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Contribution of CD95 to the activation of innate immune response

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Acknowledgment

路漫漫其修遠兮，吾將上下而求索

屈原 (公元前 343-前 278),《離騷》

The way ahead is foggy and boundless.
I shall explore it from beginning to end for the truth.
Qu Yuan (343 - 278 BC), The Sorrow of Parting

This thesis should be dedicated to all the people helped me during my PhD study. Without your help, I couldn't reach this far.

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ABBREVIATIONS

ABBEVIATIONS

Abbreviation	Explanation
AICD	activation-induced cell death
APCs	antigen presenting cells
BCRs	B-cell receptors
BTK	Bruton tyrosine kinase
CalDAG-GEFI	calcium and DAG-regulated GEFI
CCL	CC chemokine ligand
CCR	CC-chemokine receptor
CD95L	CD95 ligand
CXCL	CXC chemokine ligand
CXCR	CXC-chemokine receptor
DAMPs	damage-associated molecular patterns
DAP12	NDAX activation protein of 12 kDa
DAPI	4',6-diamidino-2-phenylindole
DCs	dendritic cells
DD	death domain
DED	death-effector-domain
DISC	death-inducing signaling complex
ERK	extracellular signal-regulated kinase
ESL-1	E-selectin ligand-1
FACS	fluorescence activated cell sorting
FADD	Fas-Associated-Death-Domain
FcRγ	Fc receptor common γ signaling chain
GPCRs	G protein-coupled receptors
GSK-3 β	glycogen synthase kinase 3-β
HRP	horseradish peroxidase
i.p.	intraperitoneally
i.v.	intravenously
ICAMs	intercellular adhesion molecules
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-JUN N-terminal kinase
LFA-1	Lymphocyte function-associated antigen 1
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MHC	histocompatibility complex

MMPs	metalloproteinases
NF-κB	nuclear factor-κB
NK cells	natural killer cells
PAMPs	pathogen-associated molecular patterns
PECAM1	platelet endothelial cell adhesion molecule
PH domain	Pleckstrin homology domain
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLC-γ2	phospholipase C-γ2
PMA	phorbol 12-myristate 13-acetate
PRRs	pattern recognition receptors
PSGL-1	P-selectin glycoprotein lighan-1
Rap	RAS-related protein
RhoA	Ras homolog gene family, member A
ROS	reactive oxygen species
sCD95L	soluble CD95 ligand
SEM	error of the mean
SFKs	Src family kinases
SH2	SRC-homology 2
Syk	spleen tyrosine kinase
TCR	T-cell receptor
TLRs	Toll-like receptors
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
VCAMs	vascular cell-adhesion molecule
wt	wild-type

1 SUMMARY

Myeloid cell recruitment plays a pivotal role in innate immune response. It comprises a cascade of sequential cellular processes including slow rolling, activation and firm adhesion, and transendothelial cell migration. Apart from the well-known apoptotic function, CD95 serves as an inflammatory mediator by inducing production of cytokines and chemokines in a variety of cell types and triggering myeloid cell transmigration to inflammatory sites via Syk-PI3K-MMP9 signaling pathway.

Utilizing an *ex vivo* autoperfused mouse flow chamber assay, we show here that CD95 ligand (CD95L) induces neutrophil slow rolling. Engagement of CD95 with CD95 ligand in myeloid cells activates the signaling pathway of Syk-BTK-PLC-γ2, which is essential for selectin-induced integrin activation in neutrophil slow rolling. Furthermore, activation of Rap1, which serves as a direct mediator for integrin activation, by CD95 signaling in neutrophils suggests the involvement of integrin activation in CD95-induced slow rolling. In line with this hypothesis, integrin activation upon CD95L treatment was detected by performing active integrin reporter antibodies binding assay and soluble ICAM1 binding assay. In addition to activating integrin, CD95 recruits integrin and forms microclusters upon CD95L stimulation. Our results indicate that CD95 signaling activates integrin in mediating neutrophils slow rolling.

We also found the involvement of endothelia cells and Ly6C^{hi} monocytes - the classical inflammatory monocytes- in CD95-mediated innate response. Inducible deletion of CD95L in endothelial cells (*CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$) impaired neutrophil recruitment in a thioglycollate-induced peritonitis model. Unlike TNF-α, deletion of CD95 in endothelial cells (*CD95*$^{f/f;Ve\text{-}CadherinERT2/4cre}$) had no impact on the expression levels of adhesion molecules and neutrophil recruitment. Moreover, CD95 selectively induced the mobilization and recruitment of inflammatory monocytes in a CCL2-dependent manner.

In this study we show for the first time that CD95 signaling mediates neutrophil slow rolling via activation of integrin. Endothelial cells participate in this process by presenting CD95L. CD95 is also involved in the recruitment of inflammatory monocytes. Taken together with our previous findings, our studies identify a CD95 chemotactic axis pathway for innate immune cell recruitment.

1 Zusammenfassung

Myeloide Zellrekrutierung spielt eine zentrale Rolle in der angeborenen Immunantwort. Sie besteht aus einer Kaskade von zellulären Prozessen, einschließlich langsamem Rollen, Aktivierung und feste Adhäsion und transendothelialer Zellmigration. Abgesehen von der bekannten apoptotischen Funktion dient CD95 als Entzündungsmediator durch Induktion der Produktion von Cytokinen und Chemokinen in einer Vielzahl von Zelltypen und Auslösung von myeloider Transmigration zu Entzündungsstellen durch einen Syk-PI3K-MMP9-Signalweg.

Durch Verwendung eines ex vivo Autoperfusions-Flusskammer-Assays, zeigen wir hier, dass der CD95-Ligand (CD95L) langsames Rollen von Neutrophilen induziert. Engagement von CD95 mit dem CD95-Liganden in myeloiden Zellen aktiviert den Syk-BTK-PLC-γ2 Signalweg, der für Selektin-induzierte Aktivierung von Integrinen während des langsamen Rollens von Neutrophilen essentiell ist. Aktivierung von Rap1, ein direkter Vermittler für Aktivierung von Integrin, durch CD95 -Signalgebung in Neutrophilen weist auf eine Beteiligung von Integrin-Aktivierung bei CD95-induziertem langsamen Rollen hin. Im Einklang mit dieser Hypothese wurde Integrin-Aktivierung nach CD95L Behandlung mittels aktivem Integrin Reporter Antikörper Bindungstest und löslichem ICAM1 Bindungstest festgestellt. Neben der Integrin-Aktivierung rekrutiert CD95 nach CD95L-Stimulation Integrin und bildet mit diesem Mikrocluster. Unsere Ergebnisse zeigen, dass der CD95 Signalweg Integrin aktiviert und so langsames Rollen in Neutrophilen vermittelt.

Weiterhin fanden wir auch eine Beteiligung von Endothelzellen und $Ly6C^{hi}$ Monozyten- den klassischen inflammatorischen Monozyten in CD95-vermittelter nativer Immunreaktion. Induzierbare Deletion von CD95L in Endothelzellen (*CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$) beeinträchtigt die Neutrophilenrekrutierung in einem Thioglycollat - induzierten Peritonitis-Modell. Im Gegensatz zu TNF-α hat Deletion von CD95 in Endothelzellen (*CD95*$^{f/f;Ve\text{-}CadherinERT2/4cre}$) keine Auswirkungen auf die Expression von Adhäsionsmolekülen und Neutrophilen- Rekrutierung. Darüber hinaus hat CD95 die Mobilisierung und Rekrutierung von inflammatorischen Monozyten in einer CCL2 - abhängigen Weise selektiv induziert.

In dieser Studie zeigen wir zum ersten Mal , dass CD95 -Signalisierung langsames Rollen von Neutrophilen über die Aktivierung von Integrin vermittelt. Endothelzellen sind an diesem Prozess durch die Präsentation von CD95L beteiligt. CD95 ist auch bei der Rekrutierung von inflammatorischen Monozyten beteiligt. Zusammen mit unseren früheren Befunden genommen, zeigt unsere Studien eine chemotaktische CD95 Achse für angeborene Immunzellrekrutierung.

2 INTRODUCTION

2.1 The immune system

2.1.1 An overview of the immune system

The animal immune system is composed of cellular and humoral components with a high complexity that defends the host from infectious disease and injuries by identifying and eliminating pathogens and detrimental cells. In order to protect the host successfully, the primary task of the immune system is to identify self from non-self and recognize danger signals. The self-nonself distinction is employed to maintain the host's integrity in different organisms. In unicellular organisms, it manifests as protective mechanisms such as the utilization of antimicrobial peptides and production of restriction enzymes to fight against competitors for nutrients or pathogens (Rodríguez et al., 2012). After the appearance of multicellular organisms, increasingly complex immune systems have evolved, with the innate immune system presents in most multicellular organisms and the adaptive immune system only in higher vertebrates. Innate immune system is comprised of mechanisms and specialized immune cells that defend the host from pathogens in a non-specific manner depending on gremline-encoded receptors. Due to the complexity of development and longer life time compared to lower organisms, higher vertebrates have a higher risk of encountering infections and therefore an adaptive immune system has evolved, which involves specialized lymphocytes recognizing a wide range of pathogens with specific receptors and developing immunological memory (Rodríguez et al., 2012; Murphy et al., 2012).

2.1.2 The innate immune system

The innate immune system is an evolutionary ancient system. It was developed before vertebrates and invertebrates diverged. Innate defense forms the first line to protect the host from infections by other organisms. It is important for most multicellular organisms and is such a fundamental function that vertebrates, invertebrates and plants share many similarities (Kimbrell and Beutler, 2001). The innate defense is considered relatively non-specific as it is mediated by a fixed set of germline-encoded receptors. The induction of the innate response is rapid, and usually occurs within minutes to hours after infection or injury (Murphy et al., 2012). Besides functionally being the first line of

defense against infections, the innate immune system is also involved in many other physiological processes, such as tissue remodeling in development and damage repair, transport of blood lipids, and the clearance of apoptotic cells (reviewed by Seong and Matzinger, 2004).

The innate immune system is comprised of three lines of defenses to prevent an infection or eliminate it before the adaptive immune system needs to be activated. The first line comprises the physical and chemical barriers preventing the invasion of microorganisms into the interior of the body. These include the skin, the tight junctions between epithelial cells, the acidic environment of the stomach, and components of the mucus layers, such as antimicrobial enzymes and antimicrobial peptides, that inhibit growth or even kill pathogenic bacteria (Alberts et al., 2007; Murphy et al., 2012).

The second line of defense of the innate immune system depends on cell-intrinsic responses to kill the invading pathogens. Most cells that have taken up a microorganism by pathogen-induced phagocytosis will immediately direct the fusion of the phagosome with a lysosome, after which the invading microorganism will be exposed to digestive enzymes. Another ancient intrinsic defense mechanism of host cells in defense against many viral infections is the ability to degrade double-stranded RNA, which is a common intermediate in viral replication (Alberts et al., 2007; Murphy et al., 2012).

The spread of a pathogen is normally blocked by the third line of innate immune defenses, referred to as the complement system, which induces more effector cells and molecules of the innate immune system to migrate from the blood and into the tissue. The complement system consists of a number of plasma proteins that are generally synthesized by the liver, and normally circulating as inactive precursors. An encounter with pathogens or pathogen-bound antibody leads to the activation of complement, which in turn induces a cascade of reactions occurring on the surface of the pathogen and results in enhanced phagocytosis of antigens, chemotaxis of neutrophils and macrophages, lysis of pathogens by rupturing membranes, and agglutination of pathogens (Alberts et al., 2007; Murphy et al., 2012).

After microorganisms cross an epithelial barrier and start to replicate in the tissue of the host, in most cases it is instantly recognized by resident phagocytic cells and then

induces the innate immune response. Macrophages are the major phagocytes resident in normal tissue. To initiate an immune response, the first essential step is the recognition of potentially harmful microorganisms. Depending on germline-encoded receptors, the innate immune system recognizes highly conserved structural components of microbes, often referred to as pathogen-associated molecular patterns (PAMPs). PAMPs are usually essential for the normal functions of microbes, such as lipopolysaccharide (LPS) and peptidoglycan, which are bacterial cell-wall components, flagellin of bacterial flagella and viral RNAs. The receptors involved in PAMPs recognition are collectively termed as pattern recognition receptors (PRRs) (Murphy et al., 2012).

There are five classes of PRRs that have been identified: Toll-like receptors (TLRs), which detects multiple PAMPs such as LPS, flagellin, viral RNA and DNA with unmethylated CpG; NOD-like receptors (NLRs) detecting pathogen products in the cytoplasm; RIG-I-like receptors (RLRs) involving in antiviral responses; absence in melanoma 2 (AIM2)-like receptors detecting intracellular microbial DNA; and C-type lectin receptors (CLRs). PRRs also recognize non-infectious material derived from the host, which is released following tissue injury or cell death. These endogenous molecules are termed damage-associated molecular patterns (DAMPs) and have similar functions as PAMPs in activation of pro-inflammatory pathways (Chen and Nuñez, 2010).

Following ligand recognition, the PRRs activate downstream signaling pathways, such as nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK) and type I interferon pathways, which trigger changes in gene expression and result in the production of inflammatory cytokines and chemokines in tissue resident macrophages and dendritic cells (Murphy et al., 2012).

The cytokines released upon PRRs activation are interleukin 6 (IL-6), IL-12, IL-1β, and tumor necrosis factor-α (TNF-α), which are diverse in structure and have a variety of local and distant effects. IL-6 stimulates the production of new monocytes and granulocytes in the bone marrow. IL-12 activates natural killer cells (NK cells) and induces the differentiation of CD4 T cells into T_H1 cells. IL-1β, and TNF-α both induces the activation of vascular endothelial cells, resulting in increased secretion of chemokines and presentation of adhesion molecules on the endothelial lumen to

facilitate the recruitment of leukocytes (This will be discussed in further detail in the section of 2.4).

The chemoattractant cytokines released after PRRs activation in tissue resident phagocytic cells direct the chemotaxis of nearby responsive cells and are termed chemokines. Chemokines are classified into two distinct groups based on the position of cysteine residues in the N-terminus of the protein. CXC chemokine ligands, such as CXCL8, drive the recruitment of neutrophils from the blood stream to the inflamed tissue. In contrast, CC chemokine ligands trigger the migration of monocytes and lymphocytes. As an example, CCL2 attracts monocytes and induces their migration to the tissue to become tissue macrophages (Murphy et al., 2012).

Upon the activation of cytokines in endothelial cells and chemokines in circulating leukocytes, one of the most important processes of innate immunity, the recruitment of activated phagocytes, is initiated. Leukocyte recruitment from blood stream to the inflamed tissue is orchestrated by a cascade of cellular events including slow rolling, adhesion strengthening, intraluminal crawling, paracellular and transcellular migration, and migration through the basement membrane (Ley et al. 2007). These events will be described in detail in the section of 2.2.

2.1.3 The adaptive immune system

In contrast to the innate immune system, the adaptive immune system is evolutionarily young as it appeared 500 million years ago in vertebrates with jaws (gnathostomes) (Cooper and Alder, 2006). The adaptive immune system mediates specific immune responses, such as the production of antibodies against a particular pathogen or its product. The adaptive immune response is developed during the lifetime of an individual as an adaptation to infection with that pathogen which results in an immunological memory, conferring lifelong protective immunity against the same pathogen. The cells for the adaptive immune system are T and B lymphocytes which are generated in the bone marrow and mature in the thymus or bone marrow respectively. T cells are intimately involved in cell-mediated immune response, whereas B cells play a large role in the humoral immune response by secretion of antibodies (Murphy et al., 2012).

Whereas innate immunity depends on gremline-encoded receptors to recognize common features of many pathogens, the adaptive immune system utilizes receptors encoded by rearranging gene segments to recognize a wide variety of antigens. Furthermore, each mature lymphocyte differs from the others in the specificity of its antigen receptor. Lymphocytes are continuously undergoing a process akin to natural selection and only those lymphocytes that encounter antigen which binds to their receptor specifically will be activated to proliferate and differentiate into effector cells. This process is termed clonal selection, which is the central principle of adaptive immunity (Murphy et al., 2012).

T-cell receptors (TCRs) recognize antigen-derived peptides that are processed by antigen presenting cells (APCs) and presented in the major histocompatibility complex (MHC) on the surface of APCs. CD4 and CD8 are the co-receptors assisting TCRs in communicating with APCs. $CD8^+$ T cells are cytotoxic T cells (Tc) recognizing antigen presented by MHC class I^+ APCs and killing the target infected cells by releasing cytotoxins or apoptosis mediators. $CD4^+$ T cells recognize antigens presented by MHC class II^+ APCs and secrete cytokines to regulate the cell-mediated and humoral immune response (Murphy et al., 2012).

B-cell receptors (BCRs) are composed of immobilized antibodies and CD79. In contrast to TCRs, BCRs recognize the naïve form of antigens. Upon activation by encountering an antigen, B cells differentiate into plasma cells, producing large amounts of antigen-specific antibodies that contribute to the humoral immune response. Antibodies participate in host defense in three main ways: neutralization of bacterial toxins; opsonization, wherein pathogens coated with antibodies are recognized by FC receptors on phagocytes and then destroyed by phagocytosis; and complement activation mediated by antibodies binding to a pathogen (Murphy et al., 2012).

After a naïve lymphocyte has been activated, it takes 4-5 days for the completion of clonal expansion and differentiation of lymphocytes to effector cells. So the first adaptive immune response only occurs several days after the infection has commenced. Most of the lymphocytes generated by clonal expansion will eventually die. Nonetheless, a significant number of activated antigen-specific B cells and T cells persist and form the

basis of immunological memory, ensuring a rapid and effective response on re-encountering the same pathogen (Murphy et al., 2012).

2.2 Leukocyte recruitment to the site of inflammation

Leukocyte recruitment plays a pivotal role in inflammation, as all of the inflammatory processes involve or depend on leukocyte recruitment to the inflamed tissue. In response to injury or infection, locally presented stimulating factors, such as chemokines, cytokines and adhesion molecule, initiate the wave of neutrophil extravasation -in some cases also monocytes- through the vasculature into the inflamed tissue. The first wave of neutrophil recruitment is normally followed with the recruitment of monocyes and lymphocytes (Ley et al., 2007; Hajishengallis et al., 2013).

Studies accumulated from the last two decades revealed that the cellular events of leukocyte recruitment are comprised of a cascade of three major steps in sequence: slow rolling, leukocyte activation and firm adhesion, and transendothelial cell migration (Figure 1).

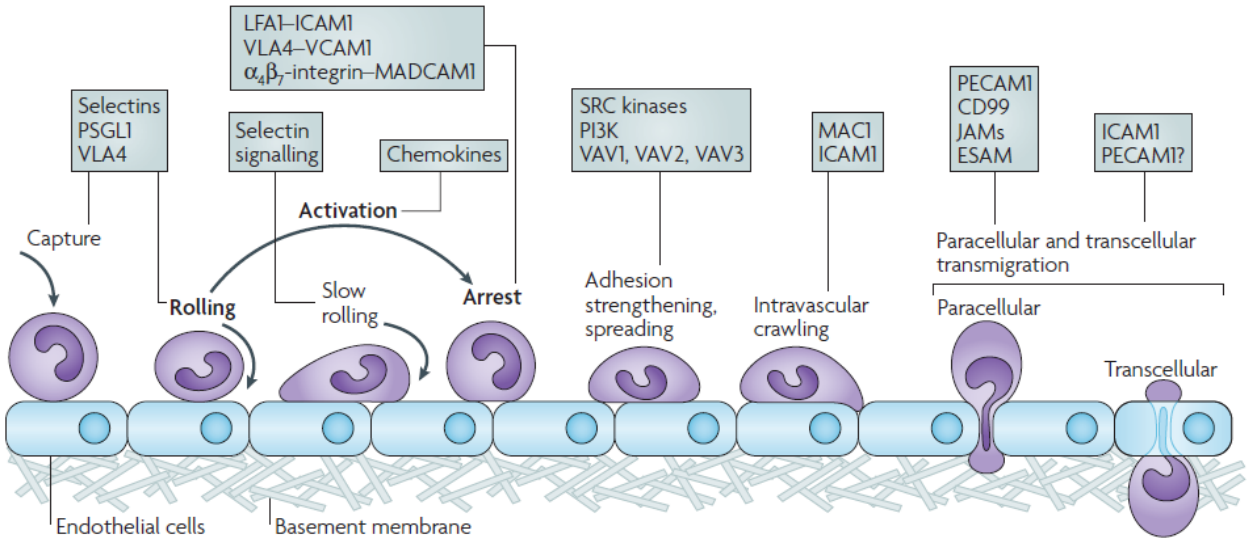

Figure 1. Cellular events of leukocytes recruitment cascade (adapted from Ley et al., 2007).

2.2.1 Slow rolling

Upon stimulation by TNF-α, IL-1β, and IL-17 during inflammation, activated endothelial cells express P-selecin, E-selectin and other adhesion molecules such as ICAMs (intercellular adhesion molecules) and VCAMs (vascular cell-adhesion molecule) on their luminal surface. Binding of selectin to its ligand, such as P-selectin glycoprotein lighan-1 (PSGL-1), CD44 and E-selectin ligand-1 (ESL-1) on leukocytes triggers the tethering (or capture) of leukocytes to the endothelium and initiates the rolling on the vessels' luminal wall (Katayama et al., 2005; Ley et al., 2007).

Selectin-mediated leukocyte rolling is dependent on shear stress: the rolling cells detach when blood flow is stopped. This phenomenon relies on the special ‘catch bond’ characteristics of selectins. The selectin-ligand bonds have rapid on and off rates and high mechanical strength to initiate tethering even through one or few bonds when certain shear stress is applied, permitting rolling in response to hydrodynamic drag (Finger et al., 1996; Marshall et al., 2003).

The signaling mechanism for selectin-mediated rolling has only been discovered recently. Selectin signaling shows similarities to immunoreceptor or integrin outside-in signaling. E-selectin engagement with ligands of PSGL-1 and CD44 induces the activation of Src family kinases (SFKs) Hck, Fgr and Lyn (Yago et al., 2010). Activated SFKs phosphorylate and activate ITAM (immunoreceptor tyrosine-based activation motif)-bearing adaptor protein FcRγ (Fc receptor common γ signaling chain) and DAP12 (NDAX activation protein of 12 kDa) (Zarbock et al., 2008). These activated adaptor proteins recruit and phosphorylate Syk (spleen tyrosine kinase), which in turn activates BTK (Bruton tyrosine kinase) (Mueller et al., 2010; Yago et al., 2010). BTK further activates the PI3K (phosphoinositide 3-kinase), PLC-γ2 (phospholipase C-γ2) and p38 MAPK pathways, mediating slow rolling through integrin activation (Figure 2).

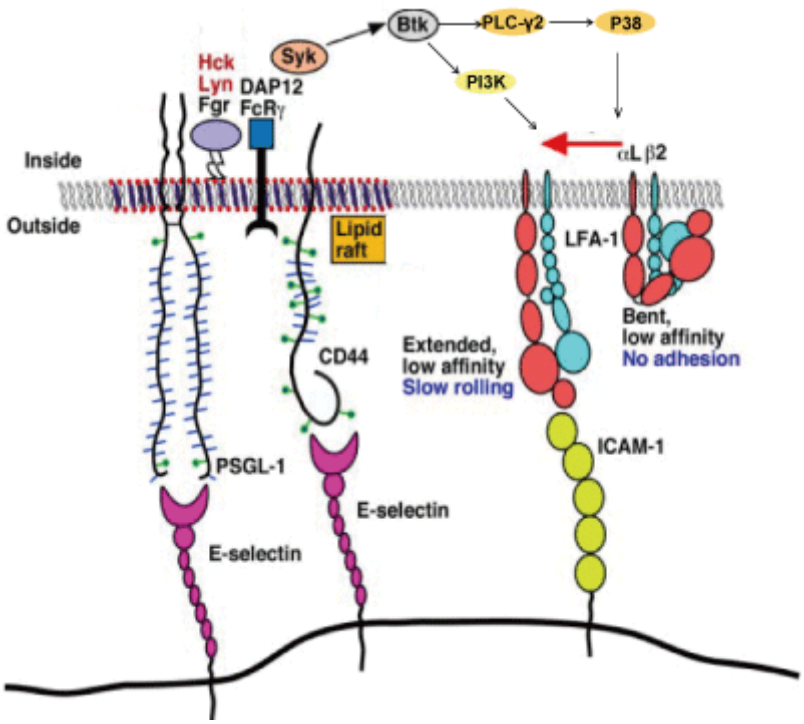

Figure 2. Selectin signaling in mediating rolling (revised from Mueller et al., 2010; Yago et al., 2010)

Integrins also play an important role in mediating slow rolling. The ligation of integrin α4 to its ligand VCAM-1 and MAdCAM-1 (mucosal vascular addressin cell adhesion molecule 1) initiates reversible lymphocyte tethering and rolling under shear stress in the absence of selectin (Berlin et al., 1995). Relying on the interaction of low-avidity integrin $\alpha_L\beta_2$ (also known as LFA-1, Lymphocyte function-associated antigen 1) with ICAM-1 (intercellular adhesion molecule 1), β_2 integrin cooperates with selectin and reduces the rolling velocity (Sigal et al., 2000; Kadono et al., 2002; Chesnutt et al., 2006). Integrin-mediated slow rolling depends on the integrin inside-out signaling, which will be discussed in further detail in later sections.

2.2.2 Leukocyte firm adhesion and activation

While rolling on the endothelium, leukocytes engage with chemokines and other cytokines presented by the endothelial cells, which triggers their chemotaxis and activation. Early studies have shown that chemokines induce rapid integrin-dependent lymphocyte arrest on vascular endothelium in *in vitro* models and physiological conditions (Campbell et al., 1998; Constantin et al., 2000). Chemokine receptors are G

protein-coupled receptors (GPCRs) and bind specifically to the corresponding chemokines. Many chemokines also bind to glycosaminoglycans (GAGs) on endothelial cell surface for efficient leukocyte recruitment (Johnson et al., 2005). Chemokine receptor-triggered GPCR signaling leads to rapid integrin activation from the low affinity state to the high ligand-binding affinity state, allowing the arrest and firm adhesion of leukocytes. This signaling cascade is termed inside-out signaling and will be described in detail later.

Activation of TCRs and BCRs also lead to activation of integrin in mediating lymphocyte firm adhesion through inside-out signaling (Katagiri et al., 2004; McLeod et al, 2004).

Leukocytes start spreading and crawling on the vascular wall once they are arrested, which is driven by integrin activation-induced cytoskeletal rearrangement, and these events are termed outside-in signaling responses. Integrin outside-in signaling is involved in functional activation of leukocytes. In neutrophils, it is essential in regulating phagocytosis and generation of reactive oxygen species (ROS) via a membrane-associated NADPH oxidase (reviewed by Schymeinsky et al., 2007). In T cells, it stabilizes the binding to APCs and induces the secretion of IL-2 and interferon-γ (Burbach et al., 2007). In platelets, on the other hand, it stabilizes the adhesion to extracellular matrices and results in the formation of thrombus (Kasirer-Friede et al., 2007).

2.2.3 Transendothelial cell migration

Transmigration through the vessel wall is the final step for leukocyte recruitment to the inflamed tissue. In this process, extravasating leukocytes need to cross over three obstacles, which are endothelial cells, the endothelial-cell basement membrane, and pericytes (Ley et al., 2007). Emigrating leukocytes induce the formation of “cuplike” transmigratory structure on endothelial cells, which is comprised of ICAM1 and VCAM1-highly enriched vertical microvilli-like projections that surround transmigrating leukocytes. These projections initiate the transmigration through the paracellular or transcellular pathway (Carman and Springer, 2004).

Leukocytes' transmigration through the loose endothelial-cell junctions is termed paracellular transmigration. Ligation of ICAM1 and VCAM1 to integrin on transmigrating

leukocytes also induces intracellular signaling in endothelial cells. Upon ligation, ICAM1 cytoplasmic domain induces the activation of RhoA (Ras homolog gene family, member A) GTPase, leading to actomyosin contraction and formation of stress fibers in endothelial cells. This in turn leads to endothelial cell contraction. In addition to the effects on cytoskeleton, RhoA also affects the integrity of adherent junctions and tight junctions between endothelial cells (Millán and Ridley, 2005).

The transcellular route is utilized only by a minority of migrating cells (Carman and Springer, 2004). Leukocytes start transcellular migration by extending pseudopodia into endothelial cells. Induced by the cytoplasmic signaling of ICAM1 ligation, ICAM1 translocates to caveolin and F-actin-rich membrane domains at the protrusion site on endothelial cells, and is then internalized and transcytosed to the basal plasma membrane through caveolae. These signaling events collectively lead to the formation of a channel through the endothelial cell where leukocyte can migrate through (Millán et al. 2006).

The last barriers hampering leukocytes transmigration are the endothelial basement membrane and pericytes. The endothelial basement membrane is composed of protein networks formed by laminins and collagen type IV that are connected by interactions with molecules such as nidogen-2 and the heparin sulphate proteoglycan perlecan (Hallmann et al., 2005). Gaps between pericytes, which have low expression level of matrix proteins, are the preferred sites for neutrophil migration due to low resistance (Sixt et al., 2001; Wang et al., 2006). This transmigration is facilitated by proteases such as metalloproteinases (MMPs) (ir-Kirk et al., 2003) and integrin $\alpha_6\beta_1$, as it is the main leukocyte receptor for laminin and can be up-regulated by the ligation of PECAM1(Platelet endothelial cell adhesion molecule, also known as CD31) to neutrophils (Dangerfield et al., 2002).

2.3 Integrin activation

Integrins are cell surface receptors composed of heterodimers of α and β subunit of type I transmembrane glycoproteins with short cytoplasmic tails. Common integrins expressed by leukocytes are $\alpha_L\beta_2$ (LFA-1), $\alpha_M\beta_2$ (Macrophage-1 antigen, Mac-1) and $\alpha_4\beta_1$ (very late antigen 4, VLA-4). The ligands for integrin $\alpha_L\beta_2$ are ICAM1, ICAM2, ICAM 3 and ICAM5, which are expressed mainly by endothelial cells. Integrin $\alpha_M\beta_2$ recognizes complement protein iC3b and cellular matrix proteins such as fibrinogen and heparin. Integrin $\alpha_4\beta_1$ recognizes VCAM-1 and fibronectin (Luo et al., 2007; Abram and Lowell, 2009).

Through the bidirectional pathways referred to as inside-out and outside-in signaling, integrins play a central role in the cascade of leukocytes recruitment. The transmitted signals by activated integrin have broad effects, such as activating leukocytes, proliferation, survival and differentiation in many other cell types (Abram and Lowell, 2009).

2.3.1 Inside-out signaling

Inside-out signaling is defined as the intracellular signaling that induces conformational changes of integrin leading to increased ligand binding affinity and clustering of integrin in the membrane, which together allow cell attachment.

Integrin adopt three states of activation: a bent form of which the ligand binding site is blocked; an extended form with intermediate ligand binding capacity; and an open form with full avidity for ligand binding (Luo et al., 2007). These forms can be detected with antibodies specifically recognizing different integrin conformations (Evans et al., 2009).

2.3.1.1 Extracellular signals for inside-out signaling

As mentioned in the section of 2.1.2, binding of chemokines or cytokines to their receptors (GPCRs) or stimulation of TCR/BCR activates integrin and lead to leukocyte recruitment (Figure 3). Signaling through other receptors, such as CD14 on monocytes and CD40 on B cells, also trigger integrin activation-induced adhesion (Humphries and Humphries, 2007; Léveillé et al., 2007).

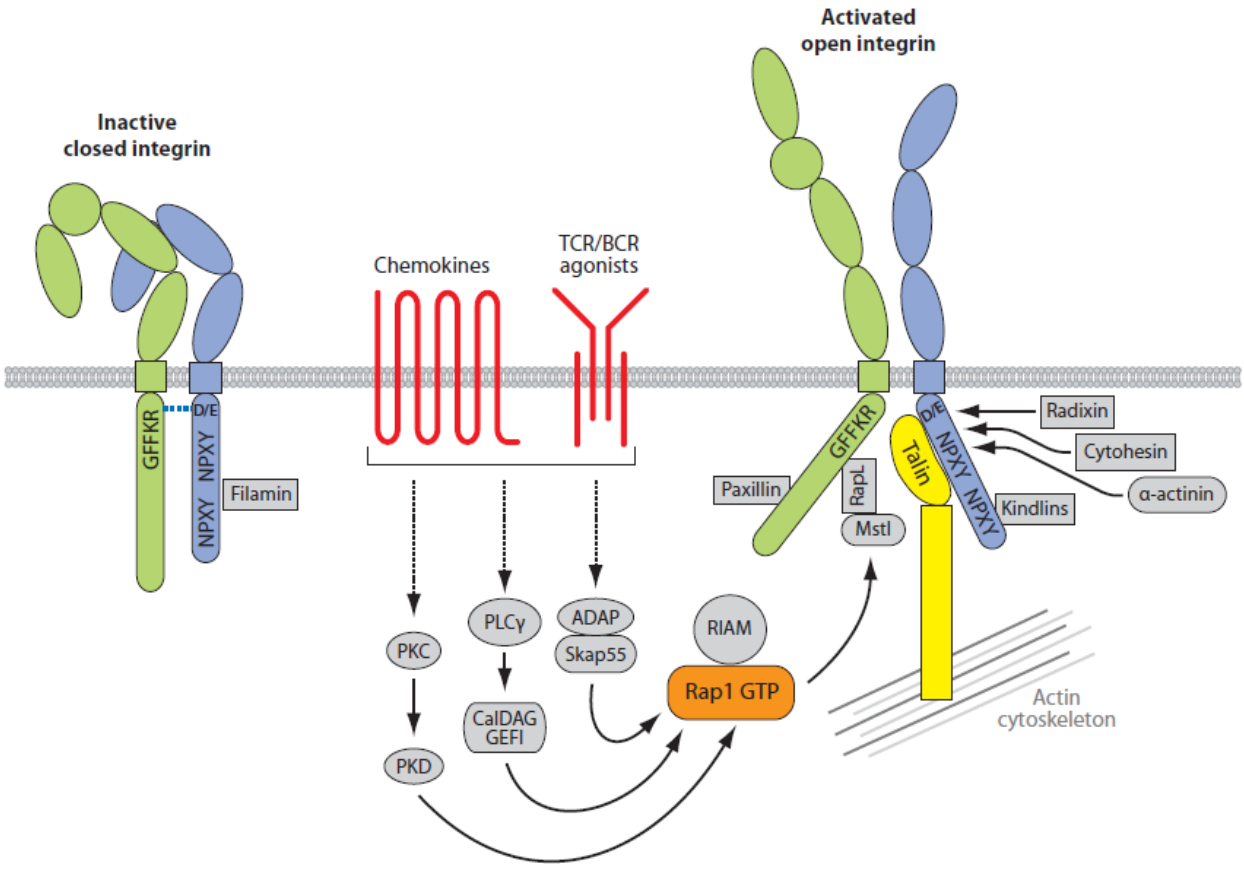

Figure 3. Integrin inside-out signaling (adapted from Abram and Lowell, 2009)

2.3.1.2 Intracellular signal transduction for inside-out signaling

Stimulation of GPCRs leads to a rapid activation of PLC signaling, which results in elevated intracellular Ca^{2+} level and the production of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). PLC-γ1 deficient Jurkat T cells showed a marked reduction of TCR-triggered adhesion to ICAM1 mediated by LFA-1 (Katagiri et al., 2004). Inhibition of PLC abolishes β_2-mediated neutrophil arrest on inflamed endothelium (Pasvolsky et al., 2007). Ca^{2+} and DAG activate guanine nucleotide exchange factors (GEFs), such as CalDAG-GEFI (calcium and DAG-regulated GEFI), which in turn allows the activation of small GTPase RAS-related protein (Rap) regulating the affinity of integrin under physiological conditions (Crittenden et al., 2004). As mentioned previously, E-seletin ligation induces integrin activation in mediating slow rolling through induction of PLC-γ2

and p38MAPK. It has been shown recently that E- selectin induced integrin activation is also CalDAG-GEF1 and Rap1 dependent (Stadtmann et al., 2011)

2.3.1.3 The key role of Rap GTPase in integrin activation

The Rap GTPases have been implicated as major regulators of the inside-out pathway in lymphocytes. Constitutively active mutants of Rap1 increase the affinity and avidity of LFA-1 on the lymphocyte membrane (Katagiri et al., 2000; Sebzda et al., 2002). Impaired activation of Rap1 is associated to the rare disorder of leukocyte adhesion deficiency III (LAD-III) in some patients (Kinashi et al., 2004). The leukocytes from these patients express normal levels of β_1, β_2 and β_3 integrin, but show impaired inside-out signaling (McDowall et al., 2003; Alon et al., 2003;). Patients with LAD-III syndrome suffer from severe and often life-threatening infections due to inefficient leukocyte recruitment. This syndrome demonstrates the importance of Rap1 in regulating integrin activation.

Besides GEFs, Rap1 can also be activated by protein kinase C (PKC), which is also responsive to Ca^{2+} and DAG. In platelets, rapid stimulation by chemokines activates Rap1 via CalDAG-GEF1, whereas sustained stimulation activates Rap1 via PKC (Cifuni et al., 2008). Protein kinase D1 (PKD1) has been demonstrated as the downstream signal for PKC induced-Rap1 activation. PKD1 recruits Rap1 to the membrane and forms a complex together with β_1 integrin cytoplasmic domain (Medeiros et al., 2005). Another pathway for Rap1 activation is through the adaptor adhesion- and degranulation- promoting adaptor protein (ADAP) and its binding partner SRC-kinase-associated protein of 55 kDa (SKAP55), which are recruited to Rap1 by Rap1-interacting adaptor molecule (RIAM) (Menasche et al., 2007).

How do Rap GTPases change the conformation of integrin? Integrins have a fairly short cytoplasmic tail. The integrin α cytoplasmic tail contains a conserved GFFKR sequence, which is crucial for a salt bridge-mediated interaction with the β subunits (Annemieke et al., 1997). Deletion or mutation of the GFFKR sequence impairs the association of α and β subunits and leads to constitutive integrin activation both *in vitro* and *in vivo* (Luo et al., 2007). It has been implicated that integrin activation is induced by phosphorylation and protein interaction with cytoplasmic tails of integrin α and β subunits, which induce the

conformational change in integrin subunits (Abram and Lowell, 2009). RIAM has been shown to recruit the big cytoskeleton protein talin to integrin β3-binding site (Watanabe et al., 2008). The direct binding of the FERM domain-containing talin-1 and kindling-3 proteins to two different NXX(Y/F) motifs of the integrin β cytoplasmic domain leads to integrin conformational changes which then propagate across the plasma membrane (Luo et al., 2007; Shattil et al., 2010). However talin-1 and kindling-3 perform differently in activating integrin. It has been shown that talin-1 is required for inducing LFA-1 extension, which relates to intermediate affinity and induced slow rolling, whereas both talin-1 and kindling-3 are required for inducing high-affinity open conformation which results in neutrophil firm adhesion and arrest (Lefort et al., 2012).

2.3.2 Outside-in signaling

The signaling triggered by ligand-induced clustering integrin in leukocytes is referred to as the outside-in signaling. As mentioned previously, integrin outside-in signaling has multiple cellular effects. In the process of leukocyte recruitment, two major functions of outside-in signaling are avidity regulation, which facilitates leukocytes adhesion, and cytoskeletal remodeling, which is involved in leukocytes crawling and transmigration (Abram and Lowell, 2009).

Outside-in signaling regulates integrin avidity through controlling the state of integrin clusters. Binding of LFA-1 to ICAM1 in T cells induces the formation of integrin microclusters which strengthen adhesion (Kim et al., 2004). Inactive LFA-1 is randomly distributed in macrophages, but upon ligation with ICAM1 LFA-1, forms nanoclusters and then macroclusters (Cambi et al., 2006).

It has been reported that Src family kinases and Syk kinase interact with the cytoplasmic domain of integrin β2 and β3 (Arias-Salgado et al., 2003). These enzymes become activated following integrin activation and often recruit more kinase molecules to the complex to initiate downstream signaling, such as Rho GTPase (Ivetic and Ridley, 2004; Kasirer-Friede et al., 2007). Rho GTPase family members, which include Rac, Rho, and Cdc42, are the primary effectors of outside-in signling, inducing the cytoskeletal rearrangements needed for firm adhesion, crawling and transmigration (Abram and Lowell, 2009; Shen et al., 2012). Among many of the Rho GTPase downstream effector

molecules, the Wiskott-Aldrich syndrome protein (WASp) plays a major role in integrin signaling in leukocytes. Deficiency of WAPs in both humans and mice leads to impaired leukocyte adhesion, poor spreading response, and reduced migration and lymphocyte activation (Notarangelo et al., 2008).

2.4 Involvement of Endothelial cells in leukocyte recruitment

Leukocyte recruitment is decisively dependent on signaling events of the interaction between activated tissue resident leukocytes/circulating leukocytes and endothelial cells.

2.4.1 Contribution of activated endothelial cells to leukocytes recruitment

Whereas leucocytes-endothelial interactions occur even without inflammation, such as during trafficking of T cells to secondary lymphoid organs and hematopoietic homeostasis, resting endothelial cells in other tissues do not interact with circulating leucocytes (Ley and Reutershan, 2006). This is because leukocyte-interactive proteins, such as P-selectin and chemokines, are sequestered in specialized secretory vesicles known as Weibel-Palade bodies in endothelial cells (Pober and Sessa, 2007). Transcription of other adhesion molecules, such as E-selectin, VCAM1 and ICAM1, are also suppressed due to the quiescent effect of basally produced nitric oxide (NO) in endothelial cells (De Caterina et al., 1995).

The inflammatory response is initiated mostly by stimulation of tissue resident innate immune cells to PAMPs or DAMPs, and results in the secretion of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, and many different chemokines. Secreted cytokines in turn induce the activation of endothelial cells in order to recruit more immune cells to the inflammation site.

Endothelial-cell activation can be classified into type I and type II activation response, according to the reaction rate to inflammation and induced gene expression (Pober and Cotran, 1990). Type I activation can be rapidly induced, but sustains shortly for only 10-12 minutes. It occurs by the binding of ligands to GPCRs on endothelial cells, such as histamine H1 receptor, resulting in downstream signals such as elevated Ca^{2+} and the

Rho pathway. Ca^{2+} elevation induces the exocytosis of Weibel-Palade bodies, which in turn brings P-selectin to the luminal surface of endothelial cells within minutes (Rondaij et al., 2006). Moreover, the rise of cytosolic Ca^{2+} also induces the formation of Ca^{2+}-calmodulin complex, which leads to the activation of myosin-light-chain kinase (MLCK). MLCK phosphorylates myosin light chain (MLC), and this phosphorylation is stabilized by Rho-dependent kinase-mediated inhibition of a phosphatase (Stevens et al., 2000). Phosphorylated MLC induces the contraction of actin filaments attaching to tight junction and adherence junction proteins, which in turn facilitates leukocyte transmigration by opening gaps between adjacent endothelial cells (Muller, 2003).

Type II activation of endothelial cells is mediated by TNF-α and IL-1β derived from activated leukocytes. Compared to type I activation, it is more persistent and induces sustained inflammatory response (Pober and Sessa, 2007). Binding of TNF-α and IL-1β leads to new gene transcription through activation of transcription factors of NF- κB and activator protein 1 (AP1) in mediating inflammatory response. For example, neutrophils recruitment is driven by chemokines and adhesion molecules, such as CXCL8 and E-selectin, that are synthesized and presented by endothelial cells via type II activation (Ley and Reutershan, 2006). Moreover, TNF-α and IL-1β open up gaps between adjacent cells and increase endothelial permeability to leukocytes via p38MAPK mediated microtubule rearrangement (Petrache et al., 2003).

2.4.2 Endogenous modulation of leukocytes recruitment by endothelial cells

Besides the effect of facilitating leukocytes recruitment by presenting adhesion molecules and chemokines, endothelial cells also secrete molecules which have inhibitory effects in modulating recruitment. One of it is Pentraxin 3(PTX-3). Several cells types release PTX-3 (in particular monocytes, dendritic cells, stromal cells and endothelial cells), in response to inflammatory signals such as TLRs engagement, TNF-α and IL-1β (Garlanda et al., 2005). It has been shown that PTX-3 inhibits neutrophil rolling by disrupting the interaction of P-selectin with its leukocyte ligand PSGL-1 via binding to P-selectin presented on endothelial cells (Deban et al., 2010).

Another modulator is the developmental endothelial locus (Del)-1, which is expressed by endothelial cells and associates with the endothelial cell surface. Del-1 was shown to be

a new ligand for LFA-1, but in contrast to ICAM-1 it antagonizes LFA-1 dependent adhesion onto endothelial cells (Eskan et al., 2012).

One more endothelial cell modulator is galectin-1. Galectin-1 shows an anti-inflammatory activity that inhibits neutrophil recruitment via downregulating the expression of adhesion molecules, such as integrin $\alpha_M\beta_2$ (Cooper et al., 2008; Gil et al., 2010).

2.5 Monocytes in innate response

In most cases, monocyte recruitment is the second wave of leukocyte recruitment in induced innate response. Monocyte recruitment plays an important role in clearance of viral, bacterial, fungal and protozol infections. It also contributes to the pathogenesis of inflammatory and degenerative diseases (Shi and Pamer, 2011).

2.5.1 Monocyte subtypes

Monocytes originate *in vivo* from hematopoietic stem cell-derived progenitors with myeloid-restricted differentiation potential. The top layers on the pyramid of monocytes differentiation include common myeloid progenitors (CMPs), granulocyte-macrophage precursors (GMPs), and macrophage/DC progenitors (MDPs) (Figure 4, Lawrence and Natoli, 2010). MDPs differentiate into monocytes and common dendritic cells (DCs) precursors (CDPs). Recently a new monocyte progenitor derived from MDPs, termed common monocyte progenitor (cMoP), has been identified to be present in bone marrow and spleen, where it generates major monocyte subsets (Hettinger et al., 2013). Monocytes give rise to tissue-resident macrophages and DCs after they penetrate into the inflamed tissue from blood.

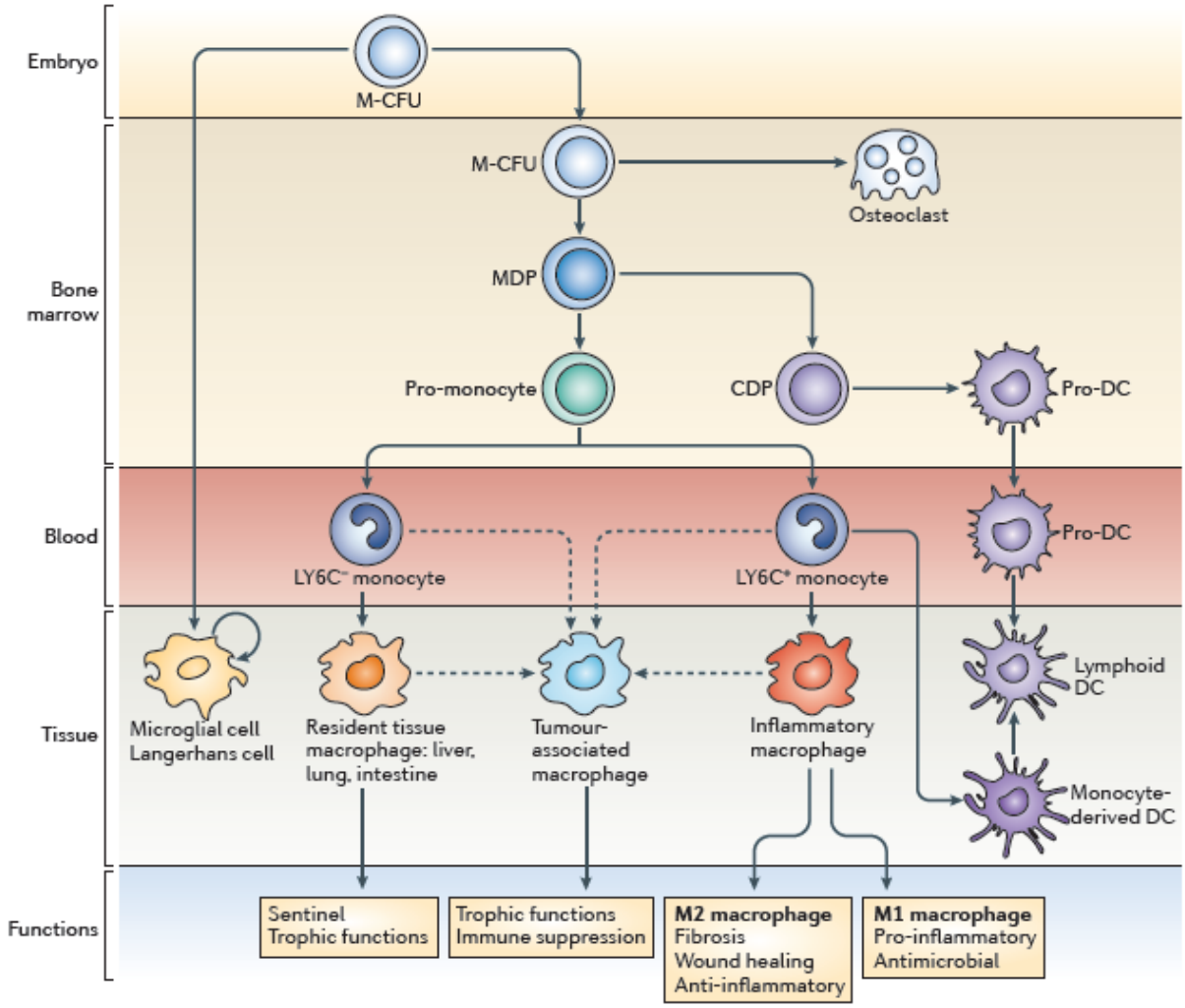

Figure 4. Monocytes development in mice. (Adapted from Lawrence and Natoli, 2010.)

In mice, monocytes are classified into two types according to the differences in expression of chemokine receptors and other surface markers. Monocytes express high levels of Ly6C and CC-chemokine receptor 2 (CCR2) but low levels of CX_3C-chemokine receptor 1 (CX_3CR1) are referred to as inflammatory or $Ly6C^{hi}$ monocytes. On the other hand, monocyte subtypes which express high level of CX_3CR1 but low levels of Ly6C and CCR2 are referred to as $Ly6C^{low}$ monocytes (Geissmann et al., 2003, 2010). Besides hematopoietic progenitors, $Ly6C^{hi}$ monocytes constitute obligatory steady-state precursors for blood resident $Ly6C^{low}$ monocytes (Yona et al., 2012).

Human monocytes are divided into three subtypes based on the surface expression levels of CD14 and CD16. $CD14^{++}CD16^{-}$ monocytes are referred to as classical

monocytes, which are similar to the mouse Ly6C^{hi} monocytes (Ziegler-Heitbrock, 2007). The CD16^{+} monocytes comprise two subtypes, the CD14^{+}CD16^{++} and CD14^{++}CD16^{+} monocytes. The CD14^{+}CD16^{++} monocytes, known as non-classic monocytes, are similar to the mouse Ly6C^{low} monocytes according to function (Ingersoll et al., 2010).

2.5.2 Distinct roles in mediating inflammation

Ly6C^{hi} monocytes

Monocyte subtypes respond differently and have distinct functions in infection or injury-induced inflammation. CC-chemokines CCL2 and CCL7 bind to CCR2, mediating Ly6C^{hi} monocyte emigration from bone marrow to blood and recruitment from blood to inflammatory sites (Serbina and Pamer, 2006; Tsou et al., 2007; Shi et al., 2011). Similar to neutrophils, Ly6C^{hi} monocytes utilize selectin and LFA-1 mediated mechanism for recruitment (Shi and Pamer, 2011). Ly6C^{hi} monocytes are termed inflammatory monocytes as they produce TNF-α and IL-1 during infection or tissues damage (Auffray et al., 2009). Studies using either adoptive transfer of monocytes or latex bead-labeled monocytes demonstrated that at least a proportion of TNF-α-producing inflammatory DCs are originated from Ly6C^{hi} monocytes (Geissmann et al., 2003; Serbina et al., 2008). Especially during inflammation upon microbe infection, Ly6C^{hi} monocytes are predominantly recruited to the infected sites and give rise to TNF- α and iNOS (inducible nitric oxide synthase)-producing (TIP) DCs for the clearance of microbes (reviewed by Shi and Pamer, 2011). Apart from differentiation into the DCs in infected tissues, recruited monocytes also mediate direct antimicrobial activity at these sites (Serbina et al., 2008).

In addition to the roles in infectious inflammation, Ly6C^{hi} monocytes are also involved in mediating sterile inflammation such as trauma, atherosclerosis and ischemia-reperfusion injury (as in cases of stroke and myocardial infarction) (Spahn and Kreisel, 2014). Inflammatory monocytes have been demonstrated to be recruited to the ischemic liver via CCR2 axis and mediate inflammation by generating ROS, TNF-α and IL-6 (Bamboat et al., 2010). In experimental atherosclerosis, Ly6C^{hi} monocytes start differentiating into macrophages after adhering to activated endothelium and inducing the accumulation of macrophages, which is decisive in the development and exacerbation of atherosclerosis

(Swirski et al., 2007). Moreover, Ly6C^{hi} monocytes also participate in the injury induced inflammation in the central nervous system. In a stroke model of intracerebral hemorrhage, Ly6C^{hi} monocytes are recruited to the brain and produce TNF to contribute to acute neurological disability (Hammond et al., 2014). Following traumatic brain injury, a rapid increase in synthesis and release of CCL2 into the cerebrospinal fluid (CSF) by the choroid plexus epithelium has been observed (Szmydynger-Chodobska et al., 2012). Monocytes transmigrating through the blood-CSF barrier along the paracellular pathway have also been shown in this report.

Ly6C^{low} monocytes

Whereas Ly6C^{hi} monocytes selectively traffic to the site of inflammation, Ly6C^{low} monocytes patrol blood vessels and enter non-inflamed tissues in steady-state conditions depending on integrin LFA-1 and CX_3CR1 (Geissmann et al., 2003). This patrolling behavior is required for rapid tissue invasion at the site of infection, where the monocytes initiate an early immune response and differentate into macrophages (Auffray et al., 2007). Recent findings reveal that Ly6C^{low} monocytes behave as “housekeepers” of the vasculature, patrolling and scanning capillaries and scavenging cellular debris in a TLR7 dependent manner (Carlin et al., 2013). Their human functional homologs, CD14^{dim}CD16^{+} monocytes (non-classical CD14^{+}CD16^{+} monocytes, as introduced previously), also demonstrate similar functions (Cros et al., 2010).

On the contrary, Ly6C^{low} monocytes perform anti-inflammatory roles. During spinal cord injury, infiltrating Ly6C^{low} monocytes are neural protective and contribute to recovery to the injury (Shechter et al., 2009; 2011). Ly6C^{low} monocytes are polarized and differentiate towards an “alternatively activated” or M2 anti-inflammatory macrophage phenotype in injured spinal cord (Kigerl et al., 2009), where they express IL-10 allowing an overall anti-inflammatory state. This limits lesion size and prevents activation of resident microglial cells (Shechter et al., 2009), and leads to secreteion of MMP-13, which enables dissolving of the glial scar to create a more permissive environment for axonal regeneration (Shechter et al., 2011). Interestingly, recruitment of Ly6C^{low} monocytes to the injured spinal cord is trafficked through a remote blood-CSF barrier, the brain-ventricular choroid plexus (Shechter et al., 2013). Moreover, during myocardial infarction, the acute inflammatory response in patients is accompanied with an early

peak of circulating inflammatory $CD16^-$ monocytes, which is followed by an increase in circulating $CD16^+$ monocytes (Tsujioka et al., 2009). This kinetic of monocyte recruitment also appears in mouse models of myocardial infarction. Whereas the first wave of $Ly6C^{hi}$ monocytes facilitates the removal of dead cardiac myocytes, the later wave of $Ly6C^{low}$ monocytes promotes the attenuation of inflammation and tissue repair (Nahrendorf et al., 2007).

2.6 CD95 in inflammation

Recent works of our lab revealed that CD95 (also called Fas or Apo-1) mediates recruitment of myeloid cells to the inflamed site in a mouse model of spinal cord injury via the activation of Syk-PI3K-MMP9 signaling pathway (Letellier et al., 2010).

CD95 is well known as a death receptor mediating apoptosis in multiple cell types. Early monoclonal antibody screening studies led to the discovery of the apoptosis-inducing function of CD95. These studies showed that crosslinking of CD95 with a specific antibody triggered apoptosis in lymphocytes (Trauth et al., 1989; Yonehara et al., 1989).

2.6.1 CD95 as an apoptosis mediator

CD95 is a type I transmembrane receptor glycoprotein with a molecular mass of about 45-52 kDa. It belongs to the TNF receptor superfamily, which lacks any catalytic activity (Nagata and Golstein, 1995; Ashkenazi and Dixit, 1998). CD95 is termed death receptor together with other TNF family members, including TNFR1, avian CAR1, death receptor 3 (DR3), DR4, and DR5 (Ashkenazi and Dixit, 1998). The death receptors contain a homologous cytoplasmic sequence termed the “death domain” (DD), which binds to the DD in other proteins to form oligomers transducing a death signal (Weber and Vincenz, 2001).

Upon ligation with CD95 ligand (CD95L), CD95 molecules are brought together in a permissive environment dependent on factors such as lipid rafts and membrane constitution. The close proximity of CD95 DD leads to the stabilization of an open conformation of the intracellular tail, allowing interaction between CD95 molecules to

form a CD95-CD95 bridge. The CD95-CD95 bridge links a trimeric DD, which can recruit the DD bearing adaptor molecule Fas-Associated-Death-Domain (FADD, MORT-1) via homologous interactions and further stabilize the bridge (Scott et al., 2009). Then the death-effector-domain (DED) containing proteins, procaspse-8 and procaspse-10, are recruited to FADD by binding to its DED. These signal events lead to the formation of a death-inducing signaling complex (DISC) (Peter and Krammer, 2003; Strasser et al., 2009; Hughes et al., 2009. Figure 5).

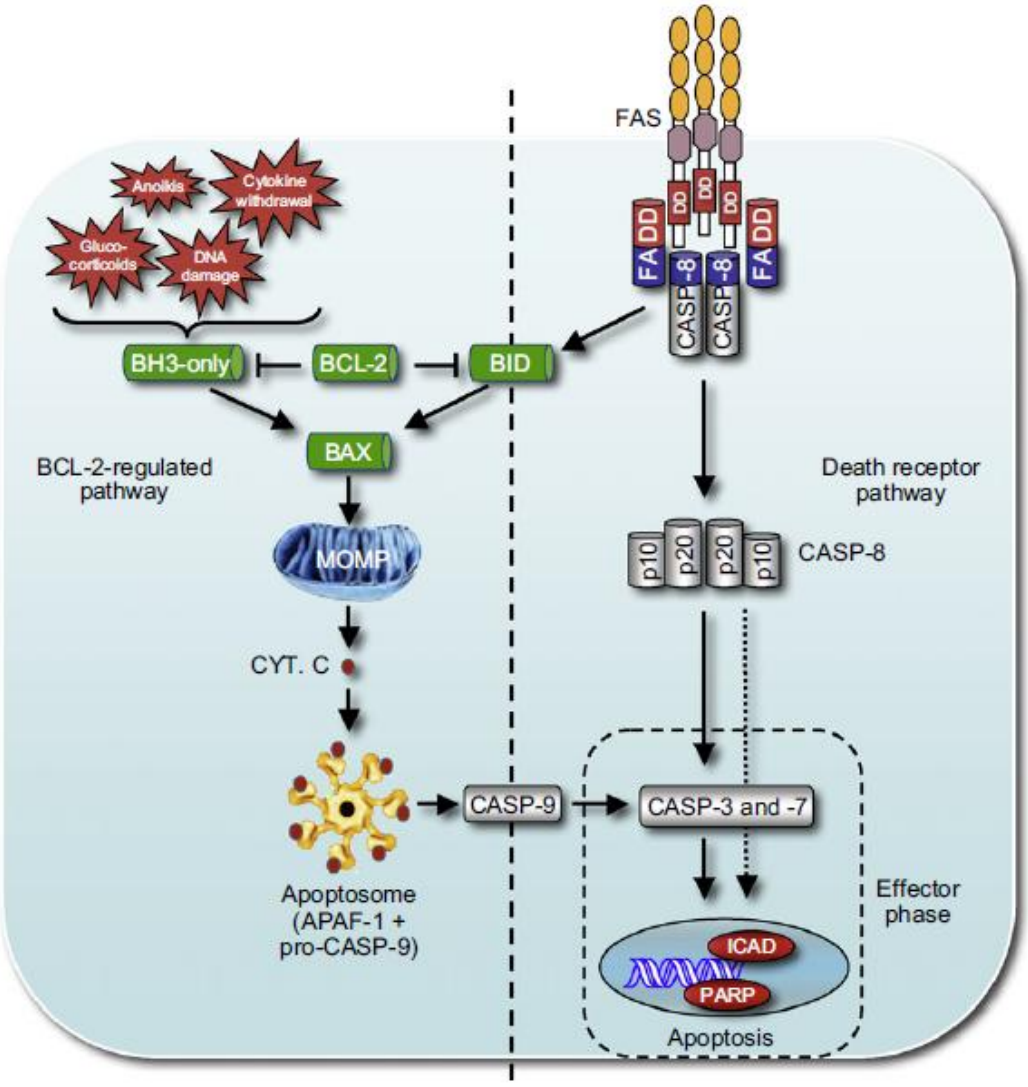

Figure 5. Death receptor-induced apoptosis signaling pathways. (Adapted from Strasser et al., 2009)

Subsequently, recruited procaspase-8 molecules in the DISC are oligomerized and activated through self-cleavage, leading to apoptosis by activating down-stream caspases. CD95 induces apoptosis through both the type I and type II pathway. In type I cells, such as activated lymphocytes, CD95 stimulation recruits high amount of procaspase-8, resulting in direct cleavage and full activation of the effector caspase, caspase-3. However in type II cells, such as hepatocytes and pancreatic β-cells, caspase cascade amplifies through caspase-8-mediated activation of pro-apoptotic BCL-2 family member BID (BH3 interacting doamain death agonist), resulting in the translocation of truncated BID (tBID) to the mitochondria. Interaction of tBID with the BCL2 proteins BAX and BAD leads to the release of cytochrome c and apoptotic protease-activating factor-1 (APAF1) from the mitochondria. Then APAF1 binds to cytochrome c and procaspase-9 to form the apoptosome, triggering the cleavage of caspase-3 and subsequently inducing apoptosis (Krammer, 2000).

CD95 mediates apoptosis in multiple cell types. In the immune system, it is involved in regulating lymphocyte maturation, repertoire selection and homeostasis (Krammer, 2000; Strasser et al., 2009). Mice carrying homozygous mutations in the genes encoding CD95 (*Fas*$^{lpr/lpr}$ or *Fas*$^{lprcg/lprcg}$) or CD95L (*FasL*$^{gld/gld}$) develop lymphadenopathy and SLE (systemic lupus erythematosu)-like autoimmune diseases (Watanabe-Fukunaga et al., 1992; Takahashi et al., 1994). In patients with autoimmune lympho-proliferative syndrome (ALPS) type Ia, heterozygous inherited mutations of CD95 gene was observed (Fisher et al., 1995). These discoveries demonstrate the critical role of CD95 in the immune system. In FADD dominant negative mice, T cell progenitor survival and differentiation into more mature pre-T cells are promoted through bypassing the selection by pre-TCR signaling (Newton et al., 2000). In mature T cells, CD95 triggers apoptosis of activated T lymphocytes. This process is termed activation-induced cell death (AICD) which plays an important role in down-regulating the immune response and the clearance of autoreactive T cells in the peripheral (Alderson et al., 1995; Green et al., 2003). Moreover, cytotoxic T cells or natural killer cells express membrane-bound CD95L to induce apoptosis of target cells such as virus-infected or damaged cells (Nagata and Golstein, 1995; Strasser et al., 2009).

2.6.2 CD95 as an inflammatory mediator

In addition to the apoptotic functions, CD95 was believed to be an anti-inflammatory molecule as it mediates the immune privilege in eye, thyroid and testis; and it alleviates inflammation by inducing AICD of activated T cells (Bellgrau et al., 1995; Griffith et al., 1995; Nagata, 1999). In line with this idea, the increased expression of CD95L in solid tumors was interpreted as a way for tumor cells to eliminate the tumor-infiltrating lymphocytes, as T cells are more sensitive to CD95-induced apoptosis, which in turn results in immune privilege for tumor cells (Green and Ferguson, 2001). Unexpectedly, the tumors overexpressed CD95L by transfection were rejected more easily after transplantation due to the high infiltration of neutrophils and other granulocytes (Arai et al., 1997; Seino et al., 1997). Instead of triggering apoptosis, CD95 ligation induces production of pro-inflammatory mediators, such as TNF-β, IL-8, IL-β, CCL2, CXCL1, CXCL3, high mobility group box 1 (HMGB1) and MMP-9, through the caspase-dependent or independent activation of AP-1 or NF-κB pathways in a variety of cell types (Park et al., 2003; Farley et al., 2006; Altemeier et al., 2007; Dupont et al., 2007; Matsumoto et al., 2007; Wang et al., 2010; Lee et al., 2011). Interestingly, a recent report shows that cells sustaining CD95-induced apoptosis release multiple cytokines and chemokines, including IL-6, IL-8, CXCL1, CCL2 and GMCSF, through RIPK1-dependent NF- κB activation to promote chemotaxis of phagocytes toward them. In this context, factors released by CD95 induction serve as “find-me” signals in the clearance of apoptotic cells (Cullen et al., 2013). Moreover, the downstream molecule of CD95, FADD is shown to be necessary in the production of type I (α/β) interferons upon dsRNA virus induced innate immune response, although the involvement of CD95 activation in this process still need to be elucidated (Balachandran et al., 2004).

CD95 is also involved in the post-translational regulation of cytokines. IL-1β family cytokines require proteolysis to gain biological activity dependent on inflammasomes controlled caspase-1. It is shown that CD95 signaling in myeloid cells activates caspase-8, leading to the maturation of IL-1β and IL-18 independently of inflammasomes or RIP3 (Bossaller et al., 2012).

In addition to mediating inflammation via inducing production and maturation of cytokines and chemokines, CD95 also acts directly on immune cells as a

chemoattractant. As shown in a previous *in vitro* study, soluble CD95L induced the transmigration of neutrophils in a transwell assay (Dupont et al., 2007). A more recent and interesting study shows that CD95L expression is triggered in peripheral myeloid cells upon injury. CD95L stimulation in myeloid cells activates PI3K and MMP-9 via recruitment and activation of Syk kinase, leading to the recruitment of myeloid cells to the injury site (Letellier et al., 2010).

2.6.3 Other non-apoptotic functions of CD95

Accumulating evidences demonstrate that CD95 also has important non-apoptotic functions, such as mediating cell survival, proliferation and migration, mostly through the activation of three major MAPKs, c-JUN N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), NF- κB and PI3K pathways (Wajant et al., 2003; Peter et al., 2007; Martin-Villalba et al., 2013).

In an early study, it has been demonstrated that CD95 induces proliferation in the presence of TCR stimulation (Alderson et al., 1993). Surprisingly, T cell proliferation driven by co-stimulation of CD95 and TCR relies on caspase activation without induction of apoptosis (Kennedy et al., 1999). Among other effects, TCR stimulation leads to the up-regulation of CD95L (Nagata and Golstein, 1995). These evidence point out an autocrine loop formed by TCR-induced CD95L and CD95-triggered T cell proliferation. In line with this, FADD deficient or dominant-negative mice show impaired TCR-induced proliferation (Zhang et al., 1998; Newton et al., 1998).

CD95 also plays as a proliferative role in liver regeneration after partial hepatectomy (Desbarats and Newell, 2000). It is believed that anti-apoptotic signaling pathways (AKT, STAT3 and NF-κB), which are important for liver regeneration, provide protection against CD95-mediated cell death and switch CD95-mediated signaling from apoptotic to non-apoptotic (Peter et al., 2007). Due to activation of NF-κB, CD95 is also reported to increase motility and invasiveness in tumor cells that resist CD95-induced apoptosis (Barnhart et al., 2004). The switch from inducing apoptosis to activation of NF-κB seems to rely on a threshold of CD95 signaling, as it has been demonstrated that heterozygous mutations in the CD95 DD lead to the inability of CD95 to induce apoptosis while still being able to efficiently activate NF-κB, ERK and P38 (Legembre et al., 2004).

In the neural system, CD95 activation induces neurite growth *in vitro* through the ERK pathway and the subsequent upregulation of neurite outgrowth mediator p35 (Desbarats et al., 2003). Instead of triggering apoptosis, CD95 engagement also activates ERK pathway in neural stem cells (Tamm et al., 2004). Moreover, CD95 activation in cultured neurons increases neurite branches in a caspase-independent and death domain-dependent manner. In an *in vivo* scenario, $Fas^{lpr/lpr}$ and $FasL^{gld/gld}$ mutants exhibit a reduced number of dendritic branches (Zuliani et al., 2006).

CD95-induced apoptosis is regularly accompanied with CD95-mediated p38 and JNK activation (Wajant et al,. 2003). Activation of p38 and JNK seems to be dependent on the activity of caspase upon CD95 stimulation (Deak et al., 1998; Low et al., 1999). It has been shown that CD95-mediated JNK/ AP1 (activator protein-1) pathway is involved in pressure overload-induced cardiac hypertrophy, which is a result of adaptive growth of the heart in response to mechanical stress (Wollert et al., 2000; Badorff et al., 2002). This response and JNK activation was completely abrogated when cardiomyocytes from $Fas^{lpr/lpr}$ mice were stimulated with CD95L (Badorff et al., 2002). Importantly, a recent work shows that CD95 has a growth-promoting role during tumorigenesis via the activation of JNK and JUN pathway. Loss of CD95 in mouse models of ovarian cancer and liver cancer reduces cancer incidence as well as tumor size (Chen et al., 2010).

Moreover, PI3K activation is also important for CD95-mediated non-apoptotic functions. Upon central nervous system injury, CD95 signaling leads to increased neural stem cell survival and neuronal differentiation via activation of the Src/PI3K/AKT/mTOT pathway (Corsini et al., 2009). In glioblastoma, CD95L is highly expressed by tumor cells and cells within the surrounding brain parenchyma. Stimulation of CD95L on glioblastoma cells recruits the Src family member Yes and PI3K to CD95, which leads to tumor cell invasion via the glycogen synthase kinase 3-β (GSK-3 β) and subsequent expression of MMP (Kleber, 2008). In addition, as mentioned previously, CD95 mediates myeloid cell recruitment via the Syk/PI3K/MMP9 pathway (Letellier et al., 2010).

2.7 Aim of the study

CD95 plays an important role in mediating myeloid cell recruitment to the inflammatory site (Letellier et al., 2010). In this study, we showed recruitment of Syk to CD95 upon CD95L stimulation and activation of the PI3K-MMP9 pathway which ultimately led to the migration of myeloid cells. As leukocyte recruitment is orchestrate by a cascade of sequential cellular processes including slow rolling, leukocyte activation and firm adhesion, and transendothelial cell migration, it remains unclear whether CD95 is also involved in regulating the early processes of rolling and firm adhesion. Neutrophils slow rolling is triggered by selectin signaling-induced integrin activation via the Syk-BTK-PLCγ-2-Rap1GTP pathway (Mueller et al., 2010; Yago et al., 2010). As CD95 activates Syk, it is of interest to investigate whether CD95 can also activate the Syk-BTK-PLCγ-2-Rap1GTP-integrin pathway in mediating myeloid cell slow rolling and adhesion.

To test whether CD95 can induce the rolling signaling, we firstly examined the phosphorylation of Syk, BTK and PLCγ-2 upon CD95L treatment in naïve cells, *Syk-/-* cells and BTK antagonist-pretreated cells. Subsequently, the effects of soluble or immobilized CD95L on neutrophil rolling were tested in an autoperfused mouse flow chamber assay. In addition, CD95 signaling-triggered integrin activation was investigated by Rap1 activation assay, active integrin reporter antibodies binding assay and soluble ICAM1 binding assay. We also examined the association of CD95 with BTK or integrin $\alpha_L\beta_2$. As endothelial cells are involved in leukocyte recruitment, we evaluated the contribution of these cells to CD95-dependent recruitment of myeloid cells. CD95L or CD95 deletion in endothelial cells were induced in *CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ or *CD95*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ mice, and myeloid cell recruitment in these mice was determined in a peritonitis model. Furthermore, the roles of CD95 signaling in mobilization and recruitment of inflammatory monocytes were examined *in vivo*.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

Chemical / Reagent / Kit	Source
acrylamide solution	Roth, Germany
Active Rap1 Pull-Down and Detection Kit	Thermo SCIENTIFIC, Germany
Amerham Hyperfilm ECL	GE Healthcare, Germany
ammonium persulphate (APS)	Merck, Germany
BCA Protein Assay	Pierce, Germany
Bovine serum albumin (BSA)	Sigma, Germany
brome phenol blue	Merck, Germany
BTK inhibitor PCI-32765 (Ibrutinib)	Biocat, Germany
Casein sodium salt from bovine milk	Sigma, Germany
collagenase I	Worthington Biochemical, USA
Complete protease inhibitor	Roche, Germany
disodium hydrogen phosphate (Na2HPO4)	Sigma, Germany
DAPI	Sigma, Germany
DMEM	Invitrogen, Germany
DNase I	Roche, Germany
Endothelial cell growth stimulant	Biomedical Technologies, USA
enhanced chemoluminescence substrate (ECL)	Perkin Elmer, USA
ethanol	Sigma, Germany
ethylene diamine tetraacetate (EDTA)	Sigma, Germany
fetal calf serum (FCS)	Biochrom, Germany
FASER Kit – APC	Miltenyi Biotec, Germany
Fluoromount-G	SouthernBiotech, Germany
glycerol	Sigma, Germany
glycine	Sigma, Germany
β-glycerophosphate	Sigma, Germany
Hank's Balanced Salt Solution (HBSS)	Invitrogen, Germany
Heparin	Sigma, Germany
HEPES	Invitrogen, Germany
Histopaque 1119	Sigma, Germany
Hybridoma-SFM	Invitrogen, Germany
hydrochloric acid (HCl)	VWR, Germany
Ketamine	Pfizer, Germany
L-Glutamine	Invitrogen, Germany
Liberase TM Research Grade	Roth, Germany
magnesium chloride (MgCl2)	Merck, Germany

methanol	Sigma, Germany
Nitrocellulose Membrane	Millipore, Germany
Nonessential amino acid	Invitrogen, Germany
Nonidet P-40 (NP-40)	Fisher Scientific, Germany
paraformaldehyde (PFA)	AppliChem, Germany
Penicillin/Streptomycin	Invitrogen, Germany
Percoll	GE Healthcare, Germany
phosphatase substrate (1mM),	Sigma, Germany
p-nitrophenylphosphate	Sigma, Germany
potassium chloride (KCl)	Merck, Germany
rat serum	Jackson ImmunoResearch, USA
RBC Lysis Buffer	eBioscience, USA
Recombinant Human E-Selectin	R&D Systems, Germany
Recombinant Moue E-Selectin	R&D Systems, Germany
Recombinant Mouse ICAM1	R&D Systems, Germany
Recombinant Mouse TNF-alpha	R&D Systems, Germany
Rompum	BAYER, Germany
RPMI	Invitrogen, Germany
Saponin	Sigma, Germany
skim milk powder	Roth, Germany
sodium azide	Sigma, Germany
sodium chloride (NaCl)	Sigma, Germany
sodium dihydrogen phosphate (NaH2PO4)	Sigma, Germany
sodium dodecyl sulphate (SDS)	Sigma, Germany
sodium fluoride (NaF)	Sigma, Germany
sodium orthovanadate	Sigma, Germany
sodium pyrophosphate	Sigma, Germany
Sodium pyruvate	Invitrogen, Germany
ß-Mercaptoethanol	Invitrogen, Germany
Super Frost slides	VWR, Germany
TEMED	Sigma, Germany
TGX Gels 4-20%	Bio-Rad, Germany
Thioglycollate	Fluka, Germany
tools for mouse surgery	Fine Science Tools, Germany
Tris base	GERBU Biotechnik, Germany
Tris HCl	GERBU Biotechnik, Germany
Trypsin	Invitrogen, Germany
Tween-20	Sigma, Germany

3.1.2 Buffers and Solutions

3.1.2.1 FACS staining and immunocytochemistry staining

PBS (20x)

NaCl	160g/l
Na_2HPO4	23g/l
NaH_2PO4	4g/l
KCl	4g/l

FACS staining buffer

PBS	1x
FCS	5%
sodium azide	1g/l

FACS staining blocking buffer

PBS	1x
rat serum	5%
sodium azide	1g/l

Erythrocytes lysis buffer

RBC Lysis Buffer	1x

Peritoneal lavage buffer

PBS	1x
EDTA	25m*M*

3.1.2.2 Protein extraction

0.2% SDS buffer

SDS in water	0.20%

NP-40 Lysis buffer

Tris-HCl	20m*M*
KCl	10m*M*
EDTA	1m*M*
NP-40	0.1%
Glycerol	10%
Protease Inhibitor Cocktail	1x
sodium orthovanadate	1x
phosphatase inhibitor Cocktail	1x

3.1.2.3 Stock solutions for inhibitors (in ddH_2O)

Sodium orthovanadate (100x)

sodium orthovanadate	200n*M*

Protease Inhibitor Cocktail (50x)

1 Protease Inhibitor Tablet in 1ml ddH_2O

Phosphatase inhibitor Cocktail (10x)

NaF	100m*M*
NaN3	100m*M*
p-nitrophenylphosphate	100m*M*
sodium pyrophosphate	100m*M*
β-glycerophosphate	100m*M*

3.1.2.4 Western Blotting

PBS-Tween (0.1%)

PBS	1x
Tween-20	1ml/l

Sample Buffer

1M Tris-HCl (pH7.4)	125 ml/l
glycerol	200 ml/l
β-mercaptoethanol	100 ml/l
SDS	40 g/l
brome phenol blue	50 mg/l

Running Buffer

Tris Base	10 g/l
glycine	30.28 g/l
SDS	150 g/l

Transfer Buffer

Tris Base	3 g/l
glycine	14.4 g/l
methanol	200ml/l

Lower Tris Buffer (4x)

Tris Base	181.7 g/l
SDS	4 g/l
Concentrated HCl	135ml/l

Upper Tris Buffer (4x)

Tris Base	60.6 g/l
SDS	4 g/l

Running Gel (per 10ml)

	10%	12%
dH_2O	4.1ml	3.5ml
Lower Tris Buffer	2.5ml	2.5ml
30% Acrylamide	3.4ml	4ml
TEMED	10µl	10µl
10% APS	100µl	100µl

Stacking Gel (per 10ml)

dH_2O	6.35ml
upper Tris Buffer	2.5ml
30% Acrylamide	1.15ml
TEMED	10µl
10% APS	100µl

Stripping Buffer

Mild stripping

Glycine	1M (PH1.8)

Harsh stripping (50°C for up to 45 minutes)

10% SDS	20ml
Tris HCl (pH 6.8, 0.5 M)	12.5ml
ß-mercaptoethanol	0.8ml
ddH_2O	67.5ml

3.1.2.5 Cell culture medium

L929, WEHI3B, U937 cells

RPMI 1640

FCS	10%

KIM127 cell

Hybridoma-SFM

Macrophages

RPMI 1640 advanced Medium (+ non-essential amino-acids & sodium pyruvate)

FCS	10%

ß-Mercaptoethanol (55mM)	0.1%
Penicillin/Streptomycin	1%
L-Glutamine	1%
L929 supernatant	20%

Neutrophils

RPMI 1640	
FCS	10%
Penicillin/Streptomycin	1%
WEHI3B conditioned medium	20%

Endothelial cells

DMEM	
FCS	20%
Penicillin/Streptomycin	1%
L-glutamine	1%
HEPES	25m*M*
Heparin	100µg/ml
Endothelial cell growth stimulant	100µg/ml
Nonessential amino acid	1%
Sodium pyruvate	1%

3.1.2.6 Antibodies

Primary antibodies

Antibody	**Source**	**Application and Concentration**
anti-phospho-Syk	Cell Signaling, USA	WB, 1:1000
anti-phospho-BTK	Cell Signaling, USA	WB, 1:1000
anti-phospho-PLC-γ2	Cell Signaling, USA	WB, 1:1000
anti-Syk	Cell Signaling, USA	WB, 1:1000
anti-BTK	Cell Signaling, USA	WB, 1:1000
anti-BTK (D3H5) biotin	Cell Signaling, USA	IP, 1:100 (5µg for 500µg lysate)
anti-PLC-γ2	Cell Signaling, USA	WB, 1:1000
anti-CD95 (M20)	Santa Cruz Biotechnology, USA	WB, 1:1000
anti-CD95 (Apo-1-1)	Enzo Life Sciences, USA	IF, 1:100
anti-CD95 biotin (Jo2)	BD Pharmingen, Germany	FC, 1:100
anti-CD95L biotin (MFL3)	BD Pharmingen, Germany	FC, 1:100
anti-Rap1	Thermo SCIENTIFIC, Germany	WB, 1:1000
anti-human CD11a (integrin α_L) biotin	eBioscience, USA	IF, 1:100

anti-mouse CD11a (integrin α_L) FITC	eBioscience, USA	FC, 1:100
anti-mouse CD11a (integrin α_L) biotin	eBioscience, USA	IP, 1:100 (5µg for 500µg lysate)
anti-mouse CD11a (integrin α_L)	Santa Cruz Biotechnology, USA	WB, 1:1000
anti-CD11b (integrin α_M) APC	BD Pharmingen, Germany	FC, 1:100
anti-CD45 APC-Cy7	BD Pharmingen, Germany	FC, 1:100
anti-Ly6G FITC	BD Pharmingen, Germany	FC, 1:100
anti-CD18 (integrin β_2) biotin	eBioscience, USA	FC, 1:100
anti-ICAM1 FTIC	eBioscience, USA	FC, 1:100
anti-ICAM2 FITC	eBioscience, USA	FC, 1:100
anti-E-selectin PE	BD Pharmingen, Germany	FC, 1:100
anti-P-selectin PE	BD Pharmingen, Germany	FC, 1:100
anti-CD31 FITC	eBioscience, USA	FC, 1:100
anti-CD31	BD Pharmingen, Germany	MACS, 1:100
anti-Human CD11/CD18 (mab24)	Hycult Biotechnology, USA	FC, 1:100

Secondary antibodies

Antibody	**Source**	**Application and Concentration**
anti-rabbit IgG HRP-conjugated	Jackson ImmunoResearch, USA	WB, 1:5000
Alexa Fluor® 647-Streptavidin	Jackson ImmunoResearch, USA	IF, 1:300
Alexa Fluor® 488 anti-rat IgG	Invitrogen, Germany	IF, 1:100
Dynabeads® Sheep Anti-Rat IgG	Invitrogen, Germany	MACS, 25µl for 10^7 cell
Dynabeads® M-280 Streptavidin	Invitrogen, Germany	IP, 40µl for 500µg lysate
Mouse Anti-Human IgG1 Fc-PE	SouthernBiotech,USA	IF, 1:100
anti-mouse IgG PE	eBioscience, USA	FC, 1:100
PE Cy7-Streptavidin	eBioscience, USA	FC, 1:300
APC-Streptavidin	eBioscience, USA	FC, 1:300

3.2 Methods

3.2.1 Animal experiments

3.2.1.1 Animals

C57BL/6N mice were purchased from Charles River Laboratories. Syk+/- mice were from Martin Turner (The Babraham Institute) and bred as heterozygous. CD95 floxed mice (University of Cologne) were bred with LysM-cre (Jackson Laboratory) mice and

VeCadherin$^{CreERT2/4}$ mice (Prof. Ralf H. Adams, University of Münster). CD95L floxed mice (a kind gift from Dr. Matthieu Lévi-Strauss) were bred with VeCadherin$^{CreERT2/4}$ mice. All animal experiments were performed in accordance with institutional guidelines of the German Cancer Research Center and were approved by the Regierungspräsidium Karlsruhe (Project Number: G188/13), Germany.

Mouse Line	**Description**	**Respective Controls**	**Experiments**
CD95$^{f/f;LysMcre}$	Deletion of CD95 in myeloid cells	cre- mice	Autoperfused mouse flow chamber assay
Syk-/-	Syk deletion in all cells	Wild-type mice	WB
CD95L$^{f/f;Ve\text{-}CadherinCreERT2/4}$	Inducible deletion of CD95L in endothelial cells	cre- mice	Thioglycollate-induced peritonitis
CD95$^{f/f;Ve\text{-}CadherinCreERT2/4}$	Inducible deletion of CD95 in endothelial cells	cre- mice	Thioglycollate-induced peritonitis

Table 1. Mouse lines used in this study

3.2.1.2 ERT2Cre-lox System

ERT2Cre-lox system is used for inducible tissue specific deletion of target genes (Figure 6, Kohan, 2008). In this system, the cre recombinase is fused to the ligand-binding domain of the estrogen receptor (LBD ER), and the expression of the fusion protein, CreERT2, is controlled by promoter of interest. In the absence of tamoxifen, CreERT2 is located in the cytoplasm. However in the presence of tamoxifen, CreER binds to tamoxifen and translocates to the nucleus, where it catalyzes recombination of the target DNA sequences flanked by loxP (lox) sites in the same orientation.

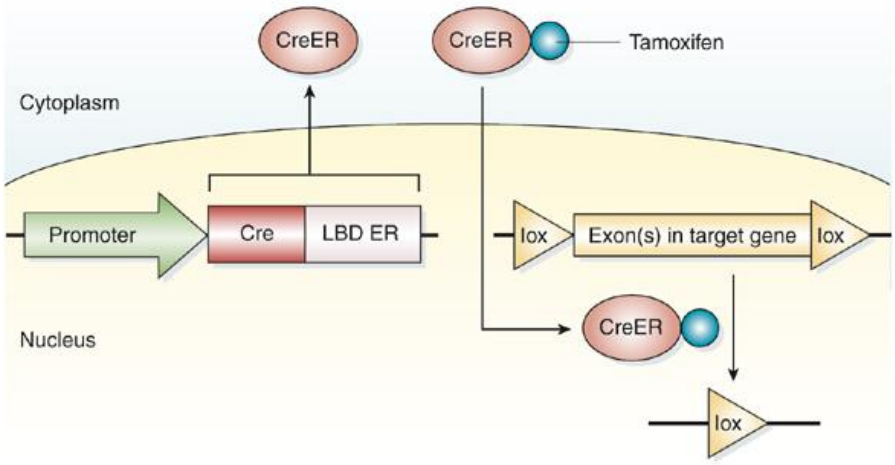

Figure 6 ERT2Cre-lox System (adapted from Kohan, 2008)

3.2.1.3 Autoperfused Mouse Flow Chamber Assay

3.2.1.3.1 Assembly of the flow chamber

Assembly of the flow chamber was modified from previously reported study (Chesnutt et al. 2006). Flow chambers were constructed from rectangular glass capillary tubing with dimensions of 20µm X 200µm (VitroCom, Mountain Lakes, NJ). Each chamber was cut to 30mm with a glass cutting stone and then placed between two pieces of glass coverslip (Menzel-Gläser, Germany) parallel to the lateral direction. Before fixing the chamber and coverslips with two component resin, the gap between flow chamber and coverslips was filled with immersion oil (AppliChen GmbH) to improve light transmission under the microscope. The free ends of the chamber were connected with a 2cm or 5cm PE 50 tubing (ID 0.58 mm, OD 0.965 mm, Becton Dickinson, Sparks, MD) individually. The connection point was sealed and fixed with two component resin. After the solidification of the resin, the chamber system was rinsed with ethanol and distilled water. To coat the flow chamber, a solution with different combinations of 15µg/ml ICAM1, 30µg/ml E-selectin and 10µg/ml CD95L in PBS was perfused through the chamber and the end parts of the flow chamber system were filled with distilled water to avoid the evaporation of the coating solution. Two hours after room temperature incubation, the flow chambers were rinsed with PBS following re-filling of 10% casein in PBS for one hour room temperature blocking. Chambers were washed with PBS and then rinsed with

10U/ml heparin. The 2cm PE tubing part of the chamber system was connected with a 150cm long, water-filled PE 50 tubing to control the shear stress in the flow chamber.

3.2.1.3.2 Visualizing and recording of rolling cells with microscopy

Male, 12 weeks old, wild-type (*wt*) mice or *CD95*$^{f/f;LysMcre}$ mice were used for autoperfused flow chamber assay. Mice were anesthetized by intraperitoneally (i.p.) injection of ketamine/rompum mixture (85 and 13 mg/kg) in saline and fixed on a paper board with sticky tape. The carotid artery was exposed and surgically sutured downstream. Afterwards the exposed artery was cannulated with a 7cm PE 10 tubing (ID 0.28 mm, OD 0.61 mm) which was pre-rinsed with heparin. The free end of the tubing was inserted into the 5cm PE 50 tubing part of the flower chamber system. The flow chamber was placed on top of the objective to visualize and video-record the rolling leukocytes (Figure 7, Olympus IX81 microscope). The water-filled PE 50 tubing was raised up to a height (stop point) at which the blood flow stopped in the flow chamber, then the tubing was put down for 30cm from the stop point. After 10 minutes of blood perfusion, one minute video (400 frames) each for three random fields of the flow chamber was recorded individually with Olympus scan^R software. Two flow chambers were used for measuring leukocytes rolling in each mouse and 3 to 4 mice were used for each group.

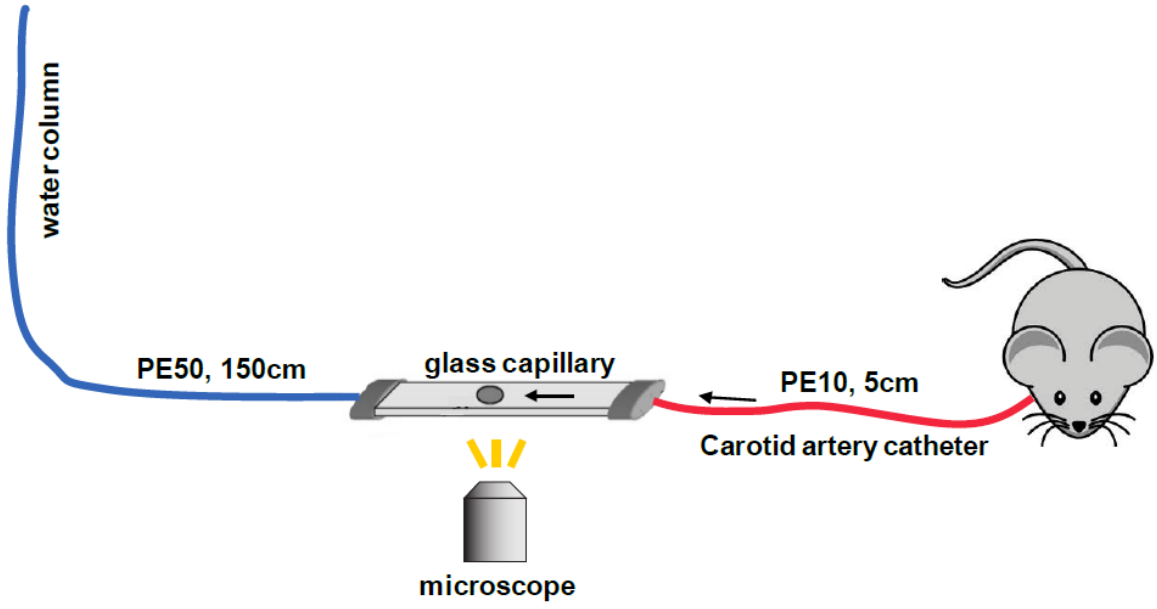

Figure 7. Scheme of the Autoperfused Mouse Flow Chamber System

3.2.1.3.3 Calculating rolling velocity

The rolling distance and the time of rolling cells were analyzed by ImageJ software, and the rolling velocity was calculated by dividing the rolling distance by the rolling time. Briefly, the rolling distance was assessed from the linear distance between the positions of each rolling cell in the start and end points of rolling. The time of rolling was assessed from the total number of frames of each rolling cell in the video.

3.2.1.4 CD95L treatment

To test the effect of soluble CD95L on leukocytes rolling in autoperfused mouse flow chamber assay, mice were intravenously (i.v.) injected with 10µg CD95L in 200µl PBS 30 minutes before connecting to the flow chamber system.

3.2.1.5 Thioglycollate-induced peritonitis

3% of thioglycollate was prepared in PBS and autoclaved 4-5 days before injecting to the mice. 1ml thioglycollate was i.p. injected to male, 12 weeks old, cre negative or positive mice of *CD95L*$^{f/f;Ve\text{-}CadherinCreERT2/4}$, *CD95*$^{f/f;Ve\text{-}CadherinCreERT2/4}$ mouse lines. It is reported that in the thioglycollate-induced peritonitis model, neutrophils recruitment peaks at 6 hours after injection whereas macrophage recruitment peaks at 72 hours (Matsukawa et al., 2005). At the indicated times, mice were sacrificed and peritoneal cells were collected by lavage with 10ml PBS containing 25m*M* EDTA. Total peritoneal cells were counted with a Neubauer hematocytometer, and the ratio of neutrophils to peritoneal cells was measured by flow cytometry.

3.2.2 Cell culture and isolation

3.2.2.1 Primary culture of macrophage from mouse bone marrow cells

Bilateral humeral, femoral and tibial bones from mice were harvested and the soft tissues on the bone were removed with a scalpel. The bones were cut off at the joint part and bone marrows were flushed out using a HBSS filled syringe connected to a 27G needle. Bone marrow cells were triturated and RBCs were lysed with 1xRBC Lysis buffer. Afterwards cells were washed, filtered through a 40µm strainer and plated in

culture flask with macrophage culture medium. On the next day, cells in suspension were transferred for further culture in petri dishes. At day 4 fresh medium was added, and after day 6 the culture medium was changed every two days. Macrophages were confluent and ready for use after 10 days *in vitro* culture. Medium was replaced to advanced RPMI (without FCS) 10 hours before stimulation.

3.2.2.2 Primary culture of Macrophage from mouse embryonic liver

Syk deficiency (*Syk-/-*) is perinatal-lethal in mice (Turner et al., 1995). Syk-/- embryos show petechiae, whereas *Syk+/-* embryos are indistinguishable from the *wt* embryo. In order to get *Syk-/-* macrophages, liver tissue was harvested from E15 embryonic livers of *wt* and *Syk-/-* embryos (embryos with petechiae). And liver cells were triturated, cultured and passaged in macrophage culture medium. After 6 days of *in vitro* culture, fetal liver hematopoietic stem cells differentiated into *Syk-/-* macrophages.

3.2.2.3 Isolation of neutrophils from mouse bone marrow cells by Percoll gradient

Neutrophil isolation is based on density gradient separation techniques (Siemsen et al., 2007). Firstly, Percoll gradients were prepared by layering 2 mL each of the 62, 55, and 50% Percoll solutions successively on top of 3 mL of 81% Percoll solution in a 15-mL falcon. Bone marrow cells were collected as mentioned above. Bone marrow cells were resuspended in 3 mL of 45% Percoll solution and carefully laid on top of the gradient (Figure 8). Afterwards the gradients were centrifuged at 1600g for 30 min with no braking during acceleration and deceleration at 10°C. The supernatant above the 62% Percoll layer was removed using a plastic transfer pipette. The cell layer located between the 81 and 62% Percoll layer was collected. Collected cells were washed and resuspended with 3ml neutrophils buffer and then laid on top of 3 mL of Histopaque 1119 in a falcon. The gradients were centrifuged at 1600g for 30 min at 10°C with no braking to remove contaminating red blood cells. The cell layer between the Histopaque and buffer layers was collected and washed with neutrophil buffer. Purity of neutrophils was assessed by FACS (fluorescence activated cell sorting) and reached >95%. Isolated neutrophils were cultured overnight with neutrophil differentiation medium to fully differentiate into mature neutrophils. Neutrophil differentiation medium contained 20% of WEHI-3B-conditioned medium which had been described to differentiate bone marrow

neutrophils (Lee et al., 1982; Garland et al., 1983). For production of WEHI-3B-conditioned medium cells were cultivated at density of 0.5 million/ml. After 1.5 day, the supernatant was collected and sterile filtrated.

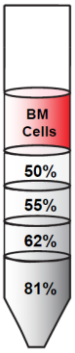

Figure 8. Scheme for Percoll gradient used in bone marrow neutrophils isolation

3.2.2.4 Isolation and culture of endothelial cells from mouse lung

Endothelial cell culture method was adapted from published protocol (Lim and Luscinskas, 2006). Mice were perfused with HBSS first and the lungs were dissected. Then the minced lung tissue was incubated with pre-warmed collagenase (10mg/ml), DNAse1 (1mg/ml) solution with gentle agitation for 45 minutes at 37°C. After digestion, tissue was triturated into single cell suspension with a 20-ml syringe connected to a 14-G metal cannula (Fisher Scientific) and cell suspension was washed and resuspended with endothelial cells culture medium. Anti-CD31 coated beads were added to every millilitre of cells suspension. After 10 min incubation at room temperature, anti-CD31 beads labelled endothelial cells were sorted with a magnetic separator. Positively selected cells were cultured with endothelial cells culture medium for 7-9 days. To improve the purity of cultured endothelial cells, primary cultured cells were detached with 0.5% trypsin and magnetically sorted with anti-CD102 beads. Sorted cells were cultured and passaged at a split ratio of no more than 1:3. Cultured endothelial cells showed cobblestone-like morphology. The purity was assessed by flow cytometry and reached >90%.

3.2.2.5 Dissociation of mouse liver cells for endothelial cell flow cytometry staining

Mice 12 weeks of age were transcardially perfused with HBSS and liberase in DMEM (5mg/ml). Minced liver tissue was incubated with liberase (5mg/ml), DNase1 (1mg/ml) in DMEM for 30 minutes at 37°C. After digestion, the tissue was triturated into single cell suspension and filtered through a 60µm strainer.

3.2.3 *In vitro* experiments

3.2.3.1 Integrin reporter antibodies binding assay

Integrin conformational change upon CD95L treatment was tested by staining with reporter antibodies recognizing specific epitopes of integrin at different statuses. To test the binding, U937 cells (10 million/ml) were premixed with anti-Human CD11/CD18 (mab24) or anti -Human CD11/CD18 (KIM127) and perfused through the human E-selectin coated flow chamber with a syringe pump (New Era Pump Systems, USA) at the flow rate of 3µl/min upon the stimulation with soluble CD95L (60ng/ml) or immobilized CD95L (10µg/ml for coating). The assembly and coating of the flow chamber was the same as described for the autoperfused mouse flow chamber assay. Cells flowed through the chamber were collected and fixed in 2% PFA. Then the fixed cells were stained with PE anti-mouse IgG and analyzed with flow cytometry.

3.2.3.2 Soluble ICAM1 binding assay

Soluble ICAM1 binding assay was performed as previously reported (Lefort et al., 2012). Bone marrow derived neutrophils were cultivated in RPMI (2% FCS) for 4 hours before stimulation. Cells were exposed to a CD95L coated or non-coated lipid membrane in the presence of ICAM1-FC (20µg/ml) and incubated at 37°C for 10 minutes. The lipid membrane was prepared as described (Kaindl et al., 2012). After incubation, cells were directly fixed with 2% PFA at 4 °C for 15 minutes, washed, and then stained with anti-human IgG1 (FC specific). The binding of soluble ICAM1 was measured by FACS.

3.2.3.3 Immunocytochemical staining and quantitative analysis

Immunocytochemical staining was performed to test the colocalization of CD95 and integrin upon CD95L treatment. U937 cells were prepared as in the integrin reporter antibodies binding assay. Fixed U937 cells were stained with anti-human CD95 (Apo-1-1) and biotinylated anti-human CD11a. Secondary antibodies used were anti-rat Alexa 488 and streptavidin- Alexa 647. Stained cells were scanned with a Leica SP5 confocal microscope. About 30 to 40 randomly selected cells from each group were analyzed for the colocalization of CD95 and integrin with the JACoP imageJ plugin according to the instruction.

3.2.3.4 Flow cytometry and cell type identification

The antibodies used for flow cytometry were described in the materials part. Staining were performed on blood cells, Percoll isolated neutrophils, peritoneal cells and cells dissociated from liver tissue.

Blood samples (100µl for each mouse) were collected from the retro orbital vein. Erythrocytes were lysed with RBC lysis buffer at room temperature for 10 minutes. For testing the cell surface level of CD95 in *CD95*$^{f/f;LysMcre}$ mice, blood cells were stained with DAPI (4',6-diamidino-2-phenylindole), anti-CD45 APC-Cy7, anti-Ly6G FITC, anti-CD11b APC and anti-CD95 (Jo2, followed with secondary staining of PE Cy7-streptavidin). For testing the cell surface integrin level of neutrophils after CD95L i.v. injection, blood cells were stained with anti-CD45 APC-Cy7, anti-Ly6G FITC, anti-CD11b APC and anti-CD11a biotin / anti-CD18 biotin (followed with secondary staining of PE-Cy7-streptavidin). The same antibodies were also used to test the cell surface integrin level of Percoll isolated neutrophils.

Peritoneal cells collected from lavage were stained with DAPI, anti-CD45 APC-Cy7, anti-Ly6G FITC, anti-CD11b APC to check the ratio of neutrophils.

To test the CD95L/CD95 deletion in endothelial cells of *CD95L*$^{f/f;Ve\text{-}CadherinCreERT2/4}$ / *CD95*$^{f/f;Ve\text{-}CadherinCreERT2/4}$ mouse lines, dissociated liver cells were stained with DAPI, anti-CD31 FITC, anti-CD45 APC-Cy7 and anti-CD95L biotin (MFL3, followed by secondary

staining of APC-streptavidin and APC-amplification staining with FASER-APC kit / anti-CD95 biotin (followed by secondary staining of PE Cy7-streptavidin).

To test the cell surface adhesion molecular level of endothelial cells from *CD95L*$^{f/f;Ve\text{-}CadherinCreERT2/4}$ / *CD95*$^{f/f;Ve\text{-}CadherinCreERT2/4}$ mouse lines, dissociated liver cells were stained with DAPI, anti-CD31 FITC, anti-CD45 APC-Cy7 and anti-ICAM1 FITC / anti-ICAM2 FITC / anti-E-selectin PE / anti-P-selectin PE.

Flow cytometry data were analyzed with Flowjo software. Neutrophils were identified according to the profile of Forward Scatter (FSC)/Sider Scatter (SSC), DAPI-negativity, and CD45, CD11b, Ly6G-positivity. Endothelial cells were identified according to the profile of FSC/SSC, DAPI, CD45-negativity, and CD31-positivity.

3.2.3.5 ICAM1 surface level on endothelial cells after CD95L treatment

The surface level of ICAM1 on endothelial cells after CD95L treatment was assessed by immunofluorecence staining. Primary cultured endothelial cells from mouse lungs were cultured in 96 wells plates. Cells were stimulated with 100ng/ml or 200ng/ml CD95L for 6 or 24 hours. Cells stimulated with 120ng/ml TNF-α for 6 or 24 hours were used as a positive control. At the indicated time points, cells were fixed with 2% PFA and stained with FITC conjugated anti-mouse ICAM1. ICAM1 level was measure with a Fluorescence Microplate Reader (Bio Tek).

3.2.3.6 Protein extraction and concentration determination

Cells were washed with PBS containing phosphatase inhibitors, pelleted, and lysed with SDS lysed buffer containing phosphatase inhibitors, proteinase inhibitors and vanadate for 30 minutes on ice. The protein concentration was determined using BCA protein assay by comparing to standardized concentrations of bovine serum albumin (BSA).

3.2.3.7 SDS-PAGE

Equal amounts of protein from cell lysates (20-50µg) in sample buffer were separated by sodiumdodecylsulphate- polyacrylamide gel electrophoresis (SDS-PAGE) on 10-12% polyacrylamide gels. Polymerization of the gels was initiated by addition of N,N,N',N'-Tetramethylethylenediamine (TEMED) and ammonium persulphate (APS) solution. The

cast running gel was overlaid with isopropanol and allowed to polymerize for 30 minutes. Then, isopropanol was removed with filter paper and the stacking gel cast in the same way. Afterwards, protein samples were loaded and electrophoresis was performed at 100V for 30 to 60 minutes.

3.2.3.8 Western Blotting

Proteins were transferred from polyacrylamide gels to nitrocellulose membranes by electroblotting. The gel and the membrane were placed between sheets of absorbent paper and immersed in transfer buffer in an electrophoresis tank. Blotting was performed at 60mA for 2 hours at 4°C. Following transfer, non-specific binding sites on the nitrocellulose membrane were blocked by incubation with 5% skim milk powder in PBS-Tween for 1 hour. Then the membranes were incubated overnight at 4°C with primary antibodies diluted in PBS-Tween containing 5% skim milk powder. Primary antibodies used are as follows: anti-phospho-Syk, anti-phospho-BTK, anti-phospho-PLC-γ2, anti-Syk, anti-BTK, anti-PLC-γ2, anti-CD95 (M20), anti-Rap1, anti-mouse CD11a (concentrations see table above). After thorough washing, antibody binding was detected via horseradish peroxidase (HRP)-conjugated secondary antibodies, with which the membranes were incubated for 1 hour at RT. The HRP signal was detected by incubation with ECL solution and subsequent exposure to Amerham Hyperfilm films.

3.2.3.9 Blot Stripping

For removal of antibody complexes from nitrocellulose membranes, they were subjected to three washes with 1M Glycine or harsh stripping buffer at 50°C for up to 45 minutes (listed in reagents part). After thorough washing with PBS-Tween and blocking, membranes were reprobed as described above.

3.2.3.10 Immunoprecipitation

CD95L treated or non-treated cells were washed with PBS containing phosphatase inhibitors, pelleted, and lysed on ice for 30 minutes with NP-40 Lysis buffer containing vanadate, inhibitors for phosphatase and proteinase (described in reagents part). Protein concentration of the lysate was determined as previously described. Lysates of 500 µg protein were used as input and the desired protein were immunoprecipitated

overnight at 4°C with the respective antibodies or the corresponding isotype controls. Afterward, 40 µl Dynabeads® M-280 Streptavidin was added to each sample and incubated for 1 hour at 4°C with rotation. Beads were washed 5 times with 1ml of lysis buffer. The immunoprecipitates were released by cooking the beads with 40 µl of 2x laemmli buffer at 95°C for 5 minutes. Immunoprecipitated samples were electrophoresed and blotted as described above.

3.2.3.11 Active Rap1 Pull-Down assay

Active Rap1 Pull-Down assay was performed according to the manufacturer's instructions. Cell lysates were prepared as described above. 100µl Glutathione Resin and 20µg of GST-RalGDS-RBD peptide were added to 500µg lysate. GTPγS and GDP incubated lysates were used as positive and negative control respectively. After one hour incubation with resin beads and peptide at 4°C, resin beads were washed 4 times,followed by incubation in 40 µl of 2x laemmli buffer at 95°C for 5 minutes. Immunoprecipitated samples were electrophoresed and blotted for anti-Rap1 as described above.

3.2.4 Statistical evaluation

Statistical analysis of all data was performed with GraphPad Prism (Version 5.01). Differences between the groups were evaluated by one-way ANOVA, Bonferroni multiple comparison post hoc test or student's *t* test where appropriate. All data were presented as mean ± standard error of the mean (SEM). Statistical significance was determined by the p-value of the statistical test and deemed as significant *$p < 0.05$; strongly significant **$p < 0.01$ and highly significant *** $p<0.001$.

4 RESULTS

4.1 CD95 activates rolling signaling

4.1.1 Phosphorylation of BTK and PLC-γ2 upon CD95L engagement

As described in the introduction, in rolling neutrophils E-selectin engagement triggers signaling cascades which cooperate with chemokine signals to facilitate neutrophil rolling and adhesion during inflammation (Zarbock et al., 2007). Upon engagement, E-selectin activates members of the SFKs, ITAM bearing adaptors, Syk, BTK, PLC-γ2, P38 and PI3Kγ (Mueller et al., 2010; Yago et al., 2010). We have previously shown that CD95L triggers the recruitment of myeloid cells to inflammatory sites in a spinal cord injury model via SFK-Syk-PI3K pathway (Letellier et al., 2010). In this study we checked whether the described CD95-pathway additionally leads to activation of BTK and PLC-γ2. To this end, we stimulated mouse macrophages cultured from bone marrow cells with CD95L and Phorbol 12-myristate 13-acetate (PMA) as a positive control. PMA is a phorbol ester that is commonly used to activate certain types of protein kinase C (PKC) which induces the activation of stimulated cells. The phosphorylation of BTK and PLC-γ2 is up-regulated after 5, 15 and 30 minutes of CD95L / PMA treatment (Figure 9).

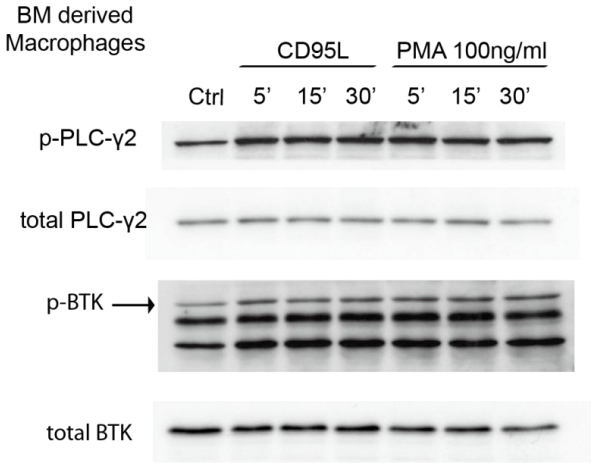

Figure 9. CD95 triggers phosphorylation of BTK and PLC-γ2 in bone marrow derived macrophages. Cultured Macrophages from mouse bone marrow cells were treated with CD95L (40ng/ml). Lysates were prepared at the indicated time points and immunoblotted for the indicated proteins.

4.1.2 CD95L engagement induced phosphorylation of PLC-γ2 is partially dependent on Syk activation

To assess the involvement of Syk in these signaling events we isolated macrophages from Syk knockout mice. *Syk-/-* macrophages can only be obtained from culture of embryonic liver cells due to perinatal-lethality (Turner et al., 1995). Macrophages were cultured from E15 embryonic livers of *wt* or *Syk-/-* embryos (embryos with petechiae). Upon CD95L treatment, the phosphorylation of PLC-γ2 was largely but not completely abolished by the lack of Syk (Figure 10 A and quantified analysis in Figure 10 B). However phosphorylation of BTK was still induced by CD95L treatment in *Syk-/-* cells as compared to *wt* cells (Figure 10 A and quantified analysis in Figure 10 C). These data indicate that CD95L-induced phosphorylation of PLC-γ2 is partially mediated via Syk.

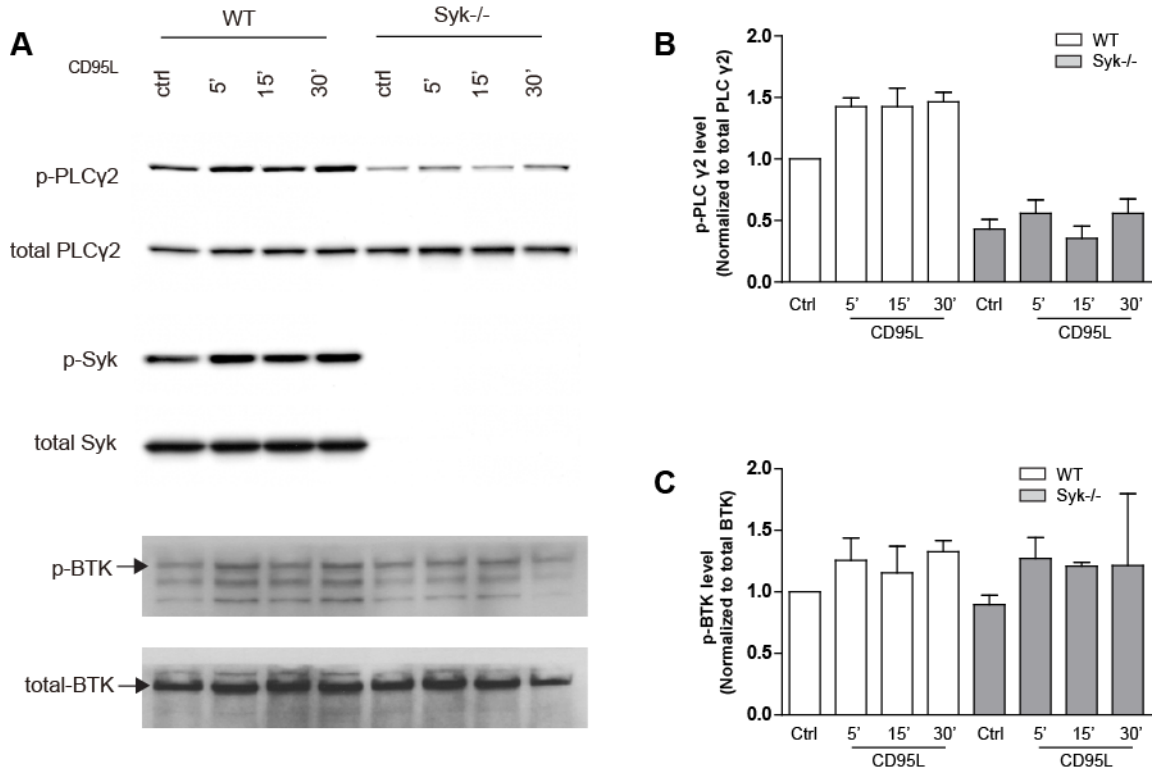

Figure 10. CD95L engagement induced phosphorylation of PLC-γ2 is partially dependent on Syk activation. (A) Cultured Macrophages from *wt* or *Syk-/-* embryonic liver cells were treated with CD95L (40ng/ml). Lysates were prepared at the indicated time points and immunoblotted for the indicated proteins. **(B)** Quantitative analysis of PLC-γ2 (B), and BTK (C) phosphorylation level in (A) from three independent experiments presented as mean ± SEM (n=3).

4.1.3 CD95 associates with BTK to activate PLC-γ2

BTK has been previously shown to bind to CD95 in B-cells via its kinase and Pleckstrin homology (PH) domain and thereby prevents the CD95-FADD interaction, which is essential for CD95 induced apoptosis signal (Vassilev et al., 1999). It is thus possible that in myeloid cells CD95 interacts directly with BTK and activates downstream signals, such as PLC-γ2, to induce myeloid cell recruitment.

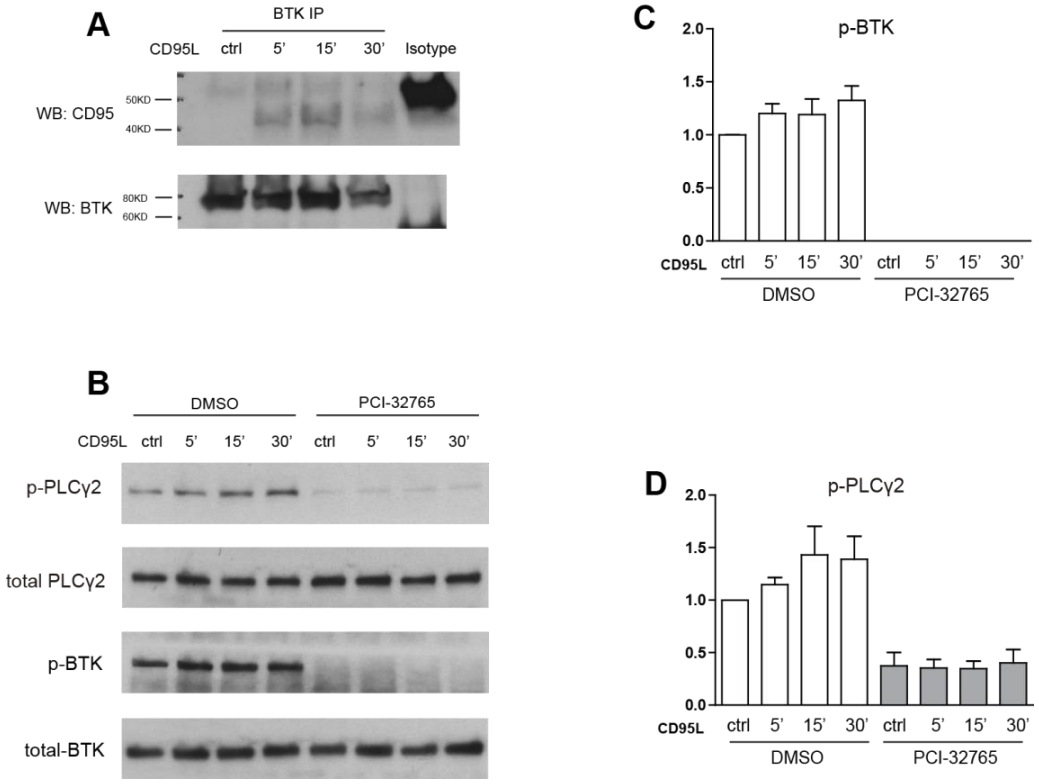

Figure 11. BTK binds to CD95 to induced phosphorylation of PLC-γ2 in myeloid cells. **(A)** Bone marrow-derived macrophages were treated with CD95L (40ng/ml). Lysates were prepared at the indicated time and immunoprecipitated with anti-BTK followed by immunoblotting with CD95 and BTK antibody. **(B)** Bone marrow-derived macrophages were treated with DMSO or BTK inhibitor PCI-32765 one hour prior to CD95L treatment (40ng/ml). Lysates were prepared at the indicated time points and immunoblotted for the indicated proteins. Quantitative analysis of BTK- **(C)** and PLC-γ2-phosphorylation **(D)** from three independent experiments presented as mean ± SEM (n=3).

To test this hypothesis we pulled down BTK by immunoprecipitation from CD95L- or control treated macrophages. Western blot analysis confirmed binding of CD95 to BTK following stimulation with CD95L (Figure 11 A). In order to further elucidate the involvement of BTK in CD95L-induced PLC-γ2 phosphorylation, macrophages were

exposed to the BTK inhibitor PCI-32765 (Ibrutinib) 1 hour prior to CD95L stimulation. Phosphorylation of BTK was fully blocked by PCI-32765 (Figure 11B, quantified analysis in C). Inhibition of BTK abolished the basal and CD95L-induced phosphorylation of PLC-γ2 (Figure 11 B, quantified analysis in D).

4.2 CD95 triggers neutrophil slow rolling

4.2.1 Autoperfused mouse flow chamber assay

In order to test the effect of CD95 activation in leukocyte slow rolling, the applied model of mouse autoperfused flow chamber was used. This is a well characterized model for studying neutrophil rolling and adhesion (Chesnutt et al., 2006). It has the advantage over the under flow static adhesion assays of studying neutrophils in whole blood, and therefore it avoids isolation-induced activation of neutrophils (Forsyth et al., 1990; Glasser et al., 1990). It has been reported that E-selectin mediates slow leukocyte rolling (Jung and Ley, 1999). In autoperfused mouse flow chamber assay, the rolling speed in E-selectin+ICAM1 coated chamber is significantly slower than the speed in chamber coated with E-selectin alone (Chesnutt et al., 2006; Zarbock et al., 2007). Using the LysMcre-GFP reporter mice, 89±2% of the rolling cells in the flow chamber have been identified as neutrophils (Chesnutt et al., 2006).

The flow chamber system was set up as described in the methods part. We first tested the neutrophil rolling in chambers coated with E-selectin or E-selectin+ICAM1. In E-selectin coated flow chamber the rolling velocity was 1.97±0.16µm/s. And in the presence of ICAM1 coating, the rolling velocity significantly decreased to 1.41±0.10µm/s (Figure 12 B). The rolling velocity is comparable to that previously reported data (Mueller et al., 2010). The number of rolling cells was similar in differently coated chambers (Figure 12 C, E-selectin chamber: 200±17, E-selectin+ICAM1 chamber 178±19). No rolling cells were observed in chambers coated with CD95L alone (data not shown).

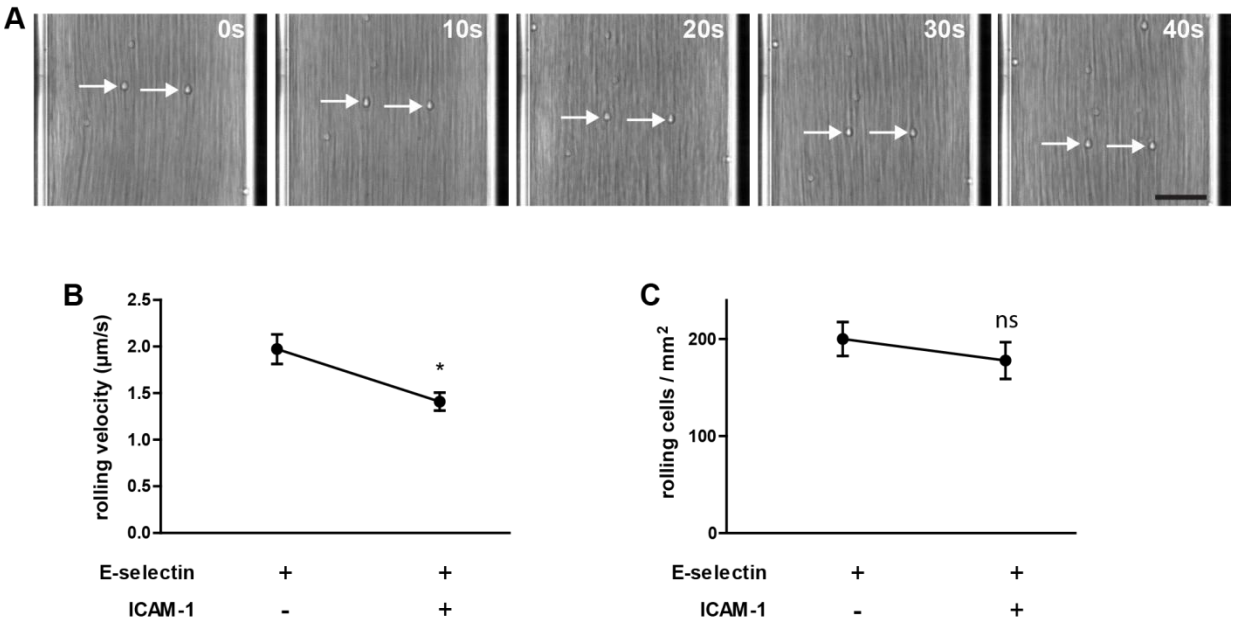

Figure 12. Mouse autoperfused flow chamber assay. The common carotid of mice was cannulated with a catheter connected to a flow chamber. The wall shear stress in the flow chamber was adjusted to 5.9 dyn/cm^2 by the water column connected to the downstream of the chamber system. **(A)** Time lapse showing the leukocytes rolling in flow chamber coated with E-selectin+ICAM-1. Arrows indicate the rolling cells. Scale bar: 50µm. The average rolling velocity **(B)** and number of rolling neutrophils **(C)** on E-selectin or E-selectin+ICAM-1 coated chambers is presented as means ± SEM. n=3, student's *t* test, *p<0.05, ns: not significant.

4.2.2 CD95L stimulation induces neutrophil slow rolling

In order to find out the involvement of CD95 in mediating neutrophil slow rolling, we performed all the autoperfused mouse flow chamber assay by using chambers coated with E-selectin and ICAM1. The rolling velocity was significantly reduced from 1.41±0.10 µm/s to 1.16±0.03 µm/s by intravenous tail (i.v.) injection of CD95L one hour prior to the assay compared to non-treated mice (Figure 13 A, C). CD95 activation has been reported to induce production of pro-inflammatory cytokines and chemokines in various cell types (Park et al., 2003; Altemeier et al., 2007). In order to exclude the possibility that CD95 mediates neutrophil slow rolling via inducing production of chemokines in other blood cells, mouse blood was perfused through the flow chambers coated with E-selectin, ICAM1 and CD95L. The rolling velocity of neutrophils in CD95L coated chamber was significantly lower than the control group (1.41±0.10 µm/s vs. 1.07±0.03 µm/s) (Figure 13 A, D). To further confirm that the observed effect was due to the action of CD95L on neutrophil's CD95, we used CD95-deficient myeloid cells (*CD95$^{f/f;LysMcre}$*). In

CD95$^{f/f;LysMcre}$ mice, coating with CD95L did not affect rolling (Figure 13 A, rolling velocity: 1.46±0.08 µm/s). We also observed increased number of rolling cells following injection of CD95L or coating with CD95L as compared to the control group(Figure 13 B). This effect was likewise attenuated in *CD95*$^{f/f;LysMcre}$ mice (Figure 13 B).

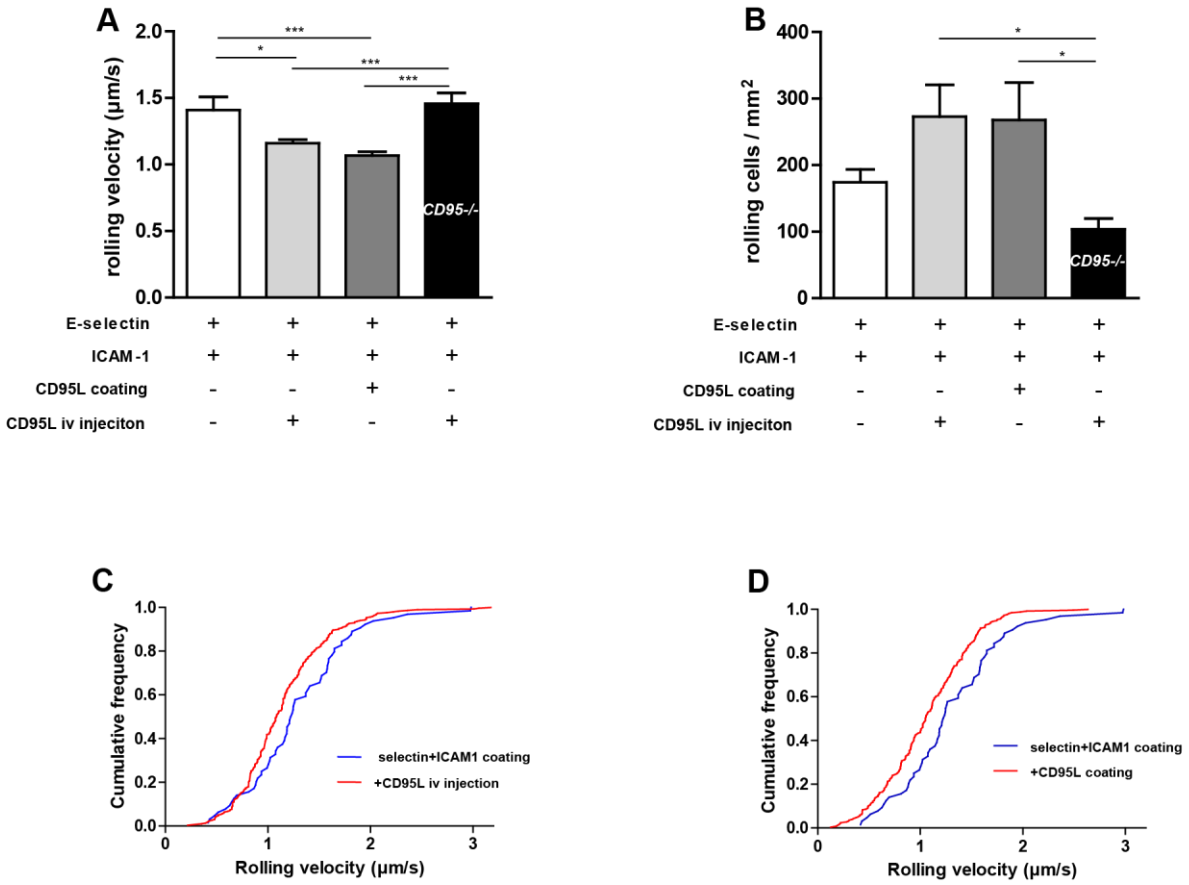

Figure 13. CD95L stimulation induces neutrophil slow rolling. The common carotid of *wt* or *CD95*$^{f/f;LysMCre+}$ mice was cannulated with a catheter connected to autoperfused flow chamber. The wall shear stress in the flow chamber was adjusted to 5.9 dyn/cm^2. The average rolling velocity **(A)** and number of rolling neutrophils **(B)** on E-selectin and ICAM-1 coated chambers with or without immobilized CD95L or after i.v. injection of CD95L is presented as means ± SEM. Two flow chambers were used for each mouse and 3 random fields from along the flow chamber were video recorded to obtain quantitative data from rolling cells. n=3-4, one-way ANOVA, Bonferroni multiple comparison post hoc test, *p<0.05, ***p<0.001. **(C, D)** Cumulative histogram shows velocity of rolling neutrophils in flow chamber coated with E-selectin+ICAM1 or E-selectin+ICAM1 following addition of immobilized CD95L **(C)** or soluble CD95L **(D)**.

4.2.3 CD95 activation or deficiency doesn't change integrin level

Neutrophil slow rolling is mainly mediated by activation of Integrin $\alpha_L\beta_2$ (LFA1) (Chesnutt et al., 2006 and Zarbock et al., 2007). In order to test whether CD95 signaling influences cell surface expression level of integrin, expression of integrin α_L, integrin α_M and integrin β_2 on neutrophils was assessed by flow cytometry in neutrophils isolated from vehicle- and CD95L- injected mice. Integrin levels were similar in neutrophils derived from control- and CD95L-injected mice (Figure 14 B, C and D).

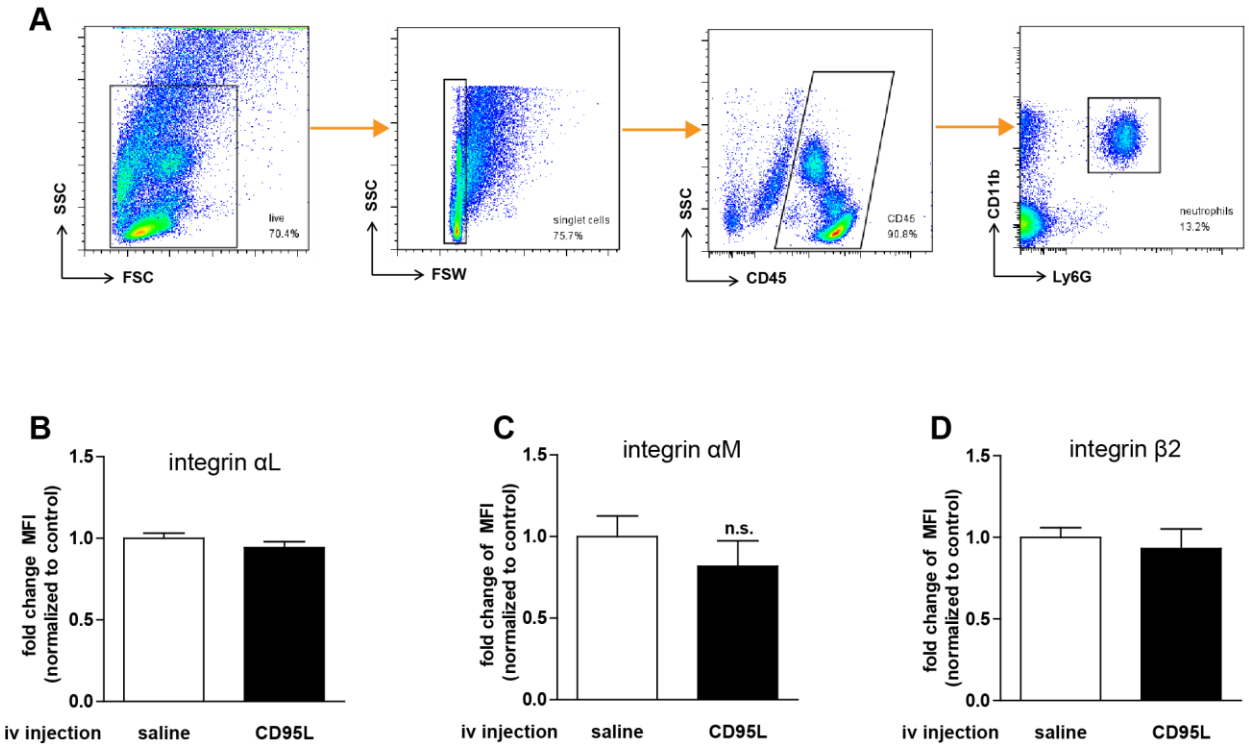

Figure 14. i.v. injection of CD95L does not influence neutrophil integrin levels. (A) Flow cytometry plot of blood neutrophils. Neutrophils were gated according to the profile of FSC/SSC, and CD45, CD11b, Ly6G positivity **(B-D)** Mice were i.v. injected with saline or CD95L (10µg). One hour later, blood samples were collected stained with antibodies of neutrophil markers and integrin subunits and analyzed by flow cytometry. Neutrophils expression levels of integrin α_L **(B)**, integrin α_M **(C)** and integrin β_2 **(D)** are presented as mean ± SEM. n=3, student's *t* test, ns: not significant.

We also checked the cell surface expression of integrin in neutrophils of *CD95*$^{f/f;LysMcre}$ mice. CD95-deficient neutrophils exhibited increased levels of integrin α_M and similar levels of integrin α_L and integrin β_2 when compared to *wt* neutrophils (Figure 15 C,D and E). Altogether these results show the effect of CD95 on slow rolling is not due to the change in cell surface expression levels of integrins.

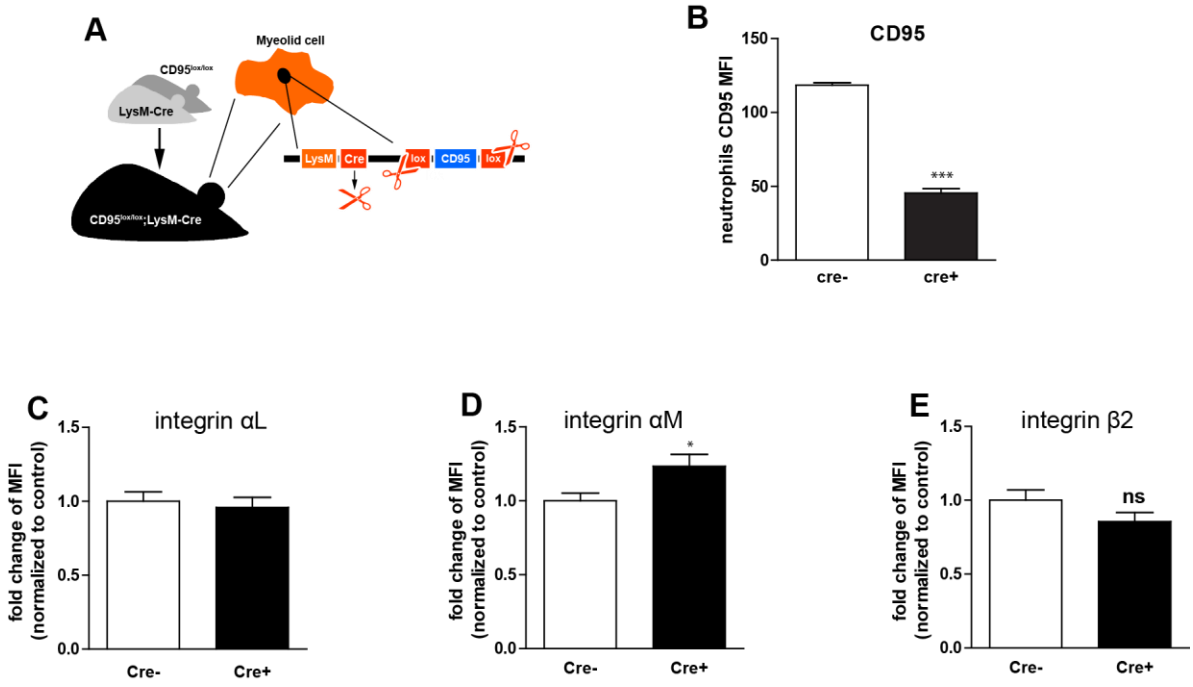

Figure 15. Deletion of CD95 in myeloid cells does not reduce neutrophil integrin levels. **(A)** Scheme of CD95 deletion in myeloid cells of *CD95*$^{f/f;LysMcre}$ mouse line. *CD95*$^{f/f}$ mice were crossed with *LysM*^{cre} mice to get CD95 deletion in myeloid cells. **(B)** Blood samples of *wt* or *CD95*$^{f/f;LysMcre}$ mice were stained with antibodies of neutrophil markers and CD95. Cell surface level of CD95 in cre- and cre+ neutrophils was analyzed by flow cytometry and presented as mean ± SEM. n=3, student's *t* test, ***p<0.001. **(C-E)** Blood samples of *wt* or *CD95*$^{f/f;LysMcre}$ mice were stained with antibodies of neutrophil markers and integrin subunits, and analyzed by flow cytometry. Neutrophils expression levels of CD11a **(C)**, CD18 **(D)** and CD11b **(E)** are presented as mean ± SEM (n=3, student's *t* test, *p<0.05, ns: not significant).

4.3 CD95 signaling activates integrin

4.3.1 Rap1 activation upon CD95L stimulation

The common final step for integrin activation is described as the Rap1a activation-dependent binding of talin1 to cytoplasmic domain of β integrin, which in turn induces the open conformational changes in integrin (Tadokoro et al., 2003; Wegener et al., 2007; Lefort et al., 2012). In order to find out whether CD95 signaling induces integrin activation, we first tested Rap1 activation upon CD95L stimulation in mouse bone marrow derived neutrophils by active Rap1 pull-down assay (Katagiri et al., 2004). In this assay, Rap1GTP was pulled down with GST-RalGDS-RBD peptide and assessed by western blot. For negative and positive controls, neutrophil lysates were treated with GDP and GTP to antagonistically bind Rap1 in the lysate before adding GST-RalGDS-RBD peptide. Rap1GTP level in GTP treated lysate was higher than in GDP treated lysate (Figure 16 A). Following CD95L stimulation, we observed significant activation of Rap1 after 15 min treatment in neutrophils (Figure 16 B, C).

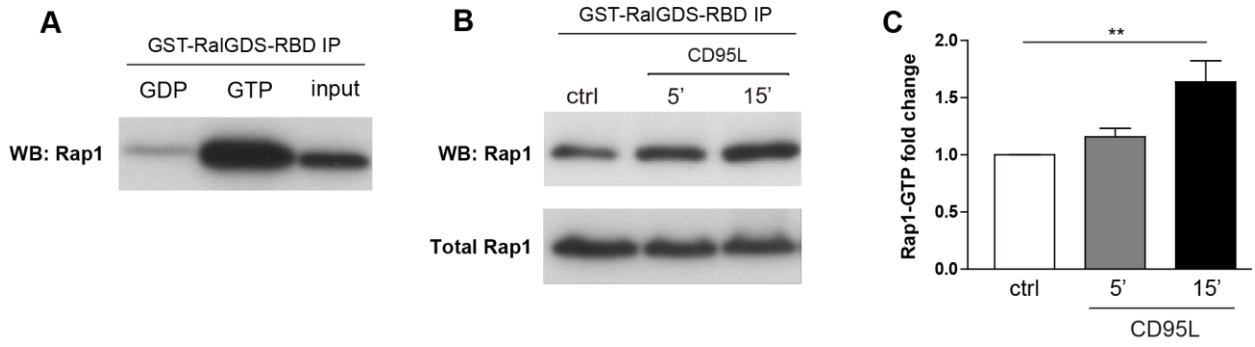

Figure 16. CD95L induces Rap1 activation. (A) Lysates from bone marrow-derived murine neutrophils were treated with GDP or GTP before the GST-RalGDS-RBD peptide affinity-precipitation. The immunoprecipitates were immunoblotted for Rap1. **(B)** Neutrophils were treated with CD95L (40ng/ml). Lysates were prepared at the indicated time points and affinity-precipitated with GST-RalGDS-RBD peptide for Rap1-GTP. The immunoprecipitates were immunoblotted for Rap1. **(C)** Quantitative analysis of Rap1-GTP activation in **(B)** from three independent experiments presented as mean ± SEM. n=3, one-way ANOVA, Bonferroni multiple comparison post hoc test, **p<0.01.

4.3.2 CD95L induces integrin activation

Different conformation status of LFA-1 can be recognized by integrin epitope specific antibodies. KIM127 recognizes an epitope of the β_2 subunit of human LFA-1 when it is extended (Beglova *et al.*, 2002), whereas mab24 binds to the epitope of I-like domain in the β_2 subunit of LFA-1 at high-affinity state (Lu *et al.*, 2001). To investigate CD95 induced integrin conformational changes, U937 cells which had been pre-incubated with the reporter antibodies were perfused through the flow chamber. Binding of reporter antibodies upon stimulation of soluble or immobilized CD95L was analyzed by flow cytometry. A significant increase of KIM127 and mab24 binding was observed in cells stimulated with soluble CD95L cells, which indicate the extension and full activation of integrin upon CD95L treatment (Figure 17 B, C).

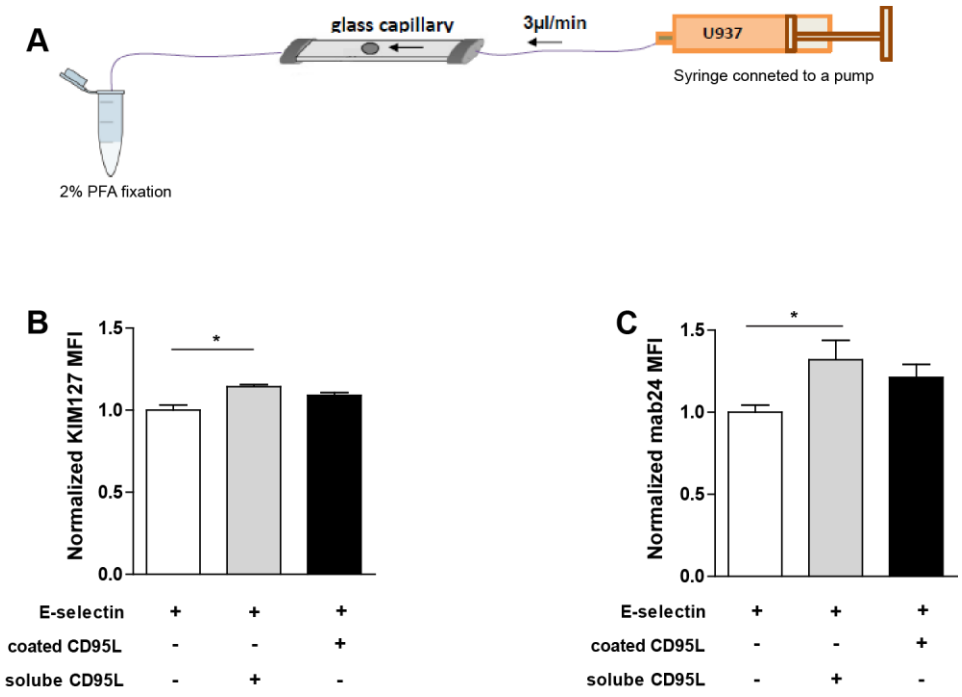

Figure 17. CD95L induces integrin activation. (A) Scheme of the flow chamber system used for integrin reporter antibodies assay. U937 cells were loaded to a syringe in the presence of reporter antibodies. Cells were perfused through the selectin or selectin + CD95L coated chamber and fixed directly in 2%PFA for further analysis. **(B-C)** Upon the treatment of soluble or immobilized CD95L, the binding of KIM127 **(B)** or mAb24 **(C)** was analyzed by flow cytometry and presented as mean ± SEM. n=3, one-way ANOVA, Bonferroni multiple comparison post hoc test, *p<0.05.

The soluble ICAM1 binding assay is another commonly used test for detecting the high affinity state of LFA-1 (Salas *et al.*, 2004; Lefort *et al.*, 2012). To further address the CD95L stimulation induced integrin activation, bone marrow derived neutrophils were incubated with ICAM1-Fc and the binding of ICAM1 was assessed with flow cytometry. CD95L activated neutrophils showed significant binding of soluble ICAM1 compared to the non-treated cells (Figure 18). Altogether these data indicate that activation of CD95 leads to activation of integrin.

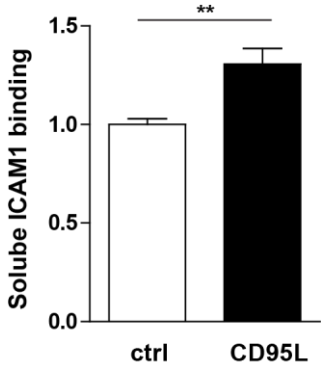

Figure 18. CD95 triggers the binding of solube ICAM1 in neutrophils. Mouse bone marrow derived neutrophils were stimulated with CD95L immobilized to lipid membranes for 10 minutes in the presence of 20µg/ml ICAM1-FC. Binding of ICAM1-FC was assessed with flow cytometry and presented as mean ± SEM. n=3, student's *t* test, **p<0.01

The cell surface levels of integrin α_L, integrin α_M and integrin β_2 were not changed in mouse bone marrow derived neutrophils after CD95L treatment. (Figure 19). This shows that CD95L stimulation induced soluble ICAM1 binding is not due to the up-regulation of integrin levels in neutrophils.

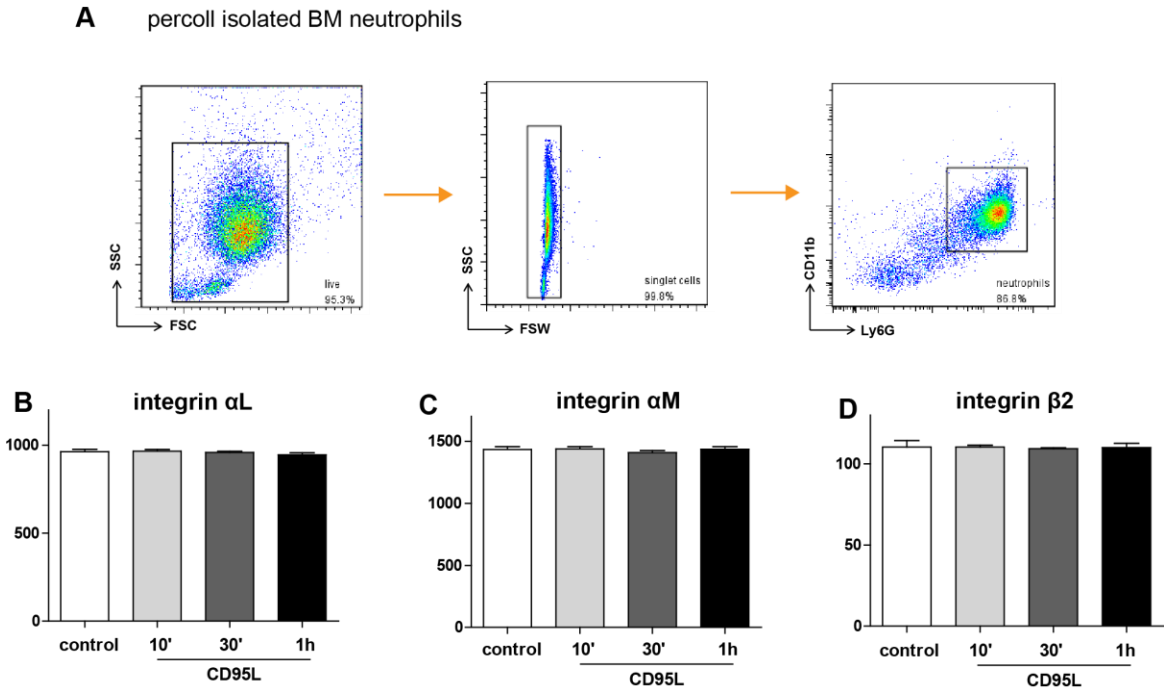

Figure 19. CD95L treatment does not influence integrin level in neutrophils in vitro. (A) Flow cytometry plot of percoll isolated-neutrophils from bone marrow. Neutrophils were gated according to the profile of FSC/ SSC, and CD45, CD11b, Ly6G positivity. **(B-D)** Bone marrow-derived neutrophils were treated with CD95L and fixed at the indicated time points. Fixed neutrophils were stained with antibodies to neutrophil markers and integrin subunits, then analyzed by flow cytometry. Neutrophils cell surface expression levels of integrin α_L **(B)**, integrin α_M **(C)** and integrin β_2 **(D)** are presented as mean ± SEM (n=3).

4.4 CD95 associates with integrin

4.4.1 Association of CD95 with integrin α_L upon CD95L treatment.

Compartmentalization of multi-molecular signaling complexes integrates extracellular signals and facilitates the activation of integrin (Bezman and Koretzky, 2007). CD44, one of the E-selectin ligand on neutrophil, has been shown to regulate CD95 via the formation of signaling complexes of CD44-erzin-actin-CD95 (Mielgo *et al.*, 2006; Mielgo *et al.*, 2007). It is possible that upon CD95L stimulation, CD95 assembles a signaling complex which associates with LFA-1 and coordinates with selectin signals to induce LFA-1 activation.

To test this hypothesis, immunoprecipitation of integrin α_L from CD95L treated mouse macrophages lysate was performed and we observed a CD95L stimulation-dependent association of CD95 and integrin (Figure 20).

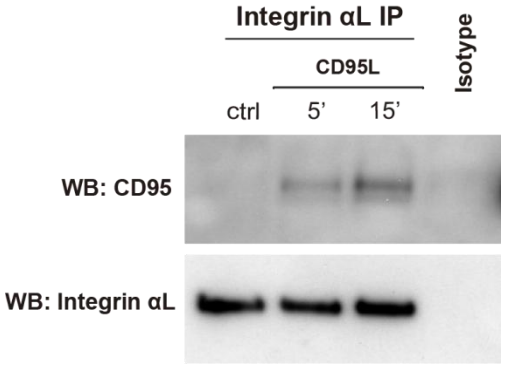

Figure 20. Association of CD95 with integrin α_L upon CD95L treatment. Bone marrow-derived macrophages were treated with CD95L (40ng /ml). Lysates were prepared at the indicated time and immunoprecipitated with anti-CD11a antibody followed by immunoblotting with CD95 and CD11a antibody.

4.4.2 Increased colocalization of CD95 and integrin upon CD95L stimulation

The colocalization of CD95 and integrin α_L was investigated by immunocytochemistry staining. We perfused control-, soluble or immobilized CD95L-treated U937 cells through an E-selectin coated flow chamber. Cells perfused through the chamber were fixed and immunostained for CD95 and integrin α_L (Figure 21 A). Increased colocalization of CD95 and Integrin α_L was observed in immobilized or soluble CD95L-treated cells as compared to control-treated ones (Figure 21 B).

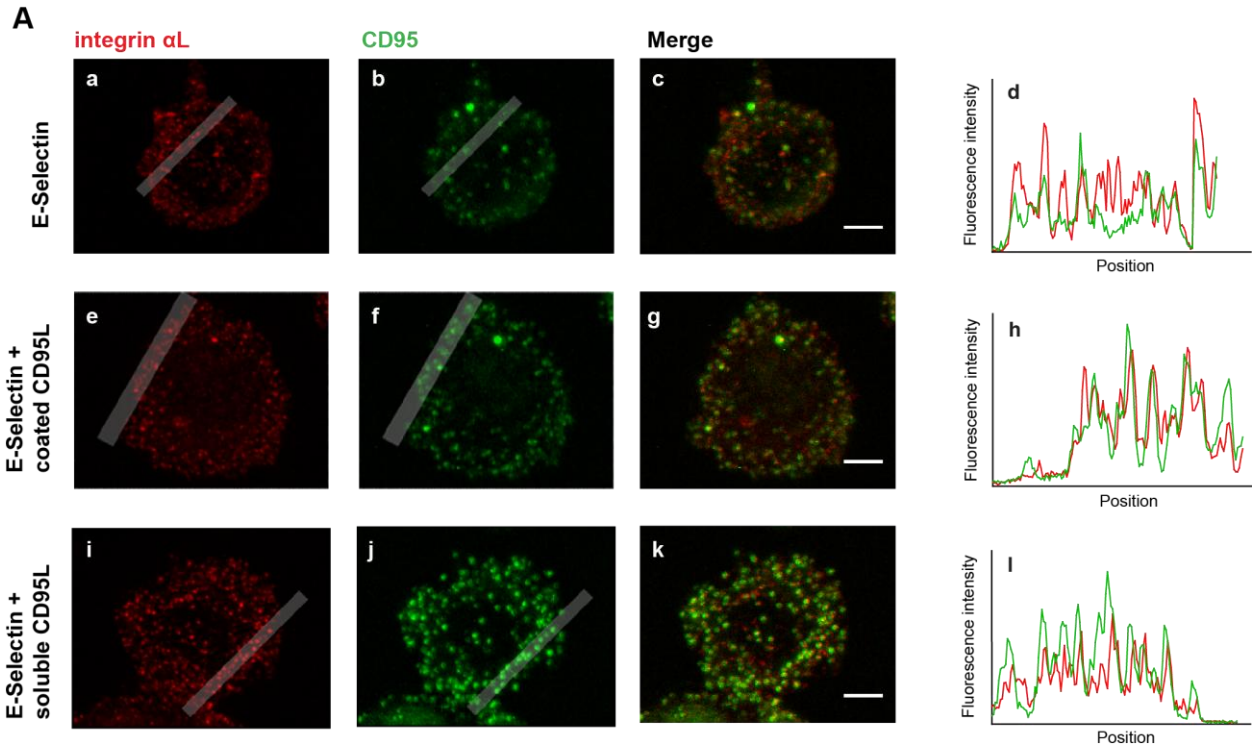

Figure 21. CD95 colocalizes with integrin α_L upon stimulation with CD95L. (A) U937 cells were perfused through human E-selectin coated flow chamber with the stimulation of immobilized CD95L (e-h) or soluble CD95L (i-l). Cells were fixed and stained with anti-CD11a (red) and anti-CD95 (green) antibodies. d, h and l show the plot profile of fluorescence intensity in transparent white square of a-b, e-f and i-j. **(B)** Pearson's coefficient analysis of CD95 and integrin colocalization in (A). Data presented as dot plot with median. Each dot represents a cell (n=33-36). one-way ANOVA, Bonferroni multiple comparison post hoc test, ***p<0.001.

4.5 Endothelial cell-derived CD95L mediates neutrophil recruitment

During inflammation, endothelial cells activated by inflammatory cytokines express adhesion molecules, such as selectin and ICAM, and synthesize chemokines and lipid chemoattractants on the luminal surface to facilitate the recruitment of leukocytes to

inflamed tissue (Ley *et al*., 2007). As shown above, immobilized CD95L induces the activation of integrin and promotes the slow rolling of neutrophils in a flow chamber system. Hence, we hypothesize that in the *in vivo* scenario, activated endothelial cells might present CD95L to facilitate neutrophil recruitment. In order to test this hypothesis, we first checked whether CD95L stimulation could activate endothelial cells in terms of increasing the cell surface level of adhesion molecules, such as ICAM1. However, we didn't observe any effect of CD95 on regulating ICAM1 level in cultured mouse endothelial cells (Figure 22).

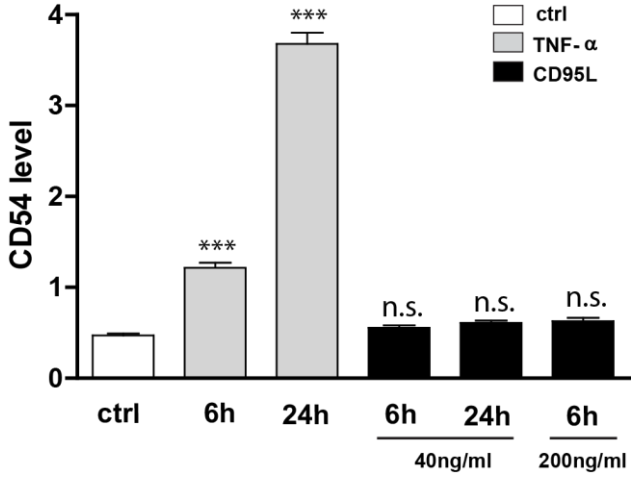

Figure 22. CD95L stimulation has no impact on cell surface ICAM1 level in endothelial cells. Mouse lung derived endothelial cells were cultured in 96 well plate and stimulated with TNF-α (100ng/ml) or CD95L (40ng/ml, 200ng/ml) for 6 or 24 hours. Cells were immuostained with anti-ICAM1 and measured with a microplate reader. Data are presented as mean ± SEM. n=3, one-way ANOVA, Bonferroni multiple comparison post hoc test, ***p<0.001, ns: not significat.

To address our hypothesis in an *in vivo* model, *Ve-Cadherin*$^{ERT2/4cre}$ and *CD95L*^{flox} mice were crossed to allow inducible deletion of CD95L in endothelial cells (Figure 23 A). Neutrophil recruitment was tested with the thioglycollate induced peritonitis model after induction with tamoxifen in *CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ mice (Figure 23 B, C). The number of peritoneal neutrophils 6 hours after thioglycollate injection was significantly reduced in endothelial-CD95L deleted mice compared to control mice (Figure 23 D). However, in mice with CD95 deletion in endothelial cells (*CD95*$^{f/f;Ve\text{-}CadherinCreERT2/4}$), we didn't observe any impairment of neutrophil recruitment (Figure 23 E).

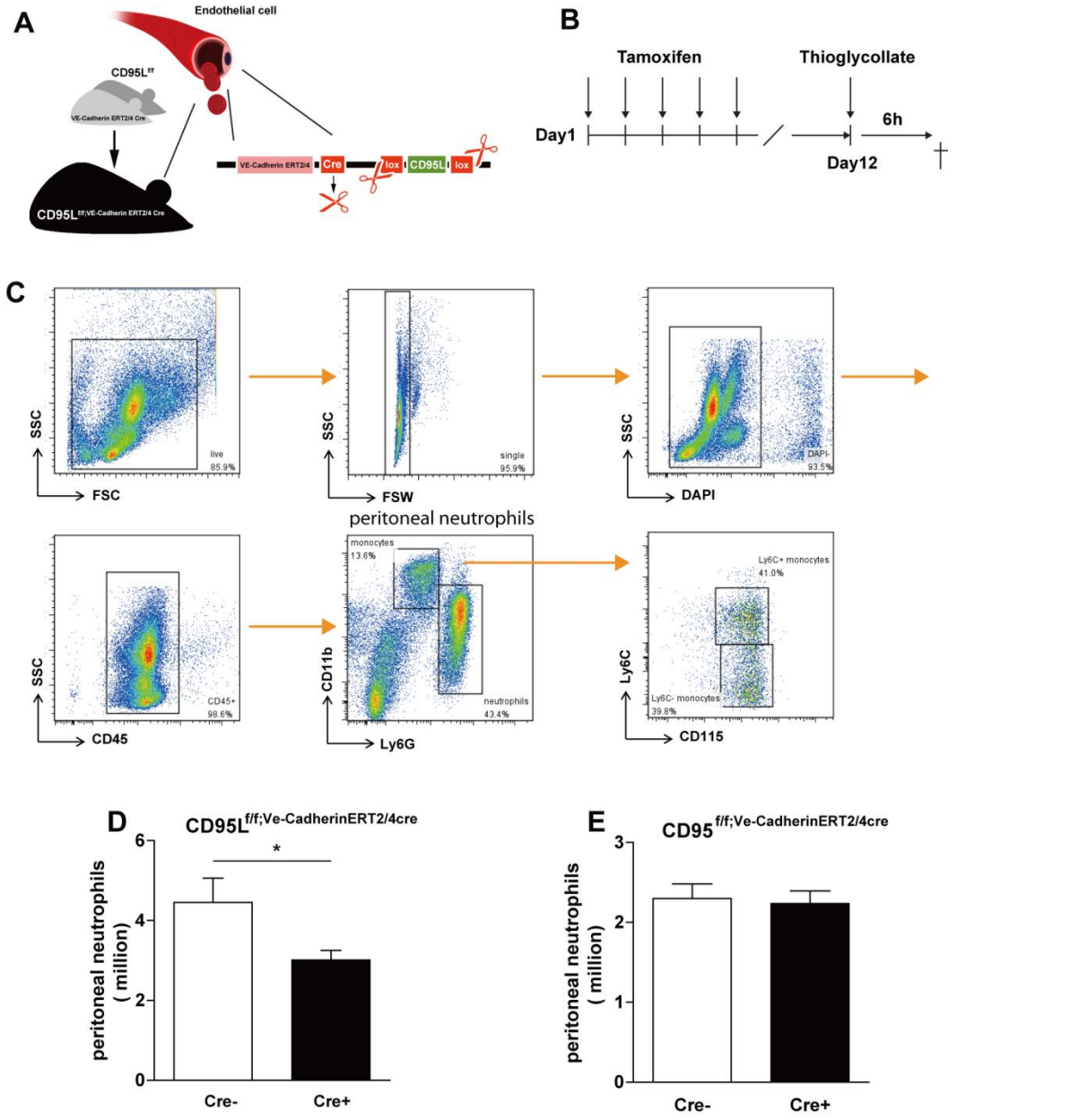

Figure 23. Endothelial cell-derived CD95L is necessary for neutrophils recruitment *in vivo*. **(A)** Scheme of inducible deletion of CD95L in endothelial cells in *CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ mouse. **(B)** Injection schedule of tamoxifen and thioglycollate is depicted. Tamoxifen (200mg/kg) was intragastrically administered to *CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ mice for 5 consecutive days. At day 12 after the first tamoxifen injection, mice were i.p. injected with thioglycollate to induce peritonitis. 6h after thioglycollate injection, peritoneal lavage was performed and peritoneal cells were stained with neutrophil markers. **(C)** Flow cytometry plot of peritoneal neutrophils 6 hours after thioglycollate injection. Neutrophils were gated according to the profile of FSC/ SSC, DAPI negativity and CD45, CD11b, Ly6G positivity. **(D)** Peritoneal neutrophils influx 6 hours after injection of thioglycollate into *wt* or *CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ mouse (n=11-14). **(E)** Peritoneal neutrophils influx 6 hours after injection of thioglycollate into WT or *CD95*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ mouse (n=16-17). Data presented as mean ± SEM, student's *t* test, *p<0.05.

The ablation of CD95L in endothelial cells of *CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ mice was confirmed by flow cytometry (Figure 24 A,B). To check the cell surface level of adhesion molecules in endothelial cells, mice livers were dissociated and immunostained for endothelial marker and anti-ICAM1/ICAM2/P-selectin/E-selectin.

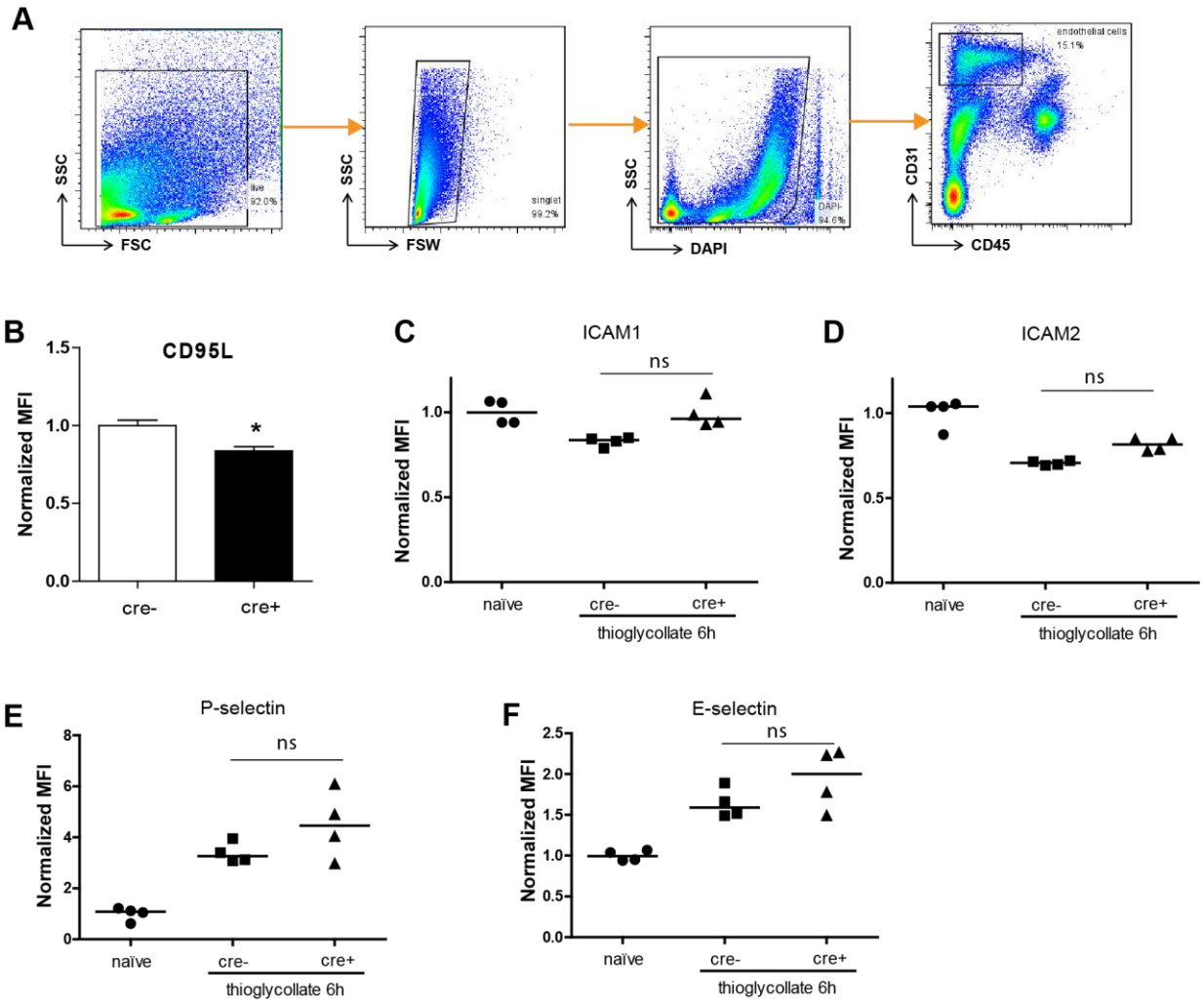

Figure 24. Inducible deletion of CD95L in endothelial cells does not influence cell surface level of ICAM and selectins. (A) Flow cytometry plot of liver endothelial cells. Dissociated liver cells from *CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ mice were stained with antibodies of endothelial cell markers and anti-CD95L/ICAM1/ICAM2/P-selectin/E-selectin. Endothelial cells were gated according to the profile of FSC/SSC, DAPI, CD45 negativity and CD31 positivity **(B)** Cell surface level of CD95L in endothelial cells of *CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ mice. Data presented as mean ± SEM, n=4, student's *t* test, *p<0.05. (C-F) Cell surface level of ICAM1 **(C)**, ICAM2 **(D)**, P-selectin **(E)** and E-selectin **(F)** in endothelial cells of naïve or thioglycollate injected *CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ mice. Data presented as dot plot with median, one-way ANOVA, Bonferroni multiple comparison post hoc test, ns: not significant.

The endothelial ICAM1 and ICAM2 levels were reduced in thioglycollate-injected mice compared with naïve mice (Figure 24 C, D). Whereas the P-selectin and E-selectin were significantly increased (Figure 24 E, F). We didn't observe a significant difference of ICAM1/ICAM2/P-selectin/E-selectin levels between CD95L-deleted and non-deleted endothelial cells (Figure 24 C-F).

The ablation of CD95 in endothelial cells of *CD95*$^{f/f;Ve\text{-}CadherinCreERT2/4}$ mice was also examined by flow cytometry (Figure 25 A). The levels of cell surface adhesion molecules ICAM1/E-selectin/P-selectin in CD95 deleted endothelial cells were similar to the CD95 non-deleted cells (Figure 25 B, C, D).

Thus CD95 and CD95L deletion in endothelia cells have no significant impact on the cell surface level of adhesion molecules of ICAM1, ICAM2, E-selectin and P-selectin.

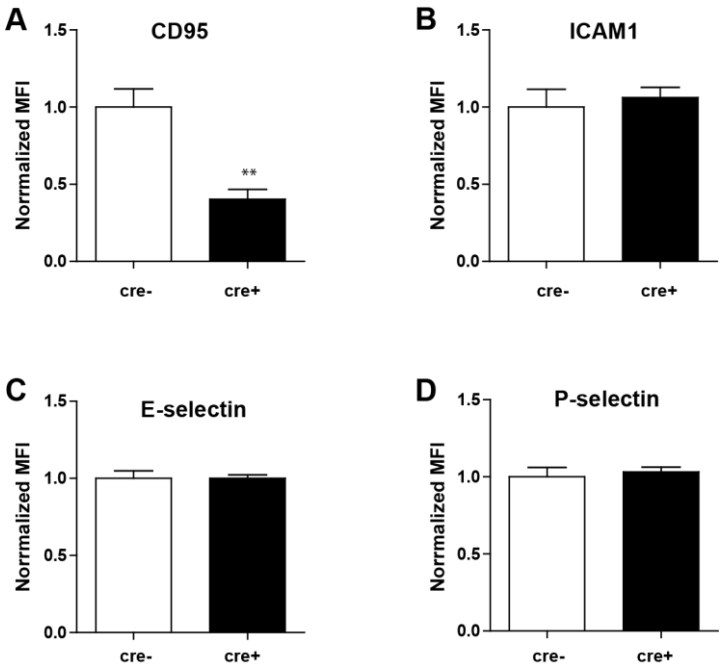

Figure 25. Inducible deletion of CD95 in endothelial cells does not influence cell surface level of ICAM1 and selectins. Dissociated liver cells from *CD95*$^{f/f;Ve\text{-}CadherinCreERT2/4}$ mice were stained with antibodies of endothelial cell marker and anti-CD95/ICAM1//P-selectin/E-selectin. **(A)** Cell surface level of CD95 in endothelial cells of *CD95*$^{f/f;Ve\text{-}CadherinCreERT2/4}$ mice. Data presented as mean ± SEM (n=2-4). **(B-D)** Cell surface levels of ICAM1 **(B)**, E-selectin **(C)** and P-selectin **(D)** in endothelial cells of *CD95*$^{f/f;Ve\text{-}CadherinCreERT2/4}$ mice. Data presented as mean ± SEM, n=4, student's *t* test, **p<0.01.

4.6 CD95 induces Ly6C^{hi} monocyte mobilization and recruitment

4.6.1 CD95 activation increases Ly6C^{hi} monocyte mobilization to blood

As described in the introduction section, neutrophil recruitment is normally followed by the wave of monocyte recruitment during innate response. In order to clarify whether CD95 signaling mediates innate immune response via recruitment of monocytes as well, we first examined the mobilization of Ly6C^{hi} monocytes from the monocytes reservoir to blood after CD95L treatment. As reported in our previous publication, in an acute inflammation model of spinal cord injury, cell surface levels of CD95L elevated dramatically in blood neutrophils and monocytes after injury (Letellier et al., 2010). Following up on this finding, we performed i.v. injection of CD95L (10µg) to naïve mice and checked the levels of Ly6C^{hi} monocytes in blood by flow cytometry. Lineage makers (Lin marker: Ly6G, Nk1.1, CD3, CD19) were used to exclude neutrophils, natural killer cells (NK cells), T and B cells. Antibodies against CD115 (colony-stimulating factor receptor, M-CSFR, which is specifically expressed in cells of monocyte and macrophage lineage), CD11b, Ly6C and CD43 were used for distinguishing monocytes (Figure 26 A). At 6 hours after CD95L injection, the ratio of Ly6C^{hi} monocytes among CD45+ cells in blood raised significantly compared to saline-injected mice. At 36 hours after CD95L injection, this ratio returned to the level of control mice (Figure 26 B). Ly6C^{lo} monocyte levels were not significantly impaired after CD95L treatment, but only increased slightly 12 hours after CD95L treatment (Figure 26 C). This data indicates that CD95 activation specifically elevates Ly6C^{hi} monocyte mobilization to blood.

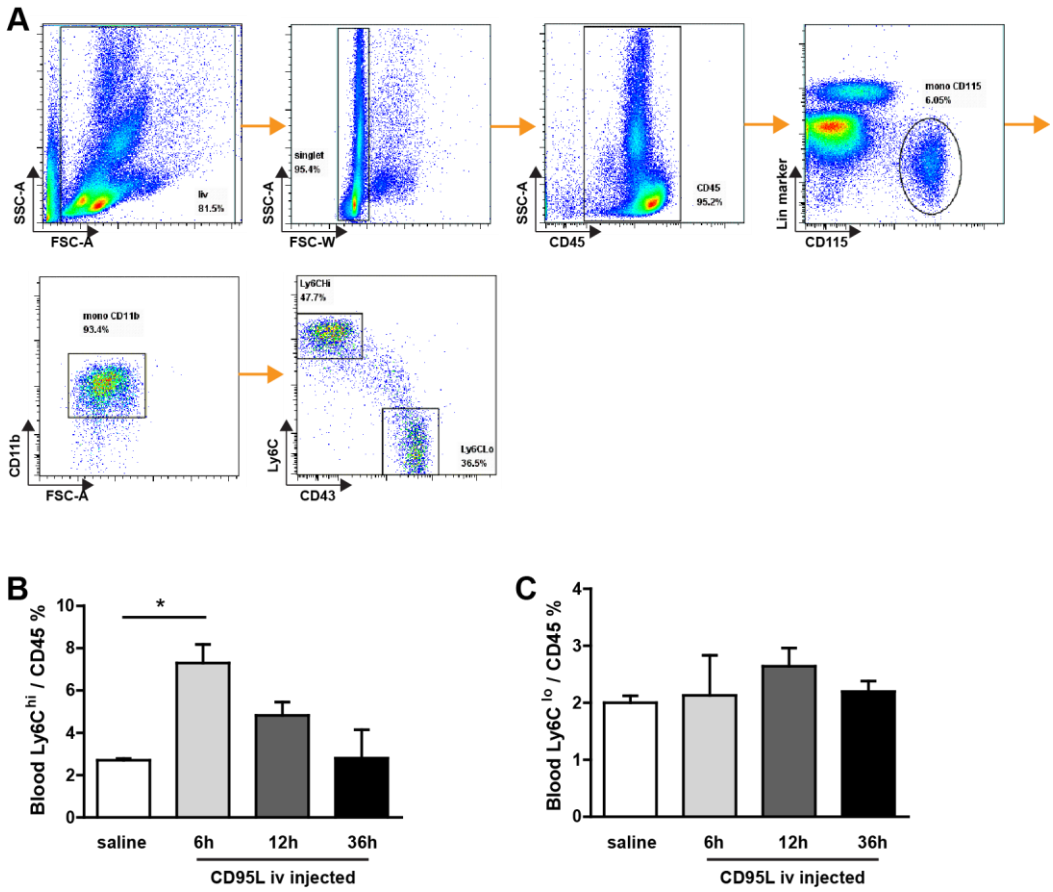

Figure 26. CD95 activation increases Ly6C^{hi} monocyte mobilization to the blood. (A) Flow cytometry plot of different population of blood monocytes. Monocytes were gated according to the profile of FSC/SSC, Lin marker negativity and CD45, CD115, CD11b, Ly6C/CD43 positivity. **(B, C)** Mice were i.v. injected with saline or CD95L (10µg). Blood samples were collected at the indicated time points and stained with monocytes markers. The ratio of Ly6C^{hi} **(B)** and Ly6C^{lo} **(C)** monocytes were assessed with flow cytometry. Data are presented as mean ± SEM. n=3, one-way ANOVA, Bonferroni multiple comparison post hoc test, *p<0.05.

4.6.2 CD95 activation increases Ly6C^{hi} monocyte recruitment to lymph nodes

Once monocytes extravasate into the tissue, the differentiation towards macrophages or DCs starts. DCs are strong APCs which serve as a bridge to connect innate immunity and adaptive immunity during inflammation. In lymph nodes, DCs are mostly derived from monocytes (Randolph et al., 2008). It is interesting to know whether CD95-mobilized monocytes are recruited to lymph nodes to become the precursors for DCs. In order to test this hypothesis, lymph nodes of mice were collected after CD95L treatment and lymph node cells were stained with monocytes markers (Figure 27 A). At 6 hours

after CD95L i.v. injection, we observed increased ratio of Ly6C^{hi} monocytes among CD45+ cells in the lymph nodes. After 36 hours injection, the ratio returned to the level of saline injected control mice, similar to the observation in the blood, although the trend was not statistically significant (Figure 27 B). Also consistent with the results from blood samples, Ly6C^{lo} monocyte levels in the lymph nodes were not influenced after CD95L treatment (Figure 27 C). Taken together, CD95 activation mobilized Ly6C^{hi} monocytes to blood and increased the recruitment of Ly6C^{hi} monocytes to lymph nodes.

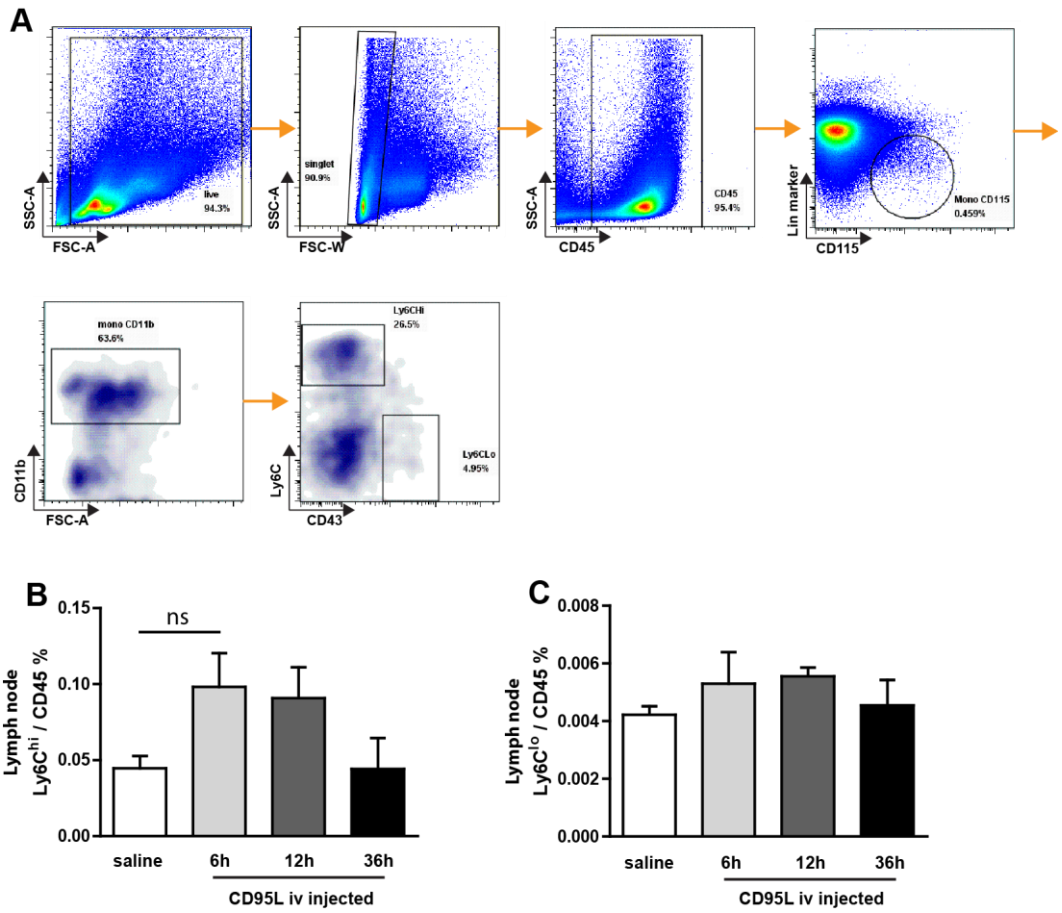

Figure 27. CD95 activation increases Ly6C^{hi} monocyte recruitment to lymph nodes. **(A)** Flow cytometry plot of different population of lymph node monocytes. Lymph node monocytes were gated according to the profile of FSC/SSC, Lin marker negativity and CD45, CD115, CD11b, Ly6C/CD43 positivity. **(B,C)** Mice were i.v. injected with saline or CD95L (10µg). Lymph nodes were collected at the indicated time points, dissociated and stained with monocyte markers. The ratio of Ly6C^{hi} **(B)** and Ly6C^{lo} **(C)** monocytes were assessed by flow cytometry. Data are presented as mean ± SEM. n=3, one-way ANOVA, Bonferroni multiple comparison post hoc test, ns: not significant.

4.6.3 Ly6C^{hi} monocytes express relatively high level of CD95

To investigate how CD95 activation selectively mobilize and recruit Ly6C^{hi} monocytes but not Ly6C^{lo} monocytes, we checked the cell surface levels of CD95 in different monocyte population from blood and bone marrow by flow cytometry. The blood Ly6C^{hi} monocytes showed significantly higher cell surface level of CD95 than Ly6C^{lo} monocytes in blood and Ly6C^{hi} monocytes in bone marrow. However, bone marrow Ly6C^{hi} monocytes and Ly6C^{lo} monocytes had relatively similar cell surface levels of CD95 (Figure 28 A). Peritoneal macrophages showed significantly higher cell surface levels of CD95 than blood Ly6C^{hi} monocytes (Figure 28 B).

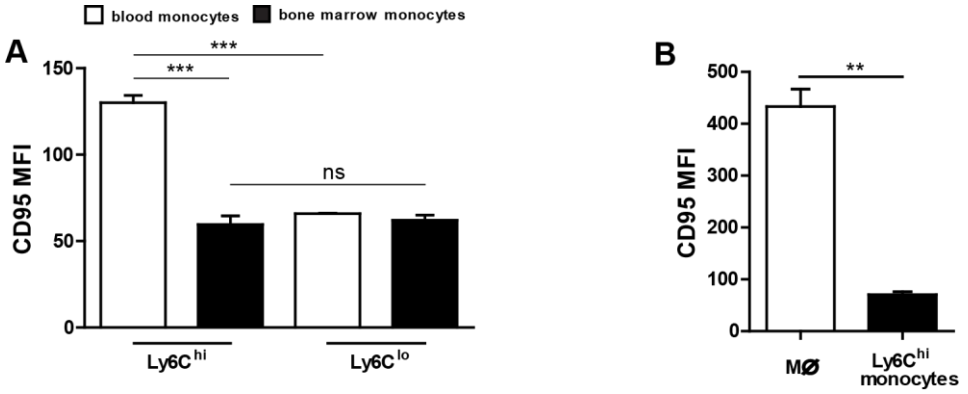

Figure 28. Blood Ly6C^{hi} monocytes express relative high level of CD95. Blood cells, bone marrow cells **(A)** and peritoneal cells **(B)** were stained with monocyte/macrophage markers and anti-CD95. CD95 levels on different monocyte populations and macrophages were assessed by flow cytometry. Data are presented as mean ± SEM. n=3, one-way ANOVA, Bonferroni multiple comparison post hoc test (A), student's *t* test (B), **p<0.01, ***p<0.001, ns: not significant.

4.6.4 CD95L treatment induces Ly6C^{hi} monocytