outside-in and inside-out signaling pathways are activated and regulated. Small GTPases are involved in the regulation of integrin function [64, 187-189]. $\beta 1$, $\beta 2$, and $\beta 3$ integrins have been activated through Rap1 activity in inside-out signaling pathway [190, 191], while Rho GTPases transduce the signals of integrin to the cytoskeleton of cells [192, 193]. In the other hand, integrins have also been reported to regulate Rho family GTPases [179]. However, the detailed mechanisms underlying the cross-talk between GTPases and integrins remain to be investigated.

1.11 Toll-like receptor (TLR)

In 1985, Toll gene was firstly identified by Christiane Nüsslein-Volhard in *Drosophila* [194]. The mutation of Toll gene makes the *Drosophila* flies look

unusual. TLRs include a class of proteins which recognize a wide range of pathogen-related molecular patterns and initiate the innate and adaptive immune responses. TLRs are single, transmembrane, non-catalytic receptors and they are expressed in dendritic cells and macrophages. These cells can recognize structurally-conserved molecules which are derived from microbes. The microbes can be recognized by TLRs if the microbes breach the physical barriers, e.g. the intestinal tract mucosa or skin, leading to the activation of immune cell responses. LPS, as microbial ligand, stimulates TLR and powerfully induces a variety of inflammatory chemokines and kinases.

The adapter proteins and kinases mediate TLR signaling, such as MyD88, Toll/IL-1 receptor (TIR) domain-containing adapter-inducing interferon- β (TRIF), TIRAP, and TRAM. TLRs recruit adapter molecules within the cell cytoplasm to propagate signal transduction [195-197].

MyD88-dependent and TRIF-dependent pathways are two distinct signaling pathways mediated by TLRs. Upon the dimerization of TLR receptors, the MyD88-dependent signaling is activated for all TLRs except TLR3, resulting in the activation of MAPKs and NF κ B. Ligand bound TLRs recruit the adaptor protein MyD88, which is one member of the TIR family. IRAK1, IRAK2 and IRAK4 are then recruited by MyD88, leading to the phosphorylation and activation of tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6). In turn, TRAF6 triggers the polyubiquitination of transforming growth factor- β activated kinase-1 (TAK1) and TRAF6 itself, which facilitates the binding with inhibitor of nuclear factor κ B kinase subunit β (IKK β). Upon this binding, IKK β is phosphorylated by TAK1, followed by the phosphorylation of inhibitor of κ B (I κ B) by IKK β . The degradation of I κ B takes place and allows the translocation of NF κ B into the cell nucleus, leading to the activation of transcription and the induction of inflammatory cytokines [198].

Both of TLR3 and TLR4 signaling pathways are TRIF-dependent, and induced by LPS and dsRNA [198]. The kinases, such as receptor-interacting protein-1 (RIP1) and TANK-binding kinase-1 (TBK1), are activated by TRIF, which forms a branch in the signaling pathway. The phosphorylation of

interferon regulatory factor 3 (IRF3) induced by TRIF/TBK1 signaling complex allows the translocation of IRF3 into the nucleus and the production of type I interferons (IFNs). In the meanwhile, polyubiquitination and activation of TAK1 and NF κ B transcription are induced by the activation of RIP1 in the same way as the MyD88-dependent pathway [198]. TLR signaling results in the suppression or induction of genes that regulate inflammatory responses. In total, more than one thousand genes are activated by TLR signaling. And the TLRs collectively constitute one of the most multi-effective gateways which tightly regulate the modulation of genes involved in an inflammatory response [198].

All of the four adaptors, MyD88, TRIF, TIRAP, and TRAM are utilized only in TLR4 signaling. TIR domain-containing adaptors MyD88 and TIRAP are recruited by the complex of TLR4, LPS and MD2, and trigger the activation of NF κ B in early phase and MAPKs. The endocytosis of the TLR4-LPS-MD2 complex happens and a signaling complex with TRIF and TRAM adaptors is formed in the endosome. This TRIF-dependent pathway again induces the production of type I IFNs and the activation of IRF3, while it also activates NF κ B activation in late phase. Both early- and late-phase activation of NF κ B are required for the production of inflammatory cytokines [198].

It has been shown that integrin α M β 2, in an inactive state, is activated by TLR stimulation [199]. In phagocytes, chemokine signaling is the classic activator for integrins. The exact mechanism of TLR-induced activation of integrin α M β 2 still remains unclear. However, the inside-out signals from PI3K and RapL pathways are involved. In turn, active integrin α M β 2 mediates outside-in signals, leading to the activation of tyrosine kinases such as Src and Syk (Figure 1.14) [200].

Figure 1.14 TLR4-induced activation of integrin $\alpha M\beta 2$

LPS-triggered TLR4 signaling activates NF κ B and the IFN pathway by MyD88 and TRIF [200]. TLR4 induces PI3K and RapL signaling pathways, that activate integrin $\alpha M\beta 2$. Inhibitors of RapL and PI3K prevent TLR4-induced activation of integrin $\alpha M\beta 2$. The activated integrin $\alpha M\beta 2$ then triggers outside-in signals leading to the activation of Src and Syk.

1.12 Aims of study

As described in sections 1.10 and 1.11, many intracellular molecules are involved in the interaction and activation of integrin. Of particular interest to us is the integrin $\alpha M\beta 2$ that plays critical roles in the function and migration of leukocytes during inflammation. This study includes 4 aims.

- (1) The contributions of SFKs and PKC δ in integrin $\alpha M\beta 2$ outside-in signaling
- (2) MEK-Erk pathway in the regulation of Foxp1
- (3) IRAK1 and the downstream MAPKs in integrin $\alpha M\beta 2$ and TLR signaling
- (4) Kindlin3 and integrin $\alpha M\beta 2$ -mediated signaling

2. Materials and Methods

2.1 Chemicals and other reagents

2.1.1 Reagents

Table 2.1 Reagents

Name	Source
BSA	Sigma-Aldrich (St. Louis, MO, USA)
HSA (human serum albumin)	
LPS	
PMA	
PVP (polyvinylpyrrolidone 10000)	
Recombinant human PKC δ	Fermentas (Burlington, CA, USA).
DNA loading dye	
DNA markers	
Protein markers	Pierce (Rockford, IL)
DSP (dithiobis succinimidyl propionate)	
GST-PAK-PBD bead	Cytoskeleton, Inc (Denver, CO, USA)
GST-Rhotekin-RBD bead	
LY2940002 (PI3K inhibitor)	Calbiochem (San Diego, CA, USA)
PD98059 (inhibitor of MEK1/2)	
Picetannol (PIC, Syk inhibitor)	
PP3 (inactive analog of PP2)	
PP2 (SFK inhibitor)	
Restriction endonucleases and other enzymes	New England Biolabs (Ipswich, MA, USA).
T4 ligase	Promega (Madison, WI, USA)
Trizol reagent	Invitrogen

Trypsin	(Carlsbad, CA, USA)
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All other general chemicals were from Sigma-Aldrich unless stated otherwise.

2.1.2 Antibodies (Abs)

Table 2.2 Antibodies (Abs)

Name	Source
AlexaFluor 488-conjugated goat anti-rabbit IgG	Invitrogen (Carlsbad, CA, USA)
Alexa Fluor 594 conjugated phalloidin	
Rabbit phospho-JNK1/2 (Thr183/Tyr185)	
Allophycocyanin (APC) conjugated goat anti-mouse IgG	BD Biosciences (San Jose, CA, USA)
Mouse anti- β -actin	
Mouse anti-Hck	
Mouse anti-phosphotyrosine (PY20)	
Mouse anti-PKC α	
Mouse anti-PKC ϵ	
Mouse anti-Syk Ab	
Fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG	Sigma-Aldrich (St. Louis, MO, USA)
Goat anti-human IgG (Fc specific)	
Goat anti-mouse IgG	
Mouse anti-talin	
Mouse IgG	
Rabbit phospho-IRAK1 Thr209	
4B4 (integrin β 1 specific)	Beckman Coulter (Fullerton, CA, USA)

Horseradish peroxidase (HRP)- conjugated donkey anti-rabbit IgG	GE Healthcare (Amersham, UK)
HRP-conjugated goat anti-rat IgG	
HRP-conjugated sheep anti-mouse IgG	
KIM185 (anti- β 2, activating Ab) [201]	Hybridomas bought from American Type Culture Collection (ATCC), Abs purified using protein G beads (GE Healthcare, Amersham, UK)
KIM127 (binds to activated β 2 integrins) [52, 202]	
LPM19c (anti- α M Ab) [203]	
MHM24 (anti- α L Ab) [204]	
Mouse anti-c-Yes	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Rabbit anti-Hck	
Rabbit anti-PKC δ (C-20)	
Mouse anti-Lyn	Abcam (Cambridge, UK)
P1F6 (integrin α V β 5 specific Ab)	
Rabbit anti- α M (EP1345Y)	
Rabbit anti-Foxp1	
Mouse phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling Technology (Beverly, MA, USA)
Rabbit anti-p38,	
Rabbit anti-SAPK/JNK1/2	
Rabbit anti-phospho-PKC δ (Tyr311)	
Rabbit anti-phospho-p38 (Thr180/Tyr182)	
Rabbit anti-HA	Delta Biolabs (Gilroy, CA, USA)
Rabbit anti-His	
Rabbit anti-GST	
Rabbit anti-IRAK1	Millipore (Bedford, MA, USA)
Rabbit anti-MAPK Erk1/2	
Rat anti-kindlin3 Ab	Generated by Asso. Prof. TAN Suet Mien lab [205] (SBS, NTU,

	SG)
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2.1.3 Ligands for integrin $\alpha M\beta 2$

Table 2.3 Ligands for integrin $\alpha M\beta 2$

Name	Source
Human ICAM-1-(D1-D5)-Fc [206]	R&D Systems (Minneapolis, MN, USA)
iC3b	Complement Technology (Tyler, TX, USA)

2.1.4 Solutions

Table 2.4 Solutions

Solutions	Recipe
Ampicillin (60 mg/ml)	60 mg/ml in distilled deionized H ₂ O (ddH ₂ O), filtered (with 0.22 μ m filter)
Kanamycin (30 mg/ml)	30 mg/ml in ddH ₂ O, filtered (with 0.22 μ m filter)
Puromycin (1.5 mg/ml)	1.5 mg/ml in ddH ₂ O, filtered (with 0.22 μ m filter)
Buffer A (subcellular fractionation)	20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 2 mM ethylene-glycol-bis (β -aminoethylether)-tetraacetic acid (EGTA), 2 mM ethylenediamine tetraacetic acid (EDTA), and protease inhibitor cocktail
Buffer B (subcellular fractionation)	20 mM Tris-HCl (pH 7.5), 1% (weight per volume, w/v) sodium dodecyl sulphate (SDS), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and protease inhibitor cocktail
Buffer C (subcellular fractionation)	10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (volume per volume, v/v) Nonidet P-40 (NP-

	40) and protease inhibitor cocktail
Coomassie blue staining buffer	50% (v/v) methanol, 10% (v/v) glacial acetic acid, 40% (v/v) ddH ₂ O, 0.05% (w/v) Coomassie blue
Coomassie blue destaining buffer	30% (v/v) methanol, 10% (v/v) glacial acetic acid, 60% (v/v) ddH ₂ O
Cytoskeletal (CSK) buffer	100 mM NaCl, 300 mM sucrose, 3 mM MgCl ₂ , 1 mM EGTA, 10 mM PIPES, pH 6.8
DNA loading dye (6×)	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol
Elution buffer for purification of Lpm19c	0.5 M acetic acid, pH3; (1 ml of acetic acid is quenched to pH7.5 by 450 µl of 1 M Tris-HCl, pH9)
50× Tris-acetate-EDTA (TAE) buffer	2 M Tris base, 5.71% (v/v) acetic acid, 0.05 M EDTA, pH 8.0
4× Resolution gel buffer	250 mM Tris, 0.4% (w/v) SDS, pH 8.8
4× Stacking gel buffer	500 mM Tris, 0.4% (w/v) SDS, pH 6.8
HBSS buffer (shear flow)	400 mg/litre (mg/L) KCl, 60 mg/L KH ₂ PO ₄ , 350 mg/L NaHCO ₃ , 8g/L NaCl, 90 mg/L Na ₂ HPO ₄ ·7H ₂ O and 1 g/L Glucose (D) or Dextrose, pH 7.4
HBSS buffer additives (shear flow)	10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4), 1 mM CaCl ₂ , 1mM of MgCl ₂ and 0.5% (w/v) HSA or 5% (v/v) heat-inactivated fetal bovine serum (FBS), pH 7.4
Kinase buffer	2.5 mM HEPES (pH 7.4), 2.5 mM MgCl ₂ , 5 mM NaF and 20 µM Na ₃ VO ₄
PKC kinase buffer	20 mM Tris-HCl (pH 7.5), 5 mM MgCl ₂ and 0.2 mM CaCl ₂
Lysis buffer (harvest protein)	50 mM Tris, 150 mM NaCl, 1% (v/v) NP-40, pH 7.5

Lysis buffer (red blood cell, RBC)	0.89% (w/v) NH_4Cl
Sodium bicarbonate buffer (SBB)	0.136% (w/v) sodium carbonate, 0.735% (w/v) sodium bicarbonate, pH 9.2
Stripping buffer (western blot)	673 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH6.8) in ddH ₂ O
10× PBS (phosphate buffered saline)	1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4 , 18 mM KH_2PO_4 , pH 7.4
10× Tris buffered saline (TBS)	1.5 M NaCl, 100 mM Tris-HCl, pH 7.4
Blocking buffer (western blot with normal Ab)	PBS-T (PBS with 0.1% (v/v) tween-20) containing 5% (w/v) non-fat milk
Blocking buffer (western blot with phospho Ab)	TBS-T (TBS with 0.1% (v/v) tween-20) containing 5% (w/v) BSA
10× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) running buffer	250 mM Tris, 1.9 M glycine, 1% (w/v) SDS
Transferring buffer (western blot)	25 mM Tris, 192 mM glycine
TfBIC	30 mM KOAc, 100 mM KCl, 10 mM CaCl_2 , 50 mM MnCl_2 , 15% glycerol
TfBII	10 mM MOPS, 75 mM CaCl_2 , 10 mM KCl, 15% glycerol, pH 7.0

2.2 Molecular cloning

2.2.1 Expression plasmids

Total RNA extraction reagent Trizol was from Invitrogen. mRNA extraction kit was from Roche Diagnostic Systems (Somerville, NJ, USA). One step reverse transcription-polymerase chain reaction (RT-PCR) kit was from Qiagen (Valencia, CA, USA). All site-directed mutations were performed using

the Quikchange SDM kit from Stratagene (La Jolla, CA, USA) with relevant primer pairs.

All constructs generated were verified by DNA sequencing (1st Base, Singapore).

2.2.1.1 α M and β 2 cDNAs

Full-length integrin α M and β 2 cDNAs in the expression plasmid pcDNA3.0 (Invitrogen, Carlsbad, CA, USA) were reported previously [206, 207]. Integrin amino acids were numbered based on the mature protein. The two constructs were provided by Asso. Prof. TAN Suet Mien.

2.2.1.2 β 2 mutants

The β 2 mutants used in this study β 2N329S, β 2D709R, β 2M740, β 2S734, β 2N727, β 2R711 and β 2K702 were described previously [207, 208]. These constructs were provided by Asso. Prof. TAN Suet Mien.

2.2.1.3 p59 Hck cDNA

Human p59 Hck cDNA encoding 78-526 amino acids reported previously [209] was obtained by RT-PCR of mRNA isolated from U937 cells using relevant forward and reverse primers and cloned into expression vector pcDNA3.1 (Invitrogen) using the KpnI and XhoI restriction sites [57]. This construct was provided by Asso. Prof. TAN Suet Mien.

2.2.1.4 Mouse PKC δ cDNA

Full-length mouse PKC δ cDNA was obtained by RT-PCR of total RNA from mouse RAW 264.7 cells (kindly provided by Dr. Peter Cheung, SBS, NTU, Singapore) using the relevant primers (Table 2.5) and cloned into expression vector pEGFP-N1 (Clontech Laboratories Inc., Mountain View, CA, USA) described before [210] between the HindIII and BamHI enzyme sites.

Mouse PKC δ kinase dead (KD) cDNA was generated by introducing a mutation (K376R) using a SDM kit. The relevant forward and reverse primers were used in Table 2.5.

Table 2.5 Primer sequences for mouse PKC δ cDNA

Primers	Primer Sequence
PKC δ forward primer	5'-cccaagcttatggcacccttcctgcgcat-3'
PKC δ reverse primer	5'-cgcggatccaagtaaattgtccaggaattgct-3'
PKC δ K376R forward primer	5'-aagtactttgcaatcaggtgtctgaagaaggac-3'
PKC δ K376R reverse primer	5'-gtccttcttcagacacctgattgcaaagtactt-3'

2.2.1.5 Human IL-1 receptor-associated kinase-1 (IRAK1) cDNA

Human IRAK1 cDNA tagged with GFP at C-terminus was in the expression vector pCMV6-AC-GFP (OriGene, Rockville, MD, USA).

2.2.2 Amplification of plasmid DNA

2.2.2.1 Miniprep of plasmid DNA

For small-scale purification of plasmid DNA, 3-5 ml Lysogeny broth (LB) medium with appropriate antibiotics was inoculated with a single colony of *Escherichia coli* (*E.coli*) that contained recombinant plasmid from an agar plate and incubated at 37 °C with constant shaking overnight. A miniprep kit (Axygen, Union City, CA, USA) was used to extract plasmid DNA according to the manufacturer's instructions.

2.2.2.2 Midiprep of plasmid DNA

For large-scale preparation of plasmid DNA, a single colony of transformed *E.coli* was inoculated into 3-5 ml LB medium in 15 ml tube for 8-12 hours before being transferred to a flask containing 100 ml LB medium with antibiotics and incubated at 37 °C with constant shaking overnight. Plasmid midi kit (Axygen) was used for plasmid extraction according to the manufacturers' instructions.

2.2.3 *E.coli* cells

The *E.coli* strain DH5 α was used to amplify plasmid DNA.

2.2.3.1 Media for *E.coli* cells

All media were sterilized by autoclaving or filtered unless otherwise stated.

Table 2.6 Media for *E.coli* cells

Media	Components
LB medium	1% (w/v) Bacto-tryptone (BD), 0.5% (w/v) yeast extract (BD), 1% (w/v) NaCl
LB agar	LB medium plus 1.5% (w/v) Bacto-agar
LA broth	LB medium containing 60 µg/ml ampicillin (Sigma-Aldrich)
LA plate	LB agar plate containing 60 µg/ml ampicillin
LK broth	LB medium containing 30 µg/ml kanamycin (Gibco, Grand Island, NY)
LK plate	LB agar plate containing 30 µg/ml kanamycin
TYM medium	2% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 0.59% (w/v) NaCl, 0.246% (w/v) MgSO ₄ , pH 7.5

2.2.3.2 Preparation of *E.coli* competent cells

The *E.coli* strain DH5α was used to prepare the competent cells. A fresh plate of cells was prepared by streaking out cells from frozen stocks and grown at 37 °C overnight. An individual colony was inoculated in 10 ml LB medium without antibiotic and was cultured at 37 °C overnight. Thereafter, 5 ml of overnight culture was transferred into each of two flasks containing 250 ml LB medium followed by incubation at 37 °C in a shaking incubator until the culture reached an optical density (O.D.) 550 of 0.6, which usually takes ~2 hours. The cells were transferred to centrifuge bottles and spun at 8000 × *g* at 4 °C for 10 minutes (min). Pellets were gently resuspended in 100 ml ice cold TfBIC buffer. The cell suspension was centrifuged at 8000 × *g* at 4 °C for 10 min. The pellet was then resuspended in 20 ml ice cold TfBII buffer.

Competent cells were distributed into convenient aliquots (100 µl/vial) in pre-chilled microcentrifuge tubes. Cells were stored at -80 °C. An aliquot of the cells was used to assay for viability and competence.

2.2.3.3 Transformation of plasmid DNA

For each transformation reaction, 50-100 µl of competent cells was needed. The competent cells were thawed on ice for a few minutes. Plasmid DNA (1 µg) or ligation product (5-10 µl) was added to the cells followed by incubation on ice for 30 min. This cell-DNA mixture was incubated in a 42 °C water bath for 1 min and on ice for 2 min. LB medium (500 µl) was added to the tube of the mixture, followed by incubation at 37 °C with constant shaking at 150-200 revolutions per minute (rpm) for 30 min to 1 hour. Thereafter, the cell suspension (50-500 µl) was spread onto LB agar plates containing the appropriate antibiotics.

2.2.4 DNA and RNA quantitation

The concentration of DNA was determined using a NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA) by its absorbance at wavelength 260 nm based on the calculation: value of O.D.260 $\times$ 50 ng/µl. The O.D.280 was also read and the ratio of O.D.260/O.D.280 was calculated to estimate the purity of the DNA solutions. An O.D.260/O.D.280 ratio between 1.6 and 2.0 was considered satisfactory.

The concentration of RNA was determined using a NanoDrop2000 Spectrophotometer by its absorbance at wavelength 260 nm based on the calculation: value of O.D.260 $\times$ 40 ng/µl. The O.D.280 was also read and the ratio of O.D.260/O.D.280 was calculated to estimate the RNA purity. An O.D.260/O.D.280 ratio more than 1.8 was considered satisfactory.

2.2.5 Restriction endonuclease digestion

Restriction endonuclease digestion was carried out in a 20-30 µl reaction volume. For DNA amount not exceeding 1µg, 2-5 units of enzyme was used together with BSA from New England Biolabs (Ipswich, MA, USA) in

appropriate buffer at 37 °C for 2-3 hours. Digestions with more than one enzyme were carried out simultaneously in a suitable buffer as recommended by the manufacturer. When the buffer requirements were incompatible, restriction digestions were performed sequentially.

2.2.6 DNA electrophoresis

A 60 ml of 1-2% (w/v) agarose gel (agarose was melted in 1× TAE buffer) was regularly used for analysis of 0.1-8 kb DNA fragments. GelRed (Biotium, Hayward, CA, USA) was added at a dilution of 1:10000 followed by casting in a mini-gel apparatus. Electrophoresis was carried out in a horizontal gel apparatus with the gel submerged in 1× TAE buffer. DNA samples were mixed with 6× DNA loading dye and loaded into the wells. DNA fragments were visualized by fluorescence over a UV light (302 nm, UV transilluminator TM-20, UVP, San Gabriel, CA, USA), under which DNA/GelRed complexes fluoresce and the image was recorded with a Gel Doc 1000 imaging system (Bio-Rad, Milano, Italy).

2.2.7 Purification of DNA fragments from agarose gel

When a particular fragment of DNA in a mixture of fragments was required, e.g. in digestions, ligations or polymerase chain reaction (PCR) mixture, agarose gel electrophoresis was routinely employed to separate one DNA fragment from others. Gel slice containing the specific DNA fragment(s) to be purified was excised from the gel using a razor blade. DNA was extracted using a gel extraction kit (Axygen).

2.2.8 DNA ligation

DNA vectors with complementary ends to be used for ligation were prepared by restriction enzyme digestion and where necessary treated with alkaline phosphatase and purified using agarose gel electrophoresis. Vector DNA (~10 ng) and insert DNA (20-40 ng) were ligated by incubation at room temperature for 2-3 hours or at 4°C for overnight, using 1 unit of T4 DNA ligase (Promega, Madison, WI, USA) with ligase buffer provided together in a 20 µl

reaction volume. A reaction without insert DNA was included in the experiment as a negative control.

2.2.9 Standard polymerase chain reaction (PCR)

PCR was routinely performed in a 25-50 µl reaction volume containing 0.1-1 µg template DNA, 1 µM of each oligonucleotide primer, 40 µM of each deoxyribonucleotide triphosphate (dNTP), and 1 unit of DNA polymerase. Taq polymerase was used for PCR colony screening. KAPA HIFI DNA polymerase (KAPA biosystems, Woburn, MA, USA) was used for high fidelity DNA synthesis. PCR was performed on a DNA Thermal Cycler (MJ Research, Waltham, MA, USA).

2.2.10 Site-directed mutagenesis (SDM)

Point mutations in the expression plasmids were made by using a QuikChange SDM Kit (Stratagene, Santa Clara, CA, USA), following the manufacturer's protocols. Overlapping primers with the desired mutations were used in the long PCR cycle. The restriction enzyme Dpn I was used to digest the original template. The Dpn I treated PCR product was transformed into competent *E.coli* and plated onto LB agar plates with appropriate antibiotics. All constructs were verified by sequencing (1st Base, Singapore).

2.2.11 RNA isolation and reverse transcription-PCR (RT-PCR)

Total RNA of cells was extracted using the TRIzol (Invitrogen) following the manufacturer's protocol.

Qiagen One Step RT-PCR Kit was subsequently used for RT-PCR of the isolated RNA. The reverse transcription (RT) of mRNA to cDNA was carried out using OmniscriptTM Reverse Transcriptase at 50°C for 30 min. Thereafter, standard PCR procedures were performed to obtain the cDNA of interest.

2.2.12 Real-time quantitative PCR (qPCR) for kindlin3

Total RNA extraction was performed using Trizol reagent from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's instruction. 2 µg of total

RNA was used for room temperature in 25 μ l final volume. Real-time qPCR was performed using 4 μ l of room temperature mixture and the Syber green real-time qPCR kit from ABI (Ambion Diagnostic, USA) on a Bio-Rad CFX96 qPCR machine [211]. Primer sequences for qPCR are shown in Table 2.7. Data was analyzed using the CFX Manager Software.

Table 2.7 Primer sequences for qPCR

Primers	Primer Sequence
human kindlin3 forward primer	5'-ttccaggctgtggctgccat-3'
human kindlin3 reverse primer	5'-cccagccaagacaacctgc-3'
human β -actin forward primer	5'-gacatggagaaaatctggca-3'
human β -actin reverse primer	5'-tggggtgtgaaggctctcaa-3'

2.3 Cell biology

2.3.1 Media for cells

Table 2.8 Media for cells

Cells	Media
HEK293T (henceforth referred to 293T) and Raw 264.7 cells	Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, Logan, UT) containing 10% (v/v) heat-inactivated FBS (HyClone, Scoresby, Australiz), 100 IU/ml penicillin (HyClone), 100 μ g/ml streptomycin (HyClone)
Jurkat (E6-1), K562, K562/ α M β 2, THP1 and U937 cells	Roswell Park Memorial Institute formulation-1640 (RPMI-1640) (HyClone, Logan, UT) containing 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 1 mM sodium pyruvate
K562 and K562/ α M β 2 cells treated with control siRNA or	RPMI-1640 containing 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin, 100

kindlin3 siRNA	$\mu\text{g/ml}$ streptomycin, 1 mM sodium pyruvate and 1.5 $\mu\text{g/ml}$ puromycin (Sigma-Aldrich)
Isolated human monocytes	RPMI-1640 containing 10% (v/v) human AB serum, 100 IU/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin, and 1 mM sodium pyruvate
HMVE cells	Endothelial cell medium (PAA Laboratories, Somerset, U.K.)
SF9 (insect cells)	SF9 medium (Invitrogen) containing 0.5% (v/v) of heat-inactivated FBS
Cell freezing medium	10% (v/v) dimethyl sulfoxide (DMSO) in heat-inactivated FBS
RPMI wash buffer	RPMI-1640 containing 5% (v/v) heat-inactivated FBS and 10mM HEPES (pH 7.4)

2.3.2 Cell storage and recovery

Cells were spun at $400\text{-}800 \times g$ for 5 min, and resuspended in cell freezing medium (as described in section 2.3.1) at a concentration of 5×10^6 cells/ml, before dispensing into Cryo Vials (Greiner, Monroe, NC, USA). Cells were frozen in a NalgeneTM Cryo 1 °C freezing container (Thermo Fisher Scientific, Waltham, MA, USA) at -80 °C for 24 hours to achieve a -1 °C/min cooling rate. Thereafter, the vials were transferred into liquid nitrogen for long-term storage.

Cells were removed from the liquid nitrogen storage and quickly thawed at 37 °C in a water bath. Cells were gently transferred into 10 ml warmed culture medium and incubated at room temperature for 5 min. Cells were then centrifuged ($400\text{-}800 \times g$, 5 min) to remove DMSO. The cell pellet was resuspended in complete medium and cultured at 37 °C in a humidified 5% CO₂ incubator.

2.3.3 Mammalian cells

2.3.3.1 Cell lines

293T, U937, THP1, Jurkat (E6-1), K562 cells were bought from ATCC.

The stable K562 cells expressing WT integrin $\alpha M\beta 2$ [212] (referred as K562/ $\alpha M\beta 2$) were kindly provided by Prof. L. Zhang (University of Maryland, Baltimore, MD, USA).

Mouse Raw 264.7 cells were described in section **2.2.1.4**.

HMVE cells immortalized with human telomerase protein were kindly provided by Dr. R. Shao (University of Massachusetts at Amherst, Springfield, MA, USA) [213].

Pan human monocytes were isolated from buffy coat (from Bloodbank, Health Sciences Authority, Singapore, following regulatory guidelines) by Ficoll-paque (GE Healthcare) sedimentation. The magnetic isolation was performed using CD14 microbeads and LS column (Miltenyi Biotec, Auburn, CA, USA). The purity of the isolated monocytes was assessed by flow cytometry using anti-CD14 monoclonal antibody (mAb) (Miltenyi Biotec).

To generate K562 con siRNA, K562 k3 siRNA, K562/ $\alpha M\beta 2$ con siRNA and K562/ $\alpha M\beta 2$ k3 siRNA cells, the 3rd generation lentiviral-based siRNA transduction system was used according to manufacturer's instructions (Applied Biological Materials, BC, Canada).

2.3.3.2 Cell culture

All mammalian cells were cultured in a humidified 5% CO₂ incubator at 37 °C.

293T cells and mouse RAW 264.7 cells were cultured in complete DMEM culture medium containing 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were passaged every two days when they reach ~90% confluency. After washing once with PBS, 1-2 ml of 0.25% (w/v)

trypsin (Invitrogen) was added into cell culture flask to dislodge the adherent cells. Trypsin was subsequently quenched in complete DMEM culture medium. Cells were then seeded into tissue culture flask or dish with full DMEM culture medium.

Jurkat (E6-1), K562, K562/ α M β 2, THP1 and U937 cells were cultured in complete RPMI-1640 medium containing 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin and 1 mM sodium pyruvate. Cells were split by diluting cells with fresh medium after reaching a cell density of $>2 \times 10^6$ cells/ml.

All four stable cell lines treated with siRNA, K562 con siRNA, K562 k3 siRNA, K562/ α M β 2 con siRNA and K562/ α M β 2 k3 siRNA, were cultured in complete RPMI-1640 medium same as their parent cells K562 and K562/ α M β 2. The cell culture was maintained with 1 mg/ml of puromycin, which would be withdrawn 3 days before being used for a particular experiment.

HMVE cells were cultured in endothelial cell medium in tissue culture dish pre-coated with 0.1% (w/v) gelatin (Life Technologies, Grand Island, NY, USA). Cells were passaged every 2-4 days with 0.25% (w/v) trypsin (Invitrogen) when 90% confluency is reached.

2.3.3.3 Cell transfection

293T cells were seeded into a 6 cm cell culture dish and cultured overnight to achieve approximately 40-80% confluency. Polyfect reagent (40 μ l) (Qiagen) was added to 150 μ l serum-free medium containing 4 μ g of expression plasmids. After 5-10 min of incubation, 1 ml complete medium was added to the Polyfect and DNA mixture. The mixture was then dispensed into the culture dish containing the cells slowly. 24-48 hours after transfection, the cells in each dish were washed in PBS and dislodged by pipetting. Cells were then transferred into centrifuge tubes and spun down at $400 \times g$ for 5 min at 4 °C for subsequent analysis. The transfection efficiency was around 70-90% based on flow cytometry analysis. The viability of the cells was more than 80% after transfection as determined by trypan blue exclusion.

For K562, K562 con siRNA and K562 k3 siRNA cells, ~10 µg of integrin plasmids (αM and β2 or β2 mutant) [214] were transfected into 2×10^6 cells using the Microporator MP-100 (a pipette type electroporator) and reagents as per the manufacturer's instructions (NanoEnTek, Korea) [215]. The plasmids were mixed with the cell suspension in a total volume of 100 µl. The suspension was aspirated into the channel of a Microporator gold-tip. The optimized microporation program used for K562 cell lines was 3 pulses of 1300 V pulse voltage with a duration of 10 milliseconds (ms) for each pulse. Electroporated cells were dispensed into a well of the 6-well cell culture plate containing 2 ml of pre-warmed medium and cultured for 24-48 hours before analysis. The transfection efficiency was around 70-90% based on flow cytometry analysis. The viability of the cells was more than 80% after transfection as determined by trypan blue staining.

2.3.4 Human primary monocytes

Pan human monocytes isolated from buffy coat were maintained in RPMI-1640 medium containing 10% (v/v) human AB serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin.

2.3.5 Insect cells

SF9 cells were cultured in SF9 medium with 0.5% (v/v) of heat-inactivated FBS in shake flask at 28°C in cell culture incubator, without requirement of CO₂. When the cells reach a density of about $2-4 \times 10^6$ cells/ml, they were passaged at a seeding density of $3-5 \times 10^5$ viable cells/ml.

When the cells were used for transfection, the suspension culture was switched to adherent culture in cell culture dish or flask.

2.3.6 Flow cytometry analysis

Flow cytometry analyses of cells expressing integrins were performed as described previously [206]. Cells were stained with relevant primary Ab (10 µg/ml) in PBS for 30 min at 4°C. Cells were washed in PBS and incubated in PBS containing FITC-conjugated sheep anti-mouse IgG (1:400) or APC-

conjugated goat anti-mouse IgG (1:500) for 30 min at 4°C. Stained cells were fixed in PBS containing 1% (v/v) formaldehyde and analyzed on a BD fluorescence activated cell sorting (FACS) caliber with CellQuest software (BD Biosciences, San Jose, CA, USA) installed.

2.3.7 Cell adhesion assays

Cell adhesion on immobilized ligands (ICAM-1, BSA and iC3b) was performed essentially as described previously [206]. Cells were labeled with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), which is an acetoxymethyl ester fluorescent dye (Molecular Probes, Eugene, OR). BCECF dye-labeled cells were seeded into wells with immobilized ligands and incubated for 30 min at 37 °C. Wells were washed and fluorescence signal of adherent cells was measured on a fluorescent plate reader (FL600) (Bio-Tek Instruments, Winooski, VT, USA). The percentage of bound cells was calculated: fluorescence signal after wash/fluorescence signal before wash × 100. Under activating condition, 10 µg/ml mAb KIM185 was included. Adhesion specificity mediated by αMβ2 was assessed by including mAb LPM19c (10 µg/ml) in the assay.

For cell adhesion to immobilized Abs, a similar assay was performed, except that the well of the microtitre plate was coated with 5 µg/ml goat anti-mouse IgG in SBB (pH 9.0) overnight at 4°C. Subsequently, the well was coated with mAb MHM24, LPM19c, or control mouse IgG (10 µg/ml each) for 1 hour at 37°C.

2.3.8 Integrin cross-linking analyses

For cross-linking of integrins on U937 cells or monocytes, cells were seeded into Ab-coated microtitre wells aforementioned. After incubation for different time points at 37°C in a humidified 5% CO₂ incubator, all cells (bound and unbound) in the wells were collected and lysed for subsequent analyses.

For cross-linking experiments using F (ab')₂, the F (ab')₂ of LPM19c and control IgG were prepared using the F (ab')₂ micropreparation kit (Pierce, Rockford, IL, USA), following the manufacturer's instructions. Digestion of

mAbs by immobilized pepsin was monitored by SDS-PAGE under reducing and nonreducing conditions. F (ab')₂ was immobilized onto tissue culture dish as described previously [216]. U937 cells were plated into the F (ab')₂-coated dish and incubated for 30 min at 37°C in a humidified 5% CO₂ incubator. All cells were collected for subsequent analyses.

2.3.9 Real-time electric cell-substrate impedance sensing (ECIS)

The 16-well E-plate (Acea Biosciences, Inc., San Diego, CA, USA) with gold-electrodes at the bottom of each well was coated with 40 µl of 4 mg/ml DSP (Pierce, Thermo Fisher Scientific, USA) in DMSO. After incubation for 30 min at room temperature [205], DSP-DMSO was discarded and wells were washed twice quickly in molecular biology grade H₂O followed by coating with BSA (100 µg/ml) or iC3b (7.5 µg/ml) in PBS for 1 hour at room temperature. After E-plates were washed in PBS, 100 µl of wash buffer was added into each well and background signal was measured using a real time cell electronic system (Acea Bioscience, Inc.). 8×10⁴ cells were seeded into each well, with or without the addition of blocking mAb Lpm19c (10 µg/ml) and/or integrin-activating mAb KIM185 for the different conditions. Cell index was measured for 90 min with 1 min intervals.

2.3.10 Shear flow study

The µ-slide I^{0.4} Luer flow chamber (Ibidi GmbH, Munich, Germany) was coated with 7.5 µg/ml iC3b (Calbiochem, San Diego, CA, USA) in PBS at 4°C overnight. iC3b was removed and the chamber was blocked with 0.2% (w/v) PVP in PBS at room temperature for 1 hour to avoid non-specific binding. Blocking buffer was removed and HBSS buffer with additives (as described in section 2.1.4) was added into the chamber. Cells (6×10⁵) with or without integrin-activating mAb KIM185 (10 µg/ml) were resuspended in 1 ml HBSS buffer plus additives and infused into the chamber at 37°C using an automated syringe pump (PHD2000 syringe pump series instrument, Programmable). 3 ml of HBSS buffer with additives was then injected and passed through the chamber at a constant shear force (0.2 or 0.4 dyn/cm²). The number of

adherent cells was recorded in four different fields (1mm vs 1mm) under a 10× objective lens.

2.3.11 Live cell imaging

For live imaging of human peripheral blood monocytes adhering and migrating on HMVE cells, monocytes were labeled with 1 μ M fluorescence label Cell-Tracker Green (Molecular Probes, Eugene, OR, USA) in complete RPMI-1640 medium for 20 min at 37°C. Cells were washed twice in medium and seeded onto HMVE cells that were grown to confluence in cover-slip glass bottom tissue culture dish (MatTek, Ashland, MA, USA). The cells were viewed with a Zeiss Axiovert 200M inverted fluorescence microscope (with 20 × objective) housed in a closed system with 5% CO₂ and temperature maintained at 37°C. Images of migrating monocytes were captured with time by a CoolSnap HQ charge-coupled device camera (Roper Scientific, Tucson, AZ, USA) for 15 min. Labeled monocytes were detected with 485/525 ex/em filters. Images were generated using the software Metamorph (Molecular Devices, Sunnyvale, CA, USA).

2.3.12 Immunofluorescence analyses

U937 cells were seeded into Ab-coated microtitre wells. Cells were collected by washing the wells in PBS containing 0.5 mM EDTA. Recovered cells were spun down at 1000× *g* for 10 min onto poly-L-lysine-coated slides using a cytospin centrifuge (Thermo Electron, San Jose, CA, USA). For monocytes, cells were allowed to adhere to poly-L-lysine slides coated with mAbs for 10 min at room temperature. Cells were washed twice in PBS, followed by fixation in PBS containing 3.7% (w/v) paraformaldehyde for 5 min at room temperature. Cells were washed twice in PBS and permeabilized in modified cytoskeletal buffer containing 0.3% (v/v) Triton X-100 and protease inhibitors mixture (Roche Diagnostic Systems, Somerville, NJ, USA) for 1 min at room temperature. Cells were washed and incubated in PBS containing rabbit anti-PKC δ Ab (1 μ g/ml) for 1 hour at room temperature. Thereafter, cells were washed and incubated in PBS containing Alexa Fluor 488 goat anti-rabbit IgG (1:1000) for 30 min at room temperature in the dark. Images were

acquired using a 40× objective lens on a fluorescence microscope (Olympus, Tokyo, Japan) equipped with MetaMorph software.

2.3.13 Cell image using confocal microscopy

The 3 cm dish with micro-well was coated with BSA (100 µg/ml) or iC3b (7.5 µg/ml) in PBS at 37°C for 2 hours. After washing twice in PBS, 3×10^4 cells (K562/ α M β 2 con siRNA or K562/ α M β 2 k3 siRNA cells) with integrin-activating mAb KIM185 (10 µg/ml) were seeded in the coated dish. Cells were incubated at 37 °C for 30 min, and the floating cells were removed. The adhered cells were fixed with 3.7% paraformaldehyde at room temperature for 10 min followed by permeabilization with 0.25% Triton-X 100 in cytoskeletal buffer at room temperature for 3 min. Non-specific binding sites were then blocked with 1% BSA in PBS for 30 min. Cells were stained with 10 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (1:500) (Sigma-Aldrich) and 0.165 µM Alexa Fluor 594 conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) (1:40) for 1 hour at room temperature. Images were observed using confocal microscope LSM510 Meta with appropriate laser lines (Carl Zeiss, Inc., Thornwood, NY, USA).

2.4 Biochemistry and special assays

2.4.1 Subcellular fractionation of cells

To analyze PKC δ localization in the subcellular fractions, U937 cells were resuspended in buffer A (as described in section 2.1.4) and subjected to sonication on ice for 5 seconds. Cell lysate was centrifuged at 600× *g* to remove the nuclei and cell debris. Thereafter, cell lysate was centrifuged at 100,000× *g* for 1 hour, and the resulting supernatant was collected as the soluble fraction. The pellet was solubilized in buffer B (as described in section 2.1.4) as particulate fraction. Proteins were resolved on SDS-PAGE gel under reducing conditions, electroblotted and probed with rabbit anti-PKC δ (C-20) or anti-phospho-PKC δ Tyr311 Ab.

2.4.2 Lysis of mammalian cells

Cells were lysed in protein lysis buffer with protease inhibitor cocktail from Roche on ice for 30 min. Phosphatase inhibitor cocktail from Nacalai Tesque (Kyoto, Japan) was also added when necessary. Lysate was centrifuged at $12000\times g$ for 10 min at 4 °C. Supernatant was collected and boiled in protein loading dye containing 40 mM dithiothreitol (DTT).

2.4.3 Determination of protein concentration

The Bio-Rad protein assay, based on the method of Bradford, was used for the protein concentration determination. The dye reagent was prepared by diluting 1 volume of dye reagent concentrate with 4 volumes of ddH₂O. Five dilutions of BSA standard were prepared (the linear range of the assay for BSA is 0.1 to 1 mg/ml). 20 µl of each standard and sample solution with 980 µl of diluted dye reagent were pipetted into clean and dry test cuvettes. After incubation at room temperature for 5 min, the absorbance of each solution was measured at 595 nm. The standard curve was plotted based on absorbance at 595 nm against known protein concentration and the concentration of the unknown sample was derived from the plot.

2.4.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli [217] with modifications. Protein sample (5-15 µl for the minigel system) was mixed with an equal volume of 2 × sample loading buffer containing 40 mM DTT. Electrophoresis was carried out in SDS-PAGE electrophoresis buffer at 200 V in a Mini Electrophoresis Set (Bio-Rad).

2.4.5 Western blot

Proteins separated by SDS-PAGE were transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA). The PVDF membrane was incubated in blocking buffer (as described in section 2.1.4) at room temperature for 1 hour to block non-specific binding sites.

Thereafter, the membrane was transferred into the blocking buffer containing the relevant primary Ab at room temperature for 2-3 hours with rotation. The Ab was used at a proper dilution according to the product description of Ab.

After incubation with primary Ab, the membrane was washed for three times with PBS-T or TBS-T, followed by incubation with the blocking buffer containing HRP-conjugated goat anti-mouse IgG Ab (1:20000) (Advansta, CA, USA), HRP-conjugated goat anti-rabbit IgG Ab (1:20000) (Advansta) or HRP-conjugated goat anti-rat IgG Ab (1:5000) (GE Healthcare, UK) at room temperature for 1 hour. The membrane was washed for three times in PBS-T or TBS-T and incubated with the Western bright enhanced chemiluminescence (ECL) HRP substrate (Advansta) following the manufacturer's protocol. The membrane was exposed to an X-ray film (Konica Minolta, Chiyoda-ku, Tokyo, Japan) and developed using a Kodak X-OMAT ME-1 processor (Kodak, Rochester, NY, USA).

For membrane which required reblotting, it was incubated with stripping buffer at 55°C with shaking for 30 min. The membrane was washed in PBS-T or TBS-T six times for 5 min each, and the procedure was repeated starting with blocking step.

2.4.6 Preparation of protein A sepharose

Protein A sepharose (PAS) (1.5 g) (Amersham) was swelled in PBS and rotated at 4°C overnight. Thereafter, the PAS beads, which occupied a bed volume of approximately 6 ml, was sedimented by centrifugation ($3000 \times g$, 5 min, 4°C), washed twice in PBS and resuspended in 25 ml of PBS to obtain a 25% (v/v) bead suspension.

For the preparation of PAS-rabbit anti-rat IgG (RAR), 4 mg of RAR (Sigma-Aldrich) was added to the bead suspension (~25 ml) and the mixture was rotated at 4 °C for 1 hour. The PAS-RAR beads were washed twice in PBS and sedimented by centrifugation ($3000 \times g$, 5 min, 4 °C). The beads were then resuspended in 25 ml of PBS to obtain a 25% (v/v) bead suspension and

were stored at 4 °C. PAS-RAR beads were used for the immunoprecipitation (IP) of kindlin3.

2.4.7 Immunoprecipitation (IP) assay

To examine Hck interaction with PKC δ , U937 cells were lysed and subjected to subcellular fractionation. The particulate fraction was collected and solubilized in buffer B (as described in section 2.1.4). Hck was immunoprecipitated with rabbit anti-Hck Ab and protein A-Sepharose beads (Amersham Biosciences). The beads were washed in buffer C (as described in section 2.1.4). Proteins precipitated were resolved on SDS-PAGE gel, followed by immunoblotting with these Abs: mouse anti-Hck, rabbit anti-PKC δ (C-20), or rabbit anti-phospho-PKC δ Tyr311. Similar procedure was performed to detect Lyn interaction with PKC δ in U937 cells.

To detect a highly extended α M β 2, K562 transfectants expressing WT α M β 2 or α M β 2D709R were incubated with mAb LPM19c or the conformation reporter mAb KIM127 (10 μ g/ml each) in culture medium with or without MnCl₂ (0.5 mM) as activating agent for 30 min at 37°C [214]. Cells were washed extensively in medium and lysed in buffer C. Immune complexes in the whole-cell lysates were precipitated with protein A-Sepharose. Bound proteins were resolved on SDS-PAGE under reducing conditions. The α M protein band was detected by immunoblotting using rabbit anti- α M Ab.

To examine the association of PKC δ and IRAK1, THP1 and K562/ α M β 2 cells were lysed in protein lysis buffer. Immune complexes in total cell lysates were precipitated with anti-PKC δ Ab (C-20) Ab and protein A-Sepharose. Bound proteins were resolved on SDS-PAGE under reducing conditions. The IRAK1 protein band was detected by immunoblotting using rabbit anti-IRAK1 Ab.

2.4.8 GST pull-down assays

GST-PAK-PBD or GST-Rhotekin-RBD bead suspension (20 μ l) from Cytoskeleton (Denver, CO, USA) was added to 200 μ l of cell lysate and

incubated at 4°C for 3 hours with rotation. Beads were spun down and washed for 3 times with lysis buffer, and boiled with protein loading dye and 40 mM DTT. The eluted proteins were resolved on a 10% SDS-PAGE under reducing conditions. Anti-Rac1, anti-Cdc42, anti-RhoA, and anti-GST Abs were used to detect Rac1, Cdc42, RhoA and GST-PBD or GST-RBD. GST-PBD and GST-RBS were also detected by staining in Coomassie brilliant blue solution.

2.4.9 Expression and purification of recombinant Hck (rHck)

Hck cDNA encoding 78-526 amino acids was cloned into pFastBac Dual (Invitrogen) using the EcoRI and XbaI restriction sites to generate expression bacmid pFastBac-Dual-78-Hck with an N terminus His₆-tag (provided by Dr. Chua Geok Lin). Bacmid was first amplified in *E.coli* DH5 α , followed by amplification in Max Efficiency DH10Bac. Bacmid was transfected into SF9 insect cells using Cellfectin transfection reagent (Invitrogen). Hck expression in the transfectants was assessed by western blotting with rabbit anti-His Ab. Passage 2 (P2) virus was collected, and the optimal P2 inoculum and density of SF9 cells for Hck production were determined. Briefly, 2 $\times$ 10⁶ adherent SF9 cells in SF9 medium containing 0.5% (v/v) of heat-inactivated FBS were infected with P2 virus. Cells were collected after 3 days, lysed, and rHck was purified using Ni-NTA purification system (Qiagen).

2.4.10 In vitro kinase assay

U937 cells were lysed in buffer C containing protease inhibitor mixture for 30 min at 4°C. Cell lysate was precleared with irrelevant rabbit Ab and protein A-Sepharose beads. Hck or Lyn was precipitated with relevant Ab and protein A-Sepharose beads and washed three times in lysis buffer and two times in kinase buffer. Beads containing immunoprecipitated Hck or Lyn were incubated with 100 ng recombinant PKC δ (rPKC δ) in kinase buffer containing 3 mM ATP for 30 min at 37°C. Reaction was terminated by adding protein solubilization buffer and boiling. For PKC δ phosphorylation by rHck, 100 ng each of rPKC δ and purified rHck were used.

For γ [^{32}P] ATP PKC δ kinase assay, PKC δ in the particulate fraction of U937 cells was immunoprecipitated with rabbit anti-PKC δ Ab and protein A-Sepharose. The immunoprecipitate was washed twice in PKC kinase buffer. Each reaction was performed in PKC kinase buffer containing 20 μg histone H1 (Calbiochem, San Diego, CA) and 5 μM ATP (unlabeled ATP and 2 μCi γ [^{32}P] ATP, sp. act. 3000 Ci/mmol) (PerkinElmer, Wellesley, MA, USA) for 10 min at 30°C [218]. Reaction was terminated by adding protein solubilization buffer and boiling. Proteins were resolved on SDS-PAGE gel, followed by autoradiography.

2.4.11 SiRNA-mediated reduction of Syk, Hck and PKC δ expressions

Accell SMART pool siRNA targeting human Syk, Hck or PKC δ (0.4 nmol each) was used for 3×10^4 U937 cells in 100 μl Accell delivery medium containing 0.5% FBS to reduce expression of Syk, Hck, PKC δ as recommended by the manufacturer (Thermo Scientific Dharmacon, USA). The control Accell green non-targeting siRNA was used at the same concentration. Cells were collected 3 or 4 days later for subsequent analyses. Cell viability was at least 80% as determined by trypan blue exclusion.

siRNA targeting PKC δ in human peripheral blood monocytes was performed by incubating 2×10^6 cells in Opti-MEM1 reduced serum medium containing 33 nM PKC δ -targeting siRNA or control siRNA and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The cell suspension in polypropylene tubes was incubated for 16 hours at 37°C in a humidified 5% CO $_2$ incubator. Cell viability was ~70% as determined by trypan blue exclusion. Cells were subsequently collected for Ab cross-linking or HMVE adhesion studies. For the latter, cells were seeded onto HMVE cells grown to confluence on culture dish or culture dish without HMVE and incubated for 8 hours at 37°C in a humidified 5% CO $_2$ incubator. Cells were then collected by incubating in PBS containing 0.5 mM EDTA. Monocytes were isolated using CD14 microbeads (as described in section 2.3.3.1). Cells were lysed and Foxp1 expression was detected by immunoblotting.

2.4.12 Kindlin3 knockdown in K562 and K562/ α M β 2 cells

Stable K562 and K562/ α M β 2 cells with reduced kindlin3 were generated using the 3rd generation lentiviral-based siRNA transduction system according to the manufacturer's instructions (Applied Biological Materials, BC, Canada) [205]. Briefly, four kindlin3-targeting piLenti-RNAi-GFP plasmids were generated. The four different sequences that encode kindlin3 siRNA are: 149, TGGAGCAGATCAATCGCAA; 758, ACTACAGCTTCTTCGATTT; 1148, ACTACAAGAGCCAGGACGA; 1872, CCGAATTGTACACGAGTAT [219]. Each of these plasmids or the control siRNA encoding plasmid was co-transfected with the packaging plasmids into 293T cells to allow production of pseudo-virions, and the culture supernatant was collected.

K562 or K562/ α M β 2 cells were (1×10^6 each) resuspended in 1 ml complete RPMI-1640 growth medium supplemented with 6 μ g/ml of polybrene (Sigma-Aldrich) followed by the addition of 1 ml of pseudo-virions-containing supernatant and the cells were cultured for 1 day. The following day, cells were spun down and cultured in fresh complete RPMI-1640 medium for at least 2 days. When GFP-expressing cells were observed under a fluorescence microscope, puromycin (1.0 μ g/ml) was added to the culture to select for cells stably expressing kindlin3 siRNA. Four stable cell lines were generated which are K562 control siRNA, K562 kindlin3 siRNA (provided by Dr. Feng Chen), K562/ α M β 2 control siRNA and K562/ α M β 2 kindlin3 siRNA. The kindlin3 siRNA sequence for K562 kindlin3 siRNA cells is 149, and that for K562/ α M β 2 kindlin3 siRNA cells is 1872.

2.4.13 Statistical analysis

In all cases, student's *t* test (two tailed and paired test) was used in statistical determination of the difference observed between two groups. The difference was considered significant when $p < 0.05$.

3. Results

In the first section (3.1), we show that PKC δ mediates integrin α M β 2 signaling in monocytes. Integrin α M β 2 clustering induces the translocation of PKC δ to the plasma membrane and PKC δ phosphorylation at Tyr311, in a SFK-dependent manner. We also show that clustering of integrin α M β 2 down-regulates the expression of transcription factor Foxp1, which plays a role in monocyte differentiation.

In the second section (3.2), we provide data showing that IRAK1 interacts with PKC δ , which may be involved in the cross-talk between integrin α M β 2 signaling and TLR signaling. These two signaling pathways converge and synergize to regulate the immune response and inflammation. In this section, we also show that MEK-Erk signaling is involved in integrin α M β 2-induced Foxp1 regulation.

In the third section (3.3), we provide direct evidence that kindlin3 is required for integrin α M β 2 outside-in signaling that modulates cell spreading. Reduced kindlin3 expression in K562 cells expressing WT integrin α M β 2 or the constitutively activated integrin α M β 2N329S abrogated the adhesion and spreading capacities of these cells on ligands iC3b and BSA. We also show that kindlin3 is involved in the integrin α M β 2-Syk-Vav1 signaling axis that regulates the activities of Rac1 and Cdc42.

3.1 Clustering of integrin $\alpha M\beta 2$ triggers the phosphorylation and activation of PKC δ that regulates the expression of transcription factor Foxp1 in human monocytes

3.1.1 Clustering of integrin $\alpha M\beta 2$ triggers PKC δ phosphorylation in U937 cells

We investigated early signaling events in human monocytic U937 cells that are induced by integrin clustering. U937 cells express a high level of integrin $\alpha L\beta 2$ and a moderate level of $\alpha M\beta 2$ as determined by flow cytometry (Figure 3.1.1 A). Integrin $\alpha X\beta 2$ has minimal expression on U937 cells (data not shown). Before proceeding to the main study, we examined if U937 cells could adhere to microtitre wells coated with relevant anti-integrin mAbs. U937 cells showed a higher adhesion level on anti- $\alpha L\beta 2$ coated surface, while the adhesion levels on anti- $\alpha M\beta 2$ and control IgG coated surface significantly were reduced (Figure 3.1.1 B). This is consistent with the expression levels of these integrins on U937 cells. Whole-cell lysates were used for immunoblotting with commercial phospho-PKC δ Tyr311 Ab that cross-reacts with mouse PKC δ Tyr311 and the corresponding human PKC δ Tyr313. For consistency with the Ab used, we will refer it as Tyr311. It has been reported that phosphorylation of PKC δ at Tyr311 initiates subsequent phosphorylation reactions of other tyrosine sites that regulate PKC δ activity [220]. We found that cross-linking of integrin $\alpha M\beta 2$ induced an increased level of PKC δ phosphorylation at Tyr311, while integrin $\alpha L\beta 2$ and control IgG cross-linking did not (Figure 3.1.1 C). Cross-linking of integrin $\alpha X\beta 2$ was also performed, with no induction of PKC δ Tyr311 phosphorylation was detected (data not shown).

Previous study has shown that anti-integrin mAbs-induced responses in neutrophils rely on the engagement of FcRs by Fc portions of the integrin mAbs used [216]. To rule out the role of Fc portion, F(ab')₂ was purified and used to perform integrin cross-linking. We found that integrin $\alpha M\beta 2$ -specific F(ab')₂ induced a marked phosphorylation of PKC δ Tyr311 in U937 cells, but

control IgG F(ab')₂ did not (Figure 3.1.1 D). These data show that integrin α M β 2 clustering induces the phosphorylation of PKC δ Tyr311.

Cross-linking of integrin α M β 2 at different time points (15 to 60 min) induced a transient phosphorylation of PKC δ at Tyr311. The phosphorylation was induced at 15 min, peaked at 30 to 45 min, and reduced at 60 min following seeding of U937 cells onto anti- α M β 2 mAb coated wells (Figure 3.1.1 E). This event was dependent on SFKs, as the SFK inhibitor PP2 abolished PKC δ Tyr311 phosphorylation induced by clustering of integrin α M β 2 (Figure 3.1.1 F). The inactive analog of PP2 (PP3), Syk inhibitor (PIC) and PI3K inhibitor LY294002 (LY) were also included in this experiment, but none of them had significant effect on PKC δ Tyr311 phosphorylation (Figure 3.1.1 F). In line with the profiles of integrin cross-linking, PMA-induced PKC δ Tyr311 phosphorylation was diminished by pretreatment of PP2, but not affected by other inhibitors (Figure 3.1.1 G).

Figure 3.1.1 PKC δ Tyr311 phosphorylation upon integrin $\alpha M\beta 2$ clustering.

A. Integrin $\alpha L\beta 2$ and $\alpha M\beta 2$ expression on U937 cells as determined by FACS using anti- αL mAb MHM24 and anti- αM mAb LPM19c, respectively (gray histogram). Black histogram shows background staining using an irrelevant mAb mouse IgG. **B.** Cell adhesion assays were conducted onto anti- αL mAb MHM24, anti- αM mAb LPM19c, or control mouse IgG (Ig)-coated microtitre wells. **C.** U937 cells were seeded into mAb coated wells and then incubated at 37°C for 30 min in a humidified incubator with

5% CO₂. All cells (bound and unbound) were harvested and lysed. Whole-cell lysates were used to perform immunoblotting using phospho-PKC δ Tyr311 Ab. Total PKC δ was also detected using the same membrane after stripping off Abs. **D.** Similarly as **C**, instead of whole Ab, purified F(ab')₂ (Ig, control IgG F(ab')₂; α M, LPM9c F(ab')₂) was used to induce integrin clustering. Data are representative of three independent experiments. **E.** At different time points (15~60 min), PKC δ Tyr311 phosphorylation was detected following plating of U397 cells onto immobilized mAbs (control mouse IgG or LPM19c). **F.** Following pre-treatment of different inhibitors (20 μ M each) for 1 hour, U397 cells were then seeded into microtitre wells coated with mAb LPM19c (α M) or control IgG (Ig) for 30 min. Whole-cell lysates were used to detect the phosphorylation of PKC δ Tyr311. **G.** Cells were pre-treated with different inhibitors (20 μ M each) for 1 hour. Con*, without inhibitor. Cells were then treated with 100 ng/ml of PMA for 30 min. Con, without PMA. In **C-G**, anti- β -actin blots were included as a loading control. In **C**, **E**, **F** and **G**, the ratio of phospho-PKC δ Tyr311 and total PKC δ was quantified by densitometry and plotted as relative units (phospho-PKC δ Tyr311 intensity/ total PKC δ intensity). Data are the average of three independent experiments and shown as means $\pm$ standard deviation (SD). *1-*6, p=0.0000668656, 0.0001411594, 0.0003532966, 0.0040820249, 0.0002624184 and 0.0006968976, respectively (Student's *t* test). α L, mAb MHM24; α M, mAb LPM19c; con, control, without any additive; Ig, control IgG; PP2, SFK inhibitor; PP3, inactive analog of PP2; LY, PI3K inhibitor LY294002; PIC, Syk inhibitor picetannol.

3.1.2 PKC δ translocates to the plasma membrane upon integrin α M β 2 cross-linking

In order to determine the localization of PKC δ upon clustering of integrin α M β 2, cellular fractionation experiments were performed. We found that PKC δ was localized in the cytoplasm in resting cells, and translocated to the membrane following cross-linking of integrin α M β 2 (Figure 3.1.2 A). The phosphorylation of PKC δ Tyr311 was induced by integrin α M β 2 clustering and appeared in the membrane fraction. To verify the translocation of PKC δ , immunofluorescence staining and analyses were performed. In U937 cells treated with anti- α M Ab and PMA, PKC δ translocated to the plasma membrane, while in cells treated with anti- α L Ab and control mouse IgG, it remained in the cytoplasm (Figure 3.1.2 B). The percentage of cells in each field with PKC δ translocation was calculated and plotted (Figure 3.1.2 C). To examine the activity of translocated PKC δ , we also performed *in vitro* kinase assay. Following integrin cross-linking (con IgG, anti- α L, or anti- α M) or PMA treatment, all lysates were fractionated and immunoprecipitated with anti-PKC δ Ab. The PKC δ immunoprecipitates (IPs) were incubated with γ [32 P]ATP and the substrate histone H1 (Figure 3.1.2 D) [218]. We found that PKC δ is activated following integrin α M β 2 cross-linking or PMA treatment, while PKC δ activation was not detected under other conditions. Thus, integrin α M β 2 clustering induces PKC δ Tyr311 phosphorylation, activation and membrane translocation in U937 cells.

Figure 3.1.2 PKC δ translocates to the plasma membrane upon integrin α M β 2 clustering.

A. Same as in Figure 3.1.1 C, U937 cells were seeded into empty microtitre wells (no cross-linking) or wells coated with mAb LPM19c (α M) or control mouse IgG. All cells (bound and unbound) were collected and lysed, and then fractionated using

high-speed centrifugation. Both the cytosolic and particulate fractions of each sample were used for immunoblotting to detect the phosphorylation of PKC δ Tyr311. After stripping of the Abs, we performed reblotting using anti-PKC δ for total PKC δ detection. **B.** U937 cells were cross-linked with anti- α L mAb MHM24 or anti- α M mAb LPM19c or control mouse IgG or treated with 100 ng/ml of PMA for 30 min. Cells were then transferred onto poly-L-lysine coated slides for immunofluorescence staining using anti-PKC δ Ab. Magnification, 40 $\times$. Bar, 10 μ m. **C.** The percentage of cells with PKC δ translocated to the plasma membrane was calculated as number of cells with membrane translocated PKC δ in a field/total number of cells in the same field. Data shown are means $\pm$ SD in three different fields. **D.** U937 cells cross-linked with anti- α L mAb MHM24 or anti- α M mAb LPM19c or control mouse IgG or treated with 100 ng/ml of PMA were harvested and lysed. Following cellular fractionation, the particulate fractions were used for IP with anti-PKC δ Ab. *In vitro* kinase assay was then performed to detect PKC δ activity using γ [32 P] ATP and the substrate histone H1. Whole-cell lysates of each sample were also obtained for immunoblotting with anti-phospho-PKC δ Tyr311 Ab and reblotting with anti-PKC δ Ab. α M, mAb LPM19c.

3.1.3 SFK Hck is involved in PKC δ Tyr311 phosphorylation induced by integrin α M β 2 clustering

In order to determine the role of SFKs in integrin α M β 2-induced PKC δ Tyr311 phosphorylation, we performed co-immunoprecipitation (co-IP) assays of Hck and PKC δ using the particulate fraction of U937 cells. Hck has a high level of expression in myeloid cells, and it is located around integrin α M β 2-containing phagosomes [221]. IP assays showed that PKC δ interacts with Hck following integrin α M β 2 cross-linking in U937 cells, and PKC δ undergoes phosphorylation at Tyr311 (Figure 3.1.3 A). In IP sample of control IgG treated cells, PKC δ was not detected. Hck IPs were also used for *in vitro* kinase assay with rPKC δ (Figure 3.1.3 B). The rPKC δ was phosphorylated at Tyr311 by immunoprecipitated Hck with a higher level in α M β 2-crosslinked cells than that in cells treated with control IgG. To verify the direct phosphorylation of PKC δ by Hck, rPKC δ and rHck were used for kinase assay (Figure 3.1.3 C). Our results showed that rPKC δ was phosphorylated at Tyr311 by rHck. SFK inhibitor PP2 effectively abolished the induction of PKC δ Tyr311 phosphorylation, while PP3 (the inactive analog of PP2) did not. Subsequently, we used Hck targeting siRNA to address the role of Hck during PKC δ phosphorylation induced by integrin α M β 2 clustering. Hck expression was reduced to ~50% in U937 cells by Hck-targeting siRNA compared with non-targeting control siRNA (Figure 3.1.3 D). In Hck-targeting siRNA treated U937 cells, integrin α M β 2 clustering did not induce a significant phosphorylation of PKC δ Tyr311, while it did in non-targeting control siRNA treated cells (Figure 3.1.3 E). In both groups, total PKC δ expression levels were similar. These results show that Hck is involved in integrin α M β 2-induced PKC δ Tyr311 phosphorylation.

Figure 3.1.3 Hck interacts with PKC δ upon integrin α M β 2 clustering.

A. U937 cells were seeded into microtitre wells coated with mAb LPM19c (α M) or control IgG (Ig) as in Figure 3.1.1 C. Cellular fractionation was performed using

high-speed centrifugation. The plasma membrane containing fractions were used for IP using anti-Hck Ab or control IgG. The IPs were used in immunoblotting with anti-phospho-PKC δ Tyr311 Ab to detect PKC δ Tyr311 phosphorylation, followed by reblotting with anti-PKC δ Ab to detect total PKC δ . Immunoblotting with anti-Hck was also included in this experiment. The heavy (H) chain of Abs used in the IP is indicated. **B.** Same as in A, the immunoprecipitated Hck was used for in vitro kinase assay with rPKC δ (100 ng). The phosphorylation level of rPKC δ Tyr311 in each reaction sample was detected by immunoblotting with anti-phospho-PKC δ Tyr311 Ab. Reblotting of total rPKC δ and Hck blotting were included as loading controls. **C.** 100 ng of rHck and 100 ng of PKC δ were used for in vitro kinase assay without or with treatment of PP2 or PP3 (20 μ M each). The phosphorylation of rPKC δ Tyr311 under different conditions was detected by immunoblotting. Reblotting of total rPKC δ and Hck blotting were used as loading controls. **D.** U937 cells treated with Hck-targeting siRNA or non-targeting control siRNA were lysed and immunoblotting was performed with anti-Hck Ab. Blotting of anti- β -actin was included as a loading control. **E.** Hck-targeting siRNA or control siRNA treated U937 cells were seeded into LPM19c or control IgG coated microtitre wells. The phosphorylation of PKC δ Tyr311 was assessed by immunoblotting. Total PKC δ and β -actin blots were used as loading controls. In **B-E**, protein bands of phospho-PKC δ Tyr311 and total PKC δ or Hck and β -actin were subjected to densitometry analyses. Data are the average of three independent experiments and shown as means $\pm$ SD. *1-5, $p=0.0004903815$, 0.0002703241 , 0.0005591204 , 0.0055756161 and 0.0025380899 , respectively (Student's t test). In E, the signals of phospho-PKC δ Tyr311 in the Ig groups are close to 0. α M, mAb LPM19c; Ig, control IgG.

3.1.4 Lyn interacts with PKC δ in U937 cells

Not only Hck, other SFKs such as Lyn and c-Yes, also associate with the cytoplasmic tail of $\beta 2$ integrins [155]. In U937 cells, Hck expression is dominant, while Lyn expression is modest and c-Yes expression is minimal (Figure 3.1.4 A). T cell line Jurkat was included in the blotting of c-Yes as a control [222]. It has also been shown that the promyelocyte HL60 cells lacked the mRNA of c-Yes [223]. Thus only Lyn was examined in the following experiments. U937 cells treated with anti- αM or control IgG were collected, and used for IP assays with anti-Lyn Ab (Figure 3.1.4 B). PKC δ associated with Lyn, and was phosphorylated at Tyr311 by Lyn under integrin $\alpha M\beta 2$ cross-linking. Low level of PKC δ Tyr311 phosphorylation might be due to the moderate level of Lyn expression. In cells treated with control IgG, PKC δ was not detected in Lyn IP sample. Lyn IPs from cells treated with anti- $\alpha M\beta 2$ or control IgG were also used in *in vitro* kinase assay with rPKC δ . The level of rPKC δ Tyr311 phosphorylation was higher in $\alpha M\beta 2$ cross-linked cells, as compared to control IgG treated cells (Figure 3.1.4 C). These results show that other than Hck, Lyn can also be involved in integrin $\alpha M\beta 2$ -induced PKC δ Tyr311 phosphorylation, although Hck has a predominant expression in U937 cells.

Figure 3.1.4 Lyn interacts with PKCδ upon integrin αMβ2 clustering.

A. The expression levels of Hck, Lyn and c-Yes were determined by immunoblotting with relevant Abs. β-actin blot was included as a loading control. **B.** U937 cells were seeded into microtitre wells coated with anti-αM mAb LPM19c or control IgG. All cells (bound and unbound) were harvested and used for IP with anti-Lyn or control IgG (Ig). IPs immunoblotted with anti-phospho-PKCδTyr311 Ab and reblotted with anti-PKCδ Ab to detect total PKCδ detection. **C.** Lyn IPs as in **B** were used in *in vitro* kinase assay with 100 ng of rPKCδ. The phosphorylation of rPKCδTyr311 was detected by immunoblotting. Total rPKCδ reblot serves as the loading control. Densitometry analyses of phospho-rPKCδTyr311 and total rPKCδ were performed. Data are the average of three independent experiments and shown as means±SD. *p=0.0003616023 (Student's *t* test).

3.1.5 Effect of Syk on PKC δ phosphorylation

It was reported that the ITAM of DAP12 and the SH2 domains of Syk are involved in $\beta 2$ integrins-induced signal transduction in macrophages from mouse knockout models [224]. As a tyrosine kinase, Syk also plays important role in integrin clustering-induced signal transduction in hematopoietic cells [162]. However, our results showed that 20 μ M of PIC (Syk inhibitor) could not inhibit the phosphorylation of PKC δ Tyr311 induced by α M β 2 cross-linking (Figure 3.1.1 F). In order to verify the efficacy of the PIC treatment, we also determined the phosphorylation of Erk1/2 which is induced by $\beta 2$ integrin cross-linking [225] and is dependent on Syk [224]. The phosphorylation of PKC δ Tyr311 induced by clustering of integrin α M β 2 was not affected by the treatment of PIC (from 20 to 100 μ M) in U937 cells (Figure 3.1.5 A). By contrast, the phosphorylation of Erk1/2 was inhibited by PIC treatment that is concentration-dependent. We also used Syk-targeting siRNA to treat U937 cells and a significant reduction of endogenous Syk expression was observed as compared with non-targeting control siRNA treated cells (Figure 3.1.5 B). U937 cells treated with Syk-targeting siRNA or non-targeting control siRNA were used for integrin α M β 2 cross-linking and similar extent of PKC δ Tyr311 phosphorylation was detected in these two groups (Figure 3.1.5 C). These results show that Syk is not involved in PKC δ Tyr311 phosphorylation induced by integrin α M β 2 clustering. But, it is possible that Syk may phosphorylate other tyrosine sites in PKC δ . In fact, it has been shown that thrombin-induced Syk activation is PKC δ -dependent in endothelial cells [226].

Figure 3.1.5 Effect of Syk on PKCδTyr311 phosphorylation in U937 cells.

A. U937 cells were pre-treated with PIC at different concentrations for 1 hour, followed by cross-linking with anti αM mAb LPM19c or control IgG. The phosphorylations of Erk1/2 and PKCδTyr311 were detected with relevant Abs. Reblotting of total Erk1/2, reblotting of total PKCδ and blotting of β-actin were used for loading controls. **B.** U937 cells were treated with non-targeting control siRNA or Syk-targeting siRNA, and the expression of endogenous Syk in these cells was detected by immunoblotting using anti-Syk Ab. β-actin blot serves as a loading control. **C.** U937 cells treated with non-targeting control siRNA or Syk-targeting siRNA were used to perform cross-linking study with anti αM mAb LPM19c or control IgG. The phosphorylation of PKCδTyr311 was determined by immunoblotting with anti-phospho-PKCδTyr311 Ab. Reblotting of total PKCδ and blotting of β-actin were used for loading controls. In **B** and **C**, relative units of Syk and β-actin, or phospho-PKCδTyr311 and total PKCδ were measured and the average of three

independent experiments are expressed as means $\pm$ SD. *1-3, $p=0.0072520811$, 0.0005733252 and 0.0045333312 , respectively (Student's t test). α M, mAb LPM19c; Ig, control IgG; PIC, piceatannol.

3.1.6 Integrin β 2 cytoplasmic tail is required for PKC δ Tyr311 phosphorylation

Upon integrin ligand binding, the integrin cytoplasmic tails play important roles in integrin outside-in signaling [227]. It was also reported that SFKs such as Hck, Lyn and c-Yes associate with the β 2 integrin cytoplasmic tail [155]. Thus we mapped the region of β 2 cytoplasmic tail which is required for PKC δ Tyr311 phosphorylation. The myeloid cell line K562 expresses endogenous PKC δ but does not express β 2 integrins. Stable K562/ α M β 2 cells are K562 cells stably transfected with integrin α M β 2 (Figure 3.1.6 A). Both of these two cell lines were used for cross-linking studies. Cross-linking of integrin α M β 2 induces the phosphorylation of PKC δ Tyr311 in stable K562/ α M β 2 cells, but not in K562 cells (Figure 3.1.6 B). The time course study showed that the phosphorylation of PKC δ Tyr311 is time-dependent (Figure 3.1.6 C), and it is abolished by the inhibitor of SFK (Figure 3.1.6 D). These data are consistent with those of U937 cells in Figure 3.1.1. A series of β 2 truncated mutants were made (Figure 3.1.6 E) and co-transfected with WT α M into empty K562 cells (Figure 3.1.6 F). The transfectants were used to perform cross-linking studies by anti- α M β 2 or control IgG, and the phosphorylation of PKC δ pTyr311 was detected by immunoblotting (Figure 3.1.6 G). Our data showed that the region Asn727-Ser734 of β 2 cytoplasmic tail is required for the phosphorylation of PKC δ Tyr311 induced by integrin α M β 2. This region was also reported to be involved in the co-localization of SFK Hck and integrin α M β 2 [207].

Figure 3.1.6 Defining the region of integrin $\beta 2$ cytoplasmic tail required for PKC δ Tyr311 phosphorylation.

A. Integrin $\alpha M\beta 2$ expression in empty K562 cells and K562/ $\alpha M\beta 2$ stable cells was determined by flow cytometry analyses using anti- αM mAb LPM19c or irrelevant mAb

mouse IgG. Gray and black histograms represent staining with anti- α M and background staining with irrelevant mAb, respectively. **B.** Empty K562 cells and K562/ α M β 2 stable cells were seeded into microtitre wells coated with anti- α L, anti- α M, or control IgG as described previously. All cells (bound and unbound) were harvested and the lysates immunoblotted with anti-phospho-PKC δ Tyr311 Ab. **C.** At different time points (15-60 min), the phosphorylation of PKC δ Tyr311 in K562/ α M β 2 cells was examined. **D.** K562/ α M β 2 cells were pre-treated with the indicated inhibitor (20 μ M each) for 1 hour, and were subsequently seeded into anti- α M or control Ig coated wells and incubated for 30 min. The phosphorylation of PKC δ Tyr311 was detected using anti-phospho-PKC δ Tyr311 Ab. **E.** A panel of β 2 truncated mutants with indicated deletions was used. The residue in shaded box from α M cytoplasmic tail and that in the unshaded box from β 2 tail form a salt bridge. A dotted line is drawn to represent the boundary between the juxtamembrane residues and the intracellular residues of the TM domain [228]. **F.** K562 cells were co-transfected with α M and β 2 or β 2 truncated mutants. Anti- α M staining of K562 transfectants was determined by flow cytometry. Black and gray histograms represent background staining with irrelevant Ab mouse IgG and specific staining with anti- α M mAb LPM19c. **G.** As in **F**, the K562 transfectants were seeded into immobilized mAb control IgG (–) or LPM19c (+) coated microtitre wells and incubated for 30 min at 37°C. Phospho-PKC δ Tyr311 immunoblotting was performed. In **B**, **C**, **D** and **G**, total PKC δ was detected by reblotting with anti-PKC δ Ab. β -actin blots serve as a loading control. In **C** and **D**, densitometry analyses of protein bands (phospho-PKC δ Tyr311 and total PKC δ) from three independent experiments are performed. Means $\pm$ SD are shown. *1-3, $p=0.0014817081$, 0.0005449543 and 0.0003306961 , respectively (Student's t test).

3.1.7 Integrin α M β 2 ligand binding induces PKC δ phosphorylation

We also investigated ligand binding-induced α M β 2 signaling in the induction of PKC δ Tyr311 phosphorylation. We first examined the phosphorylation of PKC δ Tyr311 in U937 cells that were seeded onto immobilized integrin α M β 2 ligand ICAM-1 [21]. The phosphorylation of PKC δ Tyr311 was detected in U937 cells that adhered to ICAM-1 in the presence of the β 2 integrin activating mAb KIM185 [201] (Figure 3.1.7 A). The mAb KIM185 not only activates integrin α M β 2, but may cross-link α M β 2 leading to downstream signaling. An intrinsically activated integrin α M β 2D709R was generated by introducing a salt-bridge mutation (Figure 3.1.7 B) [208]. K562 cells were then co-transfected with α M and β 2 or β 2D709R and integrin expression on cell surface was determined by flow cytometry (Figure 3.1.7 C). KIM127 is a reporter mAb and it binds to extended β 2 by recognizing an epitope in the I-EGF 2-fold [229]. mAb KIM127 was used for IP assays of K562 cells transfected with integrin α M β 2 or α M β 2D709R and the IPs were immunoblotted with anti- α M Ab to detect integrin α M subunit which associates with β 2 subunit (Figure 3.1.7 D). Anti- α M mAb LPM19c was also used for IP as a positive control. We used MnCl₂, not mAb KIM185 as the activating agent for integrin activation, because using mAb KIM185 would interfere with the procedure of immunoprecipitation. In WT integrin α M β 2 transfected K562 cells treated with MnCl₂, α M protein was immunoprecipitated by the mAb KIM127. In constitutively activated integrin α M β 2D709R transfected K562 cells, α M protein band was detected with or without treatment of MnCl₂. These results verify the extended conformation of integrin α M β 2D709R. To further confirm the constitutive activity of integrin α M β 2D709R, both K562 transfectants expressing integrin α M β 2 or α M β 2D709R were used for cell adhesion assays on the ligand ICAM-1 (Figure 3.1.7 E). Consistent with the result in Figure 3.1.7 D, the activated integrin α M β 2D709R transfected K562 cells adhered constitutively to the ligand ICAM-1, while the WT integrin α M β 2 transfected K562 cells required the activating mAb KIM185. Anti- α M mAb LPM19c was used as a functional blocking mAb to show binding specificity [230].

We then detected the phosphorylation of PKC δ Tyr311 in K562 cells transfected with WT integrin α M β 2 (resting) or active integrin α M β 2D709R (constitutively activated) following seeding of cells into microtitre cells without or with coated ICAM-1 (Figure 3.1.7 F). Without ICAM-1, PKC δ Tyr311 was not phosphorylated in both transfectants even with the activating mAb KIM185. With coating of ICAM-1, PKC δ Tyr311 was phosphorylated in α M β 2D709R transfected K562 cells and in α M β 2 transfected K562 cells only in presence of KIM185 (Figure 3.1.7 F, right panel). These results show that the process of clustering is required for integrin α M β 2-induced signaling leading to the activation of PKC δ .

Figure 3.1.7 PKC δ Tyr311 phosphorylation induced by integrin α M β 2 clustering.

A. U937 cells were seeded into microtitre wells coated with ICAM-1 and incubated for 30 min at 37°C. All cells (bound and unbound) were collected and lysed. PKC δ Tyr311 phosphorylation was detected using anti-phospho-PKC δ Tyr311 Ab. 10 μ g/ml of KIM185 was used as activating mAb for cell adhesion. **B.** WT integrin α M β 2 (left) and constitutively activated integrin α M β 2D709R (right) are illustrated. The cytoplasmic residues α M Arg1120 and β 2 Asp709 form a salt bridge, while the mutation D709R disrupts the salt bridge leading to integrin α M β 2 activation. **C.** The transfection efficiency of K562 transfectants with integrin α M β 2 or α M β 2D709R was determined by flow cytometry using anti- α M mAb LPM19c (gray histogram) and irrelevant mAb mouse

IgG (black histogram). **D.** K562 transfectants expressing integrin $\alpha M\beta 2$ or $\alpha M\beta 2D709R$ were lysed and subjected to IP with mAb KIM127. The mAb LPM19c which binds to αM subunit was used as an Ab control. 0.5 mM of $MnCl_2$ was used as an activating reagent instead of mAb KIM185. **E.** Adhesion analyses were performed on immobilized ICAM-1. The activating mAb KIM185 was included for the activation of WT integrin $\alpha M\beta 2$. 10 $\mu g/ml$ of LPM19c was used as blocking mAb to demonstrate binding specificity mediated by integrin $\alpha M\beta 2$ or $\alpha M\beta 2D709R$. **F.** K562 transfectants with or without the addition of KIM185 were seeded into ICAM-1 coated or non-coated microtitre wells for 30 min at 37°C. The phosphorylation of PKC δ Tyr311 in these cells was detected by immunoblotting. In **A** and **F**, reblotting of total PKC δ and blotting of β -actin were performed as loading controls. In **F**, densitometry analyses of phospho-PKC δ Tyr311 and total PKC δ bands were performed and plotted as relative units (phospho-PKC δ Tyr311 intensity / total PKC δ intensity). Means $\pm$ SD from three independent experiments are shown. *1-*2, $p=0.0006975406$ and 0.0000184517 , respectively (Student's *t* test).

3.1.8 PKC δ is involved in the down-regulation of Foxp1 by integrin α M β 2 in U937 cells

In human promyelocyte HL60 cells, Foxp1 expression is down-regulated with PMA treatment [144]. In integrin α M β 2-crosslinked monocytes, a reduction of Foxp1 expression has been reported, but the detailed signaling pathway remains unknown [169]. Consistent with these observations, we found that PMA treatment of U937 cells induced cell differentiation and down-regulated the expression of Foxp1 after 4 hours (Figure 3.1.8 A). We hypothesize that PKC δ may play a role in the down-regulation of Foxp1 expression upon integrin α M β 2 clustering. To this end, we used siRNA method to reduce PKC δ expression in U937 cells. The expression of PKC δ was significantly reduced in cells treated with PKC δ -targeting siRNA compared with cells treated with non-targeting control siRNA (Figure 3.1.8 B). The reduction in PKC δ expression was specific as other PKC members PKC α and PKC ϵ were not affected. In non-targeting siRNA-treated U937 cells, integrin α M β 2 cross-linking induced down-regulation of Foxp1 expression compared with control IgG cross-linked cells, while in PKC δ -targeting siRNA-treated cells, integrin α M β 2 cross-linking failed to reduce Foxp1 expression (Figure 3.1.8 C). These results show that integrin α M β 2 clustering-induced Foxp1 down-regulation is PKC δ -dependent.

Figure 3.1.8 PKC δ is involved in the down-regulation of Foxp1 by integrin α M β 2 in U937 cells.

A. At different time points (0-8 hours (h)) of PMA treatment (100 ng/ml), U937 cells were harvested and lysed. The whole-cell lysates were used for immunoblotting with anti-Foxp1. **B.** U937 cells treated with PKC δ -targeting siRNA or non-targeting control siRNA were lysed and immunoblotting with anti-PKC δ Ab was performed. Blots of PKC α and PKC ϵ were also included as loading controls. **C.** Control siRNA or PKC δ -targeting siRNA treated cells were used to perform cross-linking studies with anti- α M mAb LPM19c or control IgG for 8 hours (h) at 37°C. All cells (bound and unbound) were lysed and immunoblotting was performed with anti-Foxp1 Ab. In **A-C**, β -actin blots were used as a loading control. All densitometry analyses are the average of three independent experiments and plotted as relative units (PKC δ intensity/ β -actin intensity or Foxp1 intensity/ β -actin intensity). Data shown are means $\pm$ SD. *1-3, $p=0.0006660904$, 0.0070798452 and 0.0009782794 , respectively (Student's t test).

3.1.9 Integrin α M β 2 clustering induces PKC δ Tyr311 phosphorylation and Foxp1 down-regulation in human monocytes

We also extended our study to human peripheral blood monocytes. We investigated the role of PKC δ in integrin α M β 2-induced Foxp1 down-regulation in human primary monocytes. In line with the observations in U937 cells, integrin α M β 2 cross-linking specifically induced the phosphorylation of PKC δ Tyr311 in human monocytes (Figure 3.1.9 A). PKC δ membrane translocation was observed in cells cross-linked by anti- α M β 2 mAb, but not by anti- α L β 2 mAb or control IgG (Figure 3.1.9 B). Next, PKC δ expression was reduced in cells treated with PKC δ -targeting siRNA compared with those treated with non-targeting control siRNA (Figure 3.1.9 C). PKC δ -targeting siRNA is specific because PKC α and PKC ϵ expression levels were unaffected. In control siRNA treated human monocytes, Foxp1 expression was significantly reduced by cross-linking of integrin α M β 2 (Figure 3.1.9 D). In cells treated with PKC δ -targeting siRNA, the reduction of Foxp1 expression was not detected upon integrin α M β 2 clustering.

Studies have shown that integrin α M β 2 clustering modulates monocyte differentiation by regulating Foxp1 expression [144, 145]. Monocytes adhesion to the endothelium could play an important role for their differentiation. In order to address this, human peripheral blood monocytes labeled with CellTracker Green were plated onto HMVE cells that express ICAM-1 (Figure 3.1.9 E) or empty culture dish. Monocytes that were detached from HMVE cells and those from empty culture dish were lysed to assess the expression of Foxp1 by immunoblotting (Figure 3.1.9 F, left panel). A reduction of Foxp1 expression was observed in monocytes plated onto HMVE cells compared with those seeded into empty culture dish. We also treated monocytes with PKC δ -targeting siRNA or non-targeting control siRNA (Figure 3.1.9 F, right panel). Foxp1 expression was not reduced in PKC δ siRNA-treated monocytes compared with control siRNA-treated monocytes even though both groups were seeded onto HMVE. These data provide further evidence that PKC δ plays a role in integrin α M β 2-regulated Foxp1 expression.

Figure 3.1.9 Integrin α M β 2 clustering induces PKC δ Tyr311 phosphorylation and Foxp1 down-regulation in human monocytes.

A. Integrin crosslinking was performed on monocytes using anti- α M, anti- α L, or control IgG. Whole-cell lysates were used to detect PKC δ Tyr311 phosphorylation by immunoblotting. Reblotting of total PKC δ was performed as the loading control. **B.** Immunofluorescence staining of PKC δ was performed in monocytes following anti-integrin mAb or control IgG treatment. Magnification is 40 $\times$. Bar is 10 μ m. **C.** Monocytes were treated with PKC δ -targeting siRNA or control siRNA. PKC immunoblots were performed. **D.** Monocytes treated with PKC δ -targeting siRNA or non-targeting control siRNA were used in cross-linking studies with anti- α M mAb

LPM19c or control IgG for 8 hours. All cells (bound and unbound) were collected and lysed for immunoblotting of Foxp1. **E.** CellTracker Green-labeled monocytes were plated onto HMVE cells and still images of phase contrast and fluorescence were collected. Magnification is 20 $\times$. Bar is 50 μ m. **F.** In the left panel, non-treated monocytes were allowed to adhere on HMVE cells or empty culture dish for 8 hours. In the right panel, monocytes treated with PKC δ -targeting siRNA or control siRNA were plated onto HMVE cells for 8 hours. Monocytes were then detached from the mixture with HMVE using anti-CD14 magnetic beads. The expression of Foxp1 was assessed in these monocytes by immunoblotting with anti-Foxp1 Ab. In **A**, **C**, **D** and **F**, β -actin blots serve as a loading control. All densitometry analyses of protein bands (phospho-PKC δ Tyr311/total PKC δ , PKC δ / β -actin, or Foxp1/ β -actin) are the average from three independent experiments and plotted as means $\pm$ SD. *1-*5, $p=0.0004209209$, 0.0033038260 , 0.0003989987 , 0.0006070153 and 0.0015547844 , respectively (Student's t test). Ig, control IgG.

3.1.10 Cross-linking of $\beta 1$ or $\beta 5$ integrin does not induce PKC δ Tyr311 phosphorylation

Other than $\beta 2$ integrins, we also tested cross-linking of $\beta 1$ or $\beta 5$ integrin on U937 cells (Figure 3.1.10 A) and human peripheral blood monocytes (Figure 3.1.10 B). There was no induction of PKC δ Tyr311 phosphorylation in these cells cross-linked by $\beta 1$ or $\beta 5$ integrin.

Figure 3.1.10 Cross-linking of $\beta 1$ or $\beta 5$ integrin in U937 cells and monocytes.

U937 cells (**A**) and monocytes (**B**) were subjected to integrin cross-linking in microtitre wells coated with the indicated mAbs. All cells (bound or unbound) were harvested and lysed. Whole-cell lysates were used for immunoblotting with anti-phospho-PKC δ Tyr311. Reblotting of total PKC δ and blotting of β -actin blot were used as loading controls. Ig, control IgG. Representative blots of three independent experiments are shown.

3.2 Integrin α M β 2 signaling cross-talks with LPS-induced TLR4 signaling through the interaction of IRAK1 and PKC δ

3.2.1 LPS induces the activation of p38 and JNK in THP1 cells

LPS is the most studied immunostimulatory component from bacteria and it induces systemic inflammation [231]. TLR4 selectively recognizes LPS and triggers the expression of a number of cytokines and kinases which are necessary for the activation of immune responses [232, 233]. In this study, we examined the molecules involved in TLR4-mediated signaling, such as IRAK1, MAPKs and the influence of integrin α M β 2 signaling on them.

First, we examined the MAPKs pathway in LPS-induced TLR4 activation in human monocytic THP1 cells. We found that LPS treatment induced the activation of p38 and JNK (Figure 3.2.1 A & B), but had minimal effect on Erk compared with untreated group (Figure 3.2.1 C). PKC δ Tyr311 phosphorylation was also not observed in LPS-treated THP1 cells (Figure 3.2.1 D). PMA was included as a positive control. We found that PMA treatment of THP1 cell induced the phosphorylation of p38, Erk and PKC δ Tyr311 (Figure 3.2.1 A, C & D). Surprisingly, PMA did not induce the activation of JNK in THP1 cells (Figure 3.2.1 B).

The myeloid K562 cells expressing integrin α M β 2 (K562/ α M β 2) were also used for these experiments, but none of the four kinases were phosphorylated by LPS treatment. It might be because K562 cells have no TLR4 expression on cell surface. Another human monocytic cell line U937 (in result section **3.1**) was not used for these experiments, because there is minimal expression of JNK in U937 cells as compared to that of THP1 cells (data not shown). Thus, THP1 cells will be used for the following studies.

Figure 3.2.1 LPS induces the activation of p38 and JNK in THP1 cells.

K562/αMβ2 and THP1 cells were treated without or with LPS (100 ng/ml) or PMA (100 ng/ml) at 37°C for 30 min in a humidified 5% CO₂ cell culture incubator. Cells were harvested and lysed with lysis buffer. Whole cell lysates were resolved on 10% SDS-PAGE gel under reducing conditions and immunoblotting was performed to detect the phosphorylation of p38 (**A**), JNK1/2 (**B**), Erk1/2 (**C**) and PKCδTyr311 (**D**). The membranes were stripped and reblotted to detect total p38, JNK1/2, Erk1/2 and PKCδ. One set of blots from three independent experiments is shown.

3.2.2 Integrin α M β 2 clustering induces the phosphorylation of PKC δ Tyr311 and Erk in THP1 and K562/ α M β 2 cells.

In our previous study, we found that integrin α M β 2 clustering induces the phosphorylation of PKC δ Tyr311 (Figure 3.1.1) and Erk1/2 (Figure 3.1.5) in U937 cells. To further confirm these results, we also examined these two kinases in THP1 cells that were subjected to cross-linking of integrin α M β 2. Consistent with the results in U937 cells, clustering of integrin α M β 2 induced PKC δ Tyr311 (Figure 3.2.2 D) and Erk1/2 phosphorylation (Figure 3.2.2 C) in THP1 cells. Similar results were also obtained in K562/ α M β 2 cells. However, there was no significant difference of p38 (Figure 3.2.2 A) and JNK (Figure 3.2.2 B) phosphorylation between LPM19c-treated cells and control IgG-treated cells.

Our results show that integrin α M β 2 clustering triggers the activation of Erk1/2 and PKC δ (Figure 3.2.2), whereas LPS induces the activation of p38 and JNK1/2 (Figure 3.2.1).

Figure 3.2.2 Integrin $\alpha M\beta 2$ clustering induces PKC δ Tyr311 and Erk1/2 phosphorylation.

K562/ $\alpha M\beta 2$ and THP1 cells were used to perform cross-linking studies with mAb LPM19c or mouse control IgG at 37°C for 30 min in a humidified 5% CO₂ cell culture incubator as described previously [57]. All cells (bound and unbound) were lysed in lysis buffer. Whole cell lysates were resolved on 10% SDS-PAGE gel under reducing conditions for immunoblotting to detect phosphorylation of p38 (**A**), JNK1/2 (**B**), Erk1/2 (**C**) and PKC δ Tyr311 (**D**). The membranes were stripped and reblotted to detect total p38, JNK1/2, Erk1/2 and PKC δ . Ig and αM indicate control IgG and anti- αM mAb LPM19c. One set of blots from three independent experiments is shown.

3.2.3 IRAK1 interacts with PKC δ

Upon LPS stimulation, MyD88 recruits and activates IRAK-4, which is a death domain-containing kinase [234]. The activity of IRAK-4 has been reported to be important for transmitting TLR signals, including the expression of proinflammatory cytokines [235-239]. IRAK1 is known to be phosphorylated by IRAK4 and involved in mediating signals downstream of IRAK4 [240, 241]. The knockout of IRAK1 induces partial defect in the expression of proinflammatory cytokines after LPS stimulation [242]. There is a report that suggests a role of PKC δ in the production of IL-1 β in monocytes [243], but the detailed interaction of PKC δ and IRAK1 is unclear.

Our previous study has shown that integrin α M β 2 clustering induces the phosphorylation and activation of PKC δ . To ascertain the molecules that are involved in the cross-talk between integrin α M β 2 signaling and LPS-induced signaling, we performed IP studies using THP1 and K562/ α M β 2 cells. Our results showed that IRAK1 interacts with PKC δ in non-treated cells (Figure 3.2.3 A). We used LPS or PMA to treat THP1 cells and conducted IP using anti-PKC δ Ab. We found that IRAK1 associates with PKC δ regardless of LPS, PMA, or without treatment (Figure 3.2.3 B). The phosphorylation of IRAK1 was detected in PKC δ IPs (Figure 3.2.3 B), suggesting that either phosphorylated IRAK1 interacts with PKC δ or IRAK1 was phosphorylated by its interaction with PKC δ .

We also conducted integrin α M β 2 cross-linking studies to address the interaction between PKC δ and IRAK1. In both mAb LPM19c and control IgG treated THP1 cells, interaction of PKC δ with IRAK1 was detected (Figure 3.2.3 C). In both groups, comparable levels of IRAK1 phosphorylation were also detected (Figure 3.2.3 C).

Taken together, these data show that the interaction of IRAK1 and PKC δ stably occurs in THP1 cells.

Figure 3.2.3 Interaction of IRAK1 with PKCδ.

A. Non-treated K562/αMβ2 and THP1 cells were harvested and lysed to perform IP with either anti-PKCδ Ab C20 or irrelevant Ab anti-HA. Whole cell lysates and IPs were used to perform immunoblotting with anti-PKCδ Ab C20 and anti-IRAK1 Ab. **B.** THP1 cells were treated with LPS (100 ng/ml) or PMA (100 ng/ml) or without treatment at 37°C for 30 min in a humidified 5% CO₂ cell culture incubator. Cells were harvested and lysed to perform IP with anti-PKCδ Ab C20 or irrelevant Ab anti-HA. The IPs were used to perform immunoblotting with anti-PKCδ Ab C20 and anti-phospho-IRAK1T209 Ab. Reblotting of IRAK1 was also performed with anti-IRAK1 Ab. **C.** THP1 cells were used to perform cross-linking studies with mAb LPM19c or mouse control IgG at 37°C for 30 min in a humidified 5% CO₂ cell culture incubator as described previously [57]. All cells (bound and unbound) were lysed and subjected to IP with anti-PKCδ Ab C20 or irrelevant Ab anti-HA. The IPs were resolved on SDS-PAGE and immunoblotting was performed to detect PKCδ and phospho-IRAK1T209. The membrane was stripped and reblotted to detect IRAK1. Ig and αM indicate control IgG and anti-αM mAb LPM19c. One set of blots from two independent experiments is shown.

3.2.4 Phosphorylation of IRAK1 by PKC δ

In LPS-induced TLR4 activation, MyD88 recruits and activates IRAK4 which phosphorylates IRAK1 [240, 241]. PKC δ has also been reported to be involved in TLR/IRAK1-induced production of IL-1 β [244]. Our previous results have shown that IRAK1 interacts with PKC δ . To further investigate the phosphorylation of IRAK1, IRAK1-GFP was co-transfected with WT PKC δ -EGFP or PKC δ KD-EGFP into human 293T cells. The transfectants were used to perform IP studies with anti-PKC δ Ab. Our results show that both WT PKC δ and PKC δ KD interacted with IRAK1, but the phosphorylation of IRAK1 was only detected in the transfectants of WT PKC δ (Figure 3.2.4 A). It suggests that the kinase domain of PKC δ is important to induced IRAK1 phosphorylation. It should be noted that the total expression of PKC δ KD was much lower compared with PKC δ in 293T transfectants, but the immunoprecipitate of PKC δ KD is sufficient to pull-down IRAK1.

To examine the role of PKC δ in IRAK1 phosphorylation, a PKC δ -specific inhibitor (Rottlerin) was added into the 293T transfectants with IRAK1-GFP and PKC δ -EGFP before performing IP assays. It is surprising that inhibition of PKC δ did not affect its interaction with IRAK1, and no difference was observed on the level of IRAK1 phosphorylation in both groups (Figure 3.2.4 B).

In summary, our data show that IRAK1 is phosphorylated by PKC δ , and the kinase part of PKC δ is necessary for inducing IRAK1 phosphorylation.

Figure 3.2.4 Phosphorylation of IRAK1 by PKC δ .

A. Human 293T cells were co-transfected with IRAK1-GFP and PKC δ -EGFP or PKC δ KD-EGFP. After 24 hrs incubation at 37°C in a humidified 5% CO₂ incubator, 293T transfectants were lysed and subjected to IP with anti-PKC δ mAb or irrelevant mAb mouse control IgG. **B.** Human 293T cells were co-transfected with IRAK1-GFP and PKC δ -EGFP. After 24 hrs incubation at 37°C in a humidified 5% CO₂ incubator, 293T transfectants were pre-incubated with or without Rottlerin (10 μ M) for 30 min. Cells were harvested and performed IP assay with anti-PKC δ mAb or irrelevant mAb mouse control IgG. The IPs in **(A)** and **(B)** were used for immunoblotting to detect PKC δ , phospho-IRAK1T209 and IRAK1. Ig and α M indicate control IgG and anti- α M mAb LPM19c. One set of blots from two independent experiments is shown.

3.2.5 MEK-Erk is involved in integrin α M β 2 outside-in signaling

In our previous study, we examined the early signaling events of integrin α M β 2 outside-in signaling pathway [57]. We found that clustering of integrin α M β 2 induced PKC δ phosphorylation and activation in a SFK-dependent manner. We also showed that integrin α M β 2 clustering down-regulated the expression of the transcription factor Foxp1 which is important for monocytes differentiation. However, there is a gap between PKC δ signaling and Foxp1 expression. To address how PKC δ induced the down-regulation of Foxp1 expression in monocytes differentiation, we used a highly specific inhibitor of MEK1/2, PD98059, in our integrin α M β 2 cross-linking studies of THP1 cells. We found that PD98059 did not affect integrin α M β 2-induced PKC δ Tyr311 phosphorylation (Figure 3.2.5 A), but the phosphorylation of Erk was attenuated by PD98059 (Figure 3.2.5 B), suggesting MEK-Erk signaling is involved in integrin α M β 2 clustering and plays its role downstream of PKC δ .

We also detected the expression of Foxp1 in THP1 cells upon integrin α M β 2 cross-linking or PMA treatment. Our results showed that PD98059 pre-treatment blocked Foxp1 down-regulation induced by integrin α M β 2 clustering or PMA treatment (Figure 3.2.5 C). Collectively, we have shown that integrin α M β 2-induced down regulation of Foxp1 expression in monocytes involves the SFKs-PKC δ -MEK/Erk signaling axis.

Figure 3.2.5 MEK1/2 is involved in integrin α M β 2 outside-in signaling.

THP1 cells were treated with or without PD98059 (20 μ M) for 1 hr at 37°C, followed by cross-linking with mAb LPM19c for 30 min at 37°C in a humidified 5% CO₂ incubator. All cells (bound and unbound) were lysed and subjected to immunoblotting to detect phosphorylation of PKC δ (**A**) and Erk1/2 (**B**). Total PKC δ and Erk1/2 serve as loading controls. Representative blots of two independent experiments are shown. **C**. THP1 cells treated with or without PD98059 (20 μ M) were used to perform cross-linking with LPM19c (anti- α M Ab) or control IgG (left). THP1 cells were pre-incubated with or without PD98059 (20 μ M) for 1hr, then were treated with or without addition PMA (100 ng/ml) for 30 min (right). All cells were lysed and immunoblotting was performed with anti-Foxp1 Ab. β -actin blotting serves as a loading control. α M indicates anti- α M mAb LPM19c.

3.3 Kindlin3 is involved in integrin α M β 2 outside-in signaling and the Syk-Vav1-Rac1/Cdc42 signaling axis

3.3.1 Screening of stable K562/ α M β 2 cells with reduced kindlin3 expression

To address the role of kindlin3, we made use of a stable cell line K562/ α M β 2 expressing high-level of integrin α M β 2 and kindlin3 [57, 212]. These cells are referred to as KM cells henceforth in this study. Cells were transduced with pseudo-lenti-virions containing GFP-plasmid that encodes kindlin3 siRNA (k3 siRNA) or non-targeting scramble siRNA (con siRNA) using the iLenti™ siRNA Expression System, and they will be referred to as k3-KM and ctrl-KM cells, respectively. There was a significant reduction of kindlin3 mRNA in k3-KM cells compared with that in ctrl-KM cells as determined by RT-qPCR (Figure 3.3.1 A). The protein expression level of kindlin3 in these cells was determined by immunoblotting (Figure 3.3.1 B). Kindlin3 was detected in ctrl-KM cells, while reduced kindlin3 expression was detected in k3-KM cells. It has been reported that talin and kindlin bind to integrin β cytoplasmic tail at different sites, and they have synergistic effect on integrin activation [49]. The expression levels of talin, PKC δ , α M, and Syk were similar in k3-KM and ctrl-KM cells (Figure 3.3.1 B). β -actin was included as a loading control. The expression levels of integrin α M β 2 on the surface of k3-KM and ctrl-KM cells were comparable as determined by flow cytometry using anti- α M mAb LPM19c and APC conjugated anti-mouse Ab (FL4) (Figure 3.3.1 C). To verify the capacity of integrin α M β 2 activation, we also performed the staining using the reporter mAb KIM127 which recognizes extended and activated β 2 integrin (Figure 3.3.1 D, data provided by Asso. Prof. TAN Suet Mien). With the addition of Mn^{2+} , k3-KM and ctrl-KM cells showed comparable levels of mAb KIM127 staining. Hence the potential of integrin α M β 2 to be activated was not affected by the reduction of kindlin3 expression.

Figure 3.3.1 Kindlin3 knockdown in stable K562/ α M β 2 cells.

A. Total RNA was extracted from ctrl-KM and k3-KM cells. Real-time RT-qPCR was performed for kindlin3 gene and β -actin was a inner control. The relative expression percentage (kindlin3/ β -actin) was plotted and it was set as 100% in ctrl-KM cells. **B.** Ctrl-KM and k3-KM cells were lysed and resolved in SDS-PAGE gel. Whole-cell lysates were immunoblotted with anti-kindlin3 Ab. The expression levels of talin, PKC δ , α M, and Syk were also determined in k3-KM and ctrl-KM cells by

immunoblotting with relevant Abs. Blotting of β -actin serves as a loading control. **C.** The expression of integrin α M β 2 on the cell surface was determined by flow cytometry analyses with staining of anti- α M mAb LPM19c and APC conjugated anti-mouse Ab (open histograms). Control IgG was used for background staining (shaded histograms). **D.** Ctrl-KM and k3-KM cells treated with or without 1 mM of Mn^{2+} were performed staining in the presence of mAb KIM127 at 37°C. Control IgG staining was performed as background and LPM19c staining was also included to detect α M expression. %GP is the percentage of gated positive cells, GM is geo-mean, and EI is expression index. One representative experiment out of two independent experiments is shown.

3.3.2 Kindlin3 plays a role in integrin α M β 2-mediated cell adhesion

In order to address the role of kindlin3 in cell adhesion, we performed cell adhesion assays on the ligands of integrin α M β 2, such as iC3b and BSA. Briefly, BCECF dye-labeled cells were seeded into BSA or iC3b coated wells, and incubated for 30 min at 37°C. Wells were washed and bound cells were determined by a fluorescent plate reader (as described in section **2.3.7** Cell adhesion assays).

Ctrl-KM and k3-KM cells were used to test their capacities to adhere to the ligand of integrin α M β 2, iC3b (Figure 3.3.2 A). Both ctrl-KM and k3-KM cells adhered poorly to iC3b in the absence of activating mAb KIM185. In the presence of mAb KIM185, ctrl-KM cells adhered to iC3b, while the adhesion level of k3-KM cells on iC3b was significantly reduced compared with that in ctrl-KM cells. The function-blocking mAb LPM19c (anti- α M) was included to demonstrate adhesion specificity mediated by integrin α M β 2. The denatured protein BSA is also a ligand for integrin α M β 2 [119, 245]. We also performed adhesion assays on immobilized BSA and similar adhesion profiles to that on iC3b were obtained (Figure 3.3.2 B). These data suggest that kindlin3 is important for integrin α M β 2-mediated cell adhesion onto ligands iC3b and BSA.

Figure 3.3.2 Kindlin3 plays a role in integrin $\alpha M\beta 2$ -mediated cell adhesion.

Ctrl-KM and k3-KM cells were plated onto microtitre wells coated with αM specific ligands iC3b (**A**) and BSA (**B**). Adhesion assays were performed as described previously. Plots shown are average from three independent experiments and expressed as means $\pm$ SD. αM activating mAb KIM185 and function blocking mAb LPM19c were used at 10 μ g/ml each.

3.3.3 Kindlin3 is involved in integrin α M β 2-mediated cell spreading

To examine the extent of cell spreading, ECIS which measure changes in the electrical impedance relative to current flow was used. Briefly, the method measures current flow based on microelectrodes situated at the bottom of the wells in E-plates. When cell attaches and spreads onto the microelectrode, it will impede the current flow and will be registered as cell index by the electric cell-substrate impedance system. Therefore, the larger the area of cell spreading is, the higher the value of cell index will be registered. In these experiments, the wells were coated with either iC3b or BSA. Ctrl-KM and k3-KM cells were then seeded into these wells for 90 min to monitor cell spreading.

For impedance assays on immobilized iC3b and BSA, ctrl-KM (line in green) showed higher level of cell index as compared with k3-KM cells (line in red) in the presence of mAb KIM185 activation (Figure 3.3.3). As shown, both ctrl-KM and k3-KM cells could not effectively adhere and spread on the ligands without mAb KIM185 activation. Function blocking mAb LPM19c was included and significantly reduced the adhesion and spreading to levels similar to that without activation. These data show that k3-KM cells have poorer cell adhesion and spreading capacity compared with ctrl-KM cells, suggesting that kindlin3 is required for integrin α M β 2 outside-in signaling.

Figure 3.3.3 Kindlin3 is involved in integrin $\alpha M\beta 2$ -mediated cell spreading.

E-plates were coated with integrin $\alpha M\beta 2$ specific ligands iC3b (**A**) or BSA (**B**). Impedance studies were conducted as described in materials and methods. Conditions for treatment of cells are the same as in Figure 3.3.2 **A** and **B**. mAbs KIM185 and Lpm19c were used for activating or function blocking of integrin $\alpha M\beta 2$. Ctrl-KM cells with KIM185 (line in green) showed higher cell index than k3-KM cells with KIM185 (line in red). Each time point indicates the mean of technical triplicates, with standard deviations calculated for every 1 min interval. Three independent

experiments were performed and one representative is shown.

3.3.4 Microscopy visualization of K562/ α M β 2 cells with reduced kindlin3 expression

Based on the ECIS experiments, kindlin3 has been shown to be important for integrin α M β 2-mediated cell spreading. To visualize the degree of cell spreading, fluorescence microscopy imaging of ctrl-KM and k3-KM cells that were allowed to adhere and spread on glass bottom microwell dishes coated with either BSA or iC3b was performed. Cells were fixed and permeabilized before staining with Alexa Fluor 594 conjugated phalloidin and DAPI to stain for filamentous-actin (F-actin) and nucleus, respectively.

On integrin α M β 2 ligands BSA or iC3b, ctrl-KM cells showed pronounced micro-spikes or filopodia, while less projections were observed on k3-KM cells (Figure 3.3.4 A and B, data provided by Liu Weiling). Additionally, ctrl-KM cells showed a propensity to spread onto BSA or iC3b substrate, as compared with k3-KM cells. These data suggest that kindlin3 is important for integrin α M β 2-mediated cell spreading and actin cytoskeletal dynamics.

Figure 3.3.4 Microscopy analyses of ctrl-KM and k3-KM cells on integrin $\alpha\text{M}\beta 2$ ligands.

Ctrl-KM and k3-KM cells were plated on immobilized BSA (**A**) or iC3b (**B**). F-actin was stained with Alexa-Fluor 594-conjugated phalloidin (red) and nucleus was stained with DAPI (blue). Magnification is 63 $\times$. Bar is 5 μm .

3.3.5 Kindlin3 is important for cell adhesion under shear flow.

We have shown that kindlin3 is essential for integrin $\alpha\text{M}\beta 2$ -mediated cell adhesion and spreading. Integrin outside-in signaling regulates cytoskeletal remodelling that stabilizes integrin-ligand binding. We also examined the role of kindlin3 in cell adhesion under flow conditions. Shear flow assays were performed using parallel flow chamber slides that were coated with iC3b. Ctrl-KM or k3-KM cells with mAb KIM185 were infused into the chamber at 0.4, 0.6 and 0.8 dyn/cm². The number of adhered cells in four different fields (1 mm vs 1 mm) was counted by visualization under a microscope using a 10 $\times$ objective lens. Higher number of Ctrl-KM and k3-KM cells adhered at lower shear force compared with that at higher shear force (Figure 3.3.5). However, ctrl-KM cells adhered better than k3-KM cells. These data show that kindlin3 is important for cell adhesion under shear flow condition.

Figure 3.3.5 Shear flow analysis of ctrl-KM and k3-KM cells.

The μ -slides were coated with integrin $\alpha\text{M}\beta 2$ ligand iC3b. Adhesion assays were performed under the shear stress of 0.4, 0.6 or 0.8 dynes/cm². Plots shown are average of four fields (1 mm vs 1 mm) and expressed as means $\pm$ SD. The activating mAb KIM185 was used at 10 μ g/ml.

3.3.6 Reduced kindlin3 expression attenuates the signaling capacity of constitutively activated integrin α M β 2N329S

We also examined a constitutively activated integrin α M β 2N329S that adopts open headpiece and extended conformation [214]. We made use of another stable K562 cells that do not express integrin α M β 2, but were stably expressing with kindlin3-targeting or control siRNA [205]. These cells were named K562 con siRNA and K562 k3 siRNA cells. The two groups of cells were transfected with integrin α M β 2N329S (Figure 3.3.6 A). The adhesion properties of both transfectants on integrin α M β 2 ligand iC3b were examined (Figure 3.3.6 B). It was evident that K562 con siRNA cells transfected with α M β 2N329S, showed a higher level of binding compared with that of K562 k3 siRNA cells transfected with α M β 2N329S. The adhesion specificity mediated by integrin α M β 2N329S was demonstrated using the function-blocking mAb LPM19c. Cell impedance study was also performed to measure the degree of cell spreading on iC3b. K562 con siRNA cells transfected with α M β 2N329S (blue line) showed higher level of cell index than that of K562 k3 siRNA cells transfected with α M β 2N329S (green line) (Figure 3.3.6 C). Both groups of cells adhesion and spreading were effectively abrogated in the presence of mAb LPM19c (red and purple lines).

These data show that α M β 2N329S-transfected K562 k3 siRNA cells have poorer cell adhesion and cell spreading on integrin α M β 2 ligand iC3b compared with those of α M β 2N329S-transfected K562/ α M β 2 con siRNA cells, because constitutively activated α M β 2N329S bypassed the need for inside-out activation, which requires kindlin3. These data provide direct evidence that kindlin3 is required for integrin α M β 2 outside-in signaling that impinges on cell spreading.

Figure 3.3.6 Reduced kindlin3 expression attenuates integrin α M β 2N329S signaling.

A. K562 con siRNA and K562 k3 siRNA cells were transfected with active integrin α M β 2N329S. Similar expression levels of integrin α M β 2N329S were detected by flow

cytometry. **B.** Quantification of cells that adhere to integrin α M β 2 ligand iC3b. Cell adhesion assays were performed as described previously in Figure 3.3.2. A single experiment of three independent experiments is shown. α M blocking mAb LPM19c was used at 10 μ g/ml. **C.** Reduced kindlin3 expression attenuates cell spreading on coated-iC3b. Impedance studies were conducted as described in materials and methods. K562 con siRNA cells transfected with α M β 2N329S (blue line) showed higher cell index than that of K562 k3 siRNA cells transfected with α M β 2N329S (green line). A single representative experiment of three independent experiments is shown. Each time point indicates the mean of technical triplicates for every 1 min interval. Three independent experiments were performed.

3.3.7 Kindlin3 is involved in integrin α M β 2-Syk-Vav1 signaling

We have shown that kindlin3 plays an important role in integrin α M β 2-mediated cell adhesion and spreading. Studies also reported that Syk is involved in early signaling events of β 2 integrins in hematopoietic cells, and it is required for cell spreading in polymorphonuclear leukocytes [199, 224]. In this study, we examined the phosphorylation of Syk induced by integrin α M β 2 clustering in K562/ α M β 2 (KM), ctrl-KM and k3-KM cells (Figure 3.3.7 A). All these cells treated with activating mAb KIM185 or control IgG were seeded onto immobilized iC3b for 30 min at 37°C. All cells (bound and unbound) were collected and IP assays were performed with anti-Syk Ab or irrelevant Ab control IgG. Phospho-SykTyr525/526 was detected in KM cells in presence of mAb KIM185, but not in control IgG treated KM cells (Figure 3.3.7 A, left panel). Ctrl-KM and k3-KM cells treated with mAb KIM185 were also seeded onto immobilized iC3b. We found that the phosphorylation level of SykTyr525/526 was higher in ctrl-KM cells than that of k3-KM cells (Figure 3.3.7 A, right panel).

It has been reported that Syk interacts with GEF Vav leading to Vav phosphorylation [246, 247]. In neutrophils, the Syk-Vav signaling axis was reported to be involved in cell adhesion and migration mediated by β 2 integrin [248, 249]. Vav1 is one member of Vav gene family and it is widely expressed in hematopoietic cells [250]. Therefore, in this study we checked the phosphorylation of Vav1. All of KM, ctrl-KM and k3-KM cells were subjected to the same conditions as in Figure 3.3.7 A, and IP was performed with anti-Vav1 Ab or irrelevant control IgG. Vav1 IPs were used for immunoblotting with anti-phospho-Tyr Ab. In KM cells a basal level of Vav1 tyrosine phosphorylation was observed following plating of cells on iC3b, while the phosphorylation was significantly enhanced with addition of mAb KIM185 (Figure 3.3.7 B). In mAb KIM185 treated-ctrl-KM cells, we also detected Vav1 tyrosine phosphorylation on immobilized iC3b, while the level of Vav1 phosphorylation was much lower in mAb KIM185 treated-k3-KM cells under the same conditions (Figure 3.3.7 B). Collectively, these data show that kindlin3 is involved in integrin α M β 2-Syk-Vav1 signaling.

Figure 3.3.7 Kindlin3 is involved in integrin α M β 2-Syk-Vav1 signaling.

All of KM, ctrl-KM and k3-KM cells were plated onto tissue culture dishes coated with iC3b. 10 μ g/ml of mAb KIM185 or irrelevant control IgG was added to the cells. Cells were incubated for 30 min at 37°C and then were lysed. Whole-cell lysates were used for IP assays using anti-Syk (**A**) or anti-Vav1 Abs (**B**). Irrelevant mouse IgG and rabbit IgG were included for Syk IP and Vav1 IP, respectively. Phospho-SykTyr525/526 and Tyr-phosphorylated Vav1 were detected by immunoblotting (IB) with relevant Abs. Reblots of total Syk and total Vav1 were also performed. A blot from two independent experiments is shown.

3.3.8 Kindlin3 plays a role in integrin α M β 2-induced Rho GTPases activation

It has been reported that Vav proteins are GEFs for Rho GTPases, which activate pathways and control actin cytoskeletal rearrangements [250]. We first examined the expression levels of GTPases Rac1, Cdc42 and RhoA in ctrl-KM and k3-KM cells on immobilized iC3b or empty tissue culture dishes. The mAb KIM185 was added to each group of cells. Comparable expression levels of Rac1, Cdc42 and RhoA were detected in whole cell lysates of ctrl-KM and k3-KM cells, and iC3b treatment did not affect their expression levels (Figure 3.3.8 A, C and E). Next, we made use of two kinds of beads, GST-PBD beads which bind to active Rac1 and Cdc42 and GST-RBD beads which bind to active RhoA. Pull down assays were performed using these beads and whole cell lysates (Figure 3.3.8 A). The pull-down samples were resolved on SDS-PAGE and the presence of activated Rac1, Cdc42 and RhoA was detected by immunoblotting. In the absence of iC3b, similar basal levels of activated Rac1 or Cdc42 were detected in ctrl-KM and k3-KM cells (Figure 3.3.8 B & D). On immobilized iC3b, ctrl-KM cells showed higher levels of activated Rac1 and Cdc42 as compared to those in k3-KM cells. RhoA was not activated in these cells under all conditions (Figure 3.3.8 F). Taken together, these data suggest that kindlin-3 is involved in integrin α M β 2 outside-in signaling that controls actin cytoskeletal dynamics.

Figure 3.3.8 Kindlin3 is involved in integrin α M β 2-induced Rho GTPases activation.

Ctrl-KM and k3-KM cells treated with 10 μ g/ml of mAb KIM185 were plated onto tissue culture dishes with or without iC3b coating, in the presence of mAb KIM185. **A**, **C** and **E**. Expression levels of Rac1, Cdc42 and RhoA were detected in cell lysates. Blotting of β -actin was performed as a loading control. **B**, **D** and **F**. GST pull-down assays were performed using GST-RBD or GST-PBD beads and activation of Rac1, Cdc42 and RhoA were examined by immunoblotting (IB). Representative blots from two independent experiments are shown.

4. Discussion

In result section 3.1, we examined the membrane proximal signaling events derived from integrin $\alpha\text{M}\beta 2$ clustering in human monocytic U937 cells and human primary monocytes. Our results show that PKC δ mediates integrin $\alpha\text{M}\beta 2$ signaling in human monocytes. Integrin $\alpha\text{M}\beta 2$ clustering induces the activation of PKC δ , including the translocation to the plasma membrane and phosphorylation at Tyr311. The translocation of PKC δ may allow it to localize in SFK-enriched microdomains and subsequently induce SFK-dependent phosphorylation. It has been reported that PMA or thrombin induces c-Src-dependent PKC δ phosphorylation at Tyr311 and Tyr565 in human platelets [251]. In this study, we found that SFKs (Hck and Lyn) are required for integrin $\alpha\text{M}\beta 2$ -induced PKC δ activation. The mechanism of SFKs activation may be similar to the interaction of c-Src and platelet integrin $\alpha\text{IIb}\beta 3$ [155, 252]. Clustering of integrin $\alpha\text{IIb}\beta 3$ recruits c-Src and induce its autophosphorylation and activation [252]. Thus clustering of integrin $\alpha\text{M}\beta 2$ may also induce the activation of SFKs.

We also showed that the region Asn727-Ser734 on integrin $\beta 2$ tail is required for integrin $\alpha\text{M}\beta 2$ -induced PKC δ Tyr311 phosphorylation in K562 transfectants. This observation is consistent with the previous data of our lab, showing that this region is required for the colocalization of integrin $\alpha\text{M}\beta 2$ and Hck in Chinese hamster ovary (CHO) transfectants [207]. In platelets, Lyn has been reported to mediate PKC δ Tyr311 phosphorylation by the interaction with PKC δ [253]. Our data also show Lyn is involved in the PKC δ Tyr311 phosphorylation induced by integrin $\alpha\text{M}\beta 2$ clustering. It has been shown that Hck and Lyn interact with integrin $\beta 2$ cytoplasmic tail [155, 254], therefore these two SFKs possibly perform similar function. In monocytic U937 cells, there is a high level of Hck expression and a modest level of Lyn expression. Therefore Hck may have a dominant role as compared to Lyn.

Foxp1 serves as a transcription factor and plays an important role in monocyte differentiation [169]. It has been shown that engagement of integrin $\alpha\text{M}\beta 2$ induces monocyte differentiation that regulates Foxp1 expression [145].

In U937 cells and human monocytes, we found that PKC δ is involved in integrin α M β 2-induced Foxp1 down-regulation which is abrogated by reduced PKC δ expression using siRNA. It has been reported that PKC δ is involved in MEK-Erk signaling that mediates monocyte differentiation [255].

In result section **3.2**, we examined the contribution of MEK-Erk pathway to link up the early signaling events from integrin α M β 2 to the down-regulation of Foxp1. Using specific inhibitor of MEK (PD98059), we show that MEK-Erk pathway is involved in integrin α M β 2 outside-in signaling pathway leading to Foxp1 regulation. This is consistent with the observation that PKC δ is implicated in MEK and Erk1/2-dependent monocyte differentiation [255].

At the present, we are not sure how PKC δ activation leads to Foxp1 degradation. It has been reported that Foxp3 is degraded by the action of E3 ubiquitin ligase Stub1, which interacts with Foxp3 by promoting its polyubiquitination [256]. Foxp1 may also share the same degradation mechanism with Foxp3, as they belong to the same family of Foxp.

Integrin outside-in signaling cross-talks with ITAMs signals from FcR γ and DAP12, and also requires early effectors including SFKs and Syk [49, 224]. ITAM-based Syk signaling has been reported to regulate TLRs signaling pathway [78, 257]. Therefore integrin outside-in signaling may interface with TLR signaling through cross-regulation of ITAMs. Some studies reported that β 2 integrins promote TLR signaling during inflammation [258-260], while some reports demonstrated negative inhibition of TLR-induced responses by β 2 integrins [199, 261]. The conflicting reports suggest that the cross-talk between β 2 integrins and TLR activation is complex.

As an example, it has been reported that integrin α M deficiency enhances LPS-induced TLR signaling in macrophages, using integrin α M knockout mice [199]. Integrin α M^{-/-} mice are more susceptible to LPS treatment, with producing more IL-6, IFN- β , TNF and the chemokine CXCL10, and surviving shorter time than integrin α M^{+/-} mice. TLR4 ligation activates integrin α M β 2 through inside-out signals mediated by the PI3K and RapL pathways, which feed back to inhibit TLR signaling by activating Src and Syk. Subsequently the

activated Syk interacts with and activates MyD88 and TRIF, leading to their degradation through Cbl-b. Therefore, the active integrin $\alpha\text{M}\beta 2$ triggered by TLR crosstalks with MyD88 and TRIF, leading to inhibition of TLR signaling in innate immune responses.

LPS, from the outer membrane of Gram-negative bacteria, is an activator of macrophage [262]. TLR4 selectively recognizes LPS and powerfully induces a number of inflammatory chemokines and kinases [263]. In monocytic THP1 cells, engagement of integrin $\alpha\text{M}\beta 2$ triggers TLR activation and IL-1 production, which involves a membrane proximal mediator IRAK1 [146]. IRAK1 has been reported to be recruited by MyD88 and IRAK4 during TLR4 signaling, leading to IRAK1T209 phosphorylation by IRAK4 [263]. In this study, we found that PKC δ associates with IRAK1, which results in IRAK1T209 phosphorylation. Our data show that the interaction between IRAK1 and PKC δ stably occurs regardless of integrin $\alpha\text{M}\beta 2$ clustering, LPS-induced TLR4 activation, or without treatment. It might be possible that IRAK1 is phosphorylated by PKC δ and IRAK4 alternately, and these three molecules form a complex.

Kindlin3 serves as a co-activator of integrin $\alpha\text{L}\beta 2$, together with talin, and induces a high ligand-binding affinity [264, 265]. The previous study of our lab show that kindlin3 is involved in integrin $\alpha\text{L}\beta 2$ outside-in signaling pathway [205]. In result section **3.3**, we show that reduced kindlin3 affects integrin $\alpha\text{M}\beta 2$ outside-in signaling pathway. We provide direct evidence that kindlin3 is required for integrin $\alpha\text{M}\beta 2$ -mediated cell spreading. Syk has been reported to be activated by clustering of $\beta 2$ integrins [165]. In polymorphonuclear leukocytes, it has also been shown that Syk is involved in $\beta 2$ integrin-mediated cell spreading [199, 224]. Our data show that Syk is activated by integrin $\alpha\text{M}\beta 2$ clustering and this is dependent on kindlin3. Syk interacts with Vav GEFs and causes their activation [246, 247]. In neutrophils, Syk-Vav signaling is involved in $\beta 2$ integrin-mediated cell adhesion [248, 249]. We show that Vav1 is phosphorylated by Syk activation and the phosphorylation is abolished by reduced kindlin3 expression.

Vav GEFs have been reported to be involved in the regulation of Rho GTPases activities, leading to actin remodelling [250]. Rho GTPases (such as Rho, Rac and Cdc42), have been shown to form central coordinators in cytoskeletal organization [185, 186]. Activated Rac and Cdc42 induce the formation of lamellipodia and filopodia, whereas activated Rho induces the formation of stress fibers [266-268]. Rho GTPases have been reported to be important in β 2 integrin signaling that leads to cytoskeleton remodelling [179, 192, 193]. Thus we examined the activation of Rac1, Cdc42 and RhoA in integrin α M β 2 outside-in signaling. Our results based on GST pull-down assays show that Rac1 and Cdc42 are significantly activated by integrin α M β 2 clustering and that reduced kindlin3 expression abrogated the activation of Rac1 and Cdc42. We did not detect the activation of RhoA in control siRNA and kindlin3 siRNA treated cells. It is possible that different Rho GTPases are regulated by different integrins. In CHO cell transfectants, for example, β 1 integrins induces Rac activation, but β 3 integrins induces Rho activation [269]. Additionally, RhoA has been reported suppress the activation of Rac [270, 271]. Our data based on confocal microscopy provide evidence that integrin α M β 2 clustering induces the formation of filopodia in control siRNA treated cells. However, reduced kindlin3 expression diminishes the number of filopodia in kindlin3 siRNA treated cells. These observations may be explained by the activation of Rac1 and Cdc42, which are involved in the regulation of intracellular actin dynamics.

5. Conclusion

Monocytes are important component of the innate immune system. During inflammation, monocytes from the circulation are recruited to the sites of infection. Monocytes can adhere to the endothelium by engaging molecules e.g. ICAM-1 presented on the surface of the endothelium. Effective adhesion to the endothelium requires not only activation of the monocyte integrins e.g. $\alpha\text{M}\beta 2$ but also subsequent events involving cell spreading. We have shown in section 3.3 that kindlin3 is required for integrin $\alpha\text{M}\beta 2$ -mediated cell spreading as it regulates downstream Syk-Vav1-Rac1/Cdc42 signaling axis.

Apart from cell spreading induced by integrin $\alpha\text{M}\beta 2$ outside-in signaling, we have also shown in section 3.1 that ligand-engagement triggers another signaling pathway involving SFK-PKC δ -MEK/Erk. This signaling pathway potentially regulates the expression level of Foxp1 which is a negative regulator of *c-fms* gene that encodes the M-CSF receptor. And M-CSF signaling is well established to be important in monocyte differentiation. Thus, these data suggest that adhesion of monocytes to endothelium can potentially influence the differentiation of monocytes into macrophages.

When monocytes/macrophages localize at the sites of infection, they may encounter LPS released from Gram-negative bacteria depending on the type of infection. LPS is well established to bind TLR4 and induces TLR4 signaling involving IRAK1/4 and MyD88. This ultimately regulates the expression of inflammatory cytokines. We have shown in section 3.2 that PKC δ can interact with IRAK1 and it may play a role in phosphorylating IRAK1. Thus, these data suggest that outside-in signaling of integrin $\alpha\text{M}\beta 2$ is multifaceted in that it regulates cell spreading and monocytes differentiation, and it potentially cross-talks with TLR4 signaling via PKC δ interaction with IRAK1 (Fig. 5.1).

Future studies are needed to address how the interaction of PKC δ and IRAK1 affects integrin $\alpha\text{M}\beta 2$ signaling and TLR4 responses. The truncated mutations of these two molecules will be generated to map the interaction sites. Additionally, the role of IRAK4 in TLR4 signaling and integrin $\alpha\text{M}\beta 2$ outside-in signaling which involves PKC δ will be further investigated.

Figure 5.1 Overview of integrin $\alpha_M\beta_2$ outside-in signaling and LPS-induced TLR4 activation.

A. Integrin $\alpha_M\beta_2$ clustering induces PKC δ activation in a SFK-dependent way, leading to Foxp1 down-regulation. MEK-Erk signaling is involved in this signaling cascade and plays a role downstream of PKC δ . **B.** During LPS-induced TLR4 activation, MyD88 recruits and activates IRAK4. Subsequently the activated IRAK4 triggers the phosphorylation of IRAK1, leading to the activation of p38 and JNK1/2 and the production of cytokine IL-1 β . We observed interaction of PKC δ and IRAK1 suggesting cross-talk between integrin $\alpha_M\beta_2$ signaling and LPS-induced TLR4 activation. **C.** Kindlin3 is required for integrin $\alpha_M\beta_2$ outside-in signaling, which regulates cell spreading. The Syk-Vav1 signaling downstream of kindlin3 regulates the activity of Rac1 and Cdc42, leading to cytoskeletal reorganization.

6. References

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7. Author's publications

7.1 From this study

Xue ZH, Zhao CQ, Chua GL, Tan SW, Tang XY, Wong SC, Tan SM. (2010) Integrin $\alpha M\beta 2$ clustering triggers phosphorylation and activation of protein kinase $C\delta$ that regulates transcription factor Foxp1 expression in monocytes. J. Immunol. 184(7): 3697-3709.

Xue ZH, Feng C, Liu WL, Tan SM. (2013) A role of Kindlin-3 in integrin $\alpha M\beta 2$ outside-in signaling and the Syk-Vav1-Rac/Cdc42 signaling axis. PloS One. 8(2): e56911

7.2 From other study

Feng C, Li YF, Yau YH, Lee HS, Tang XY, **Xue ZH**, Zhou YC, Lim WM, Cornvik TC, Ruedl C, Shochat SG, Tan SM. (2012) Kindlin-3 mediates integrin $\alpha L\beta 2$ outside-in signaling, and it interacts with the scaffold protein receptor for activated-C kinase 1 (RACK1). J Biol Chem. 287(14), 10714-10725

8. Posters, awards and invited talks

Poster presentation

Xue ZH and Tan SM. Integrin $\alpha M\beta 2$ clustering triggers phosphorylation and activation of protein kinase $C\delta$ that regulates transcription factor Foxp1 expression in monocytes.

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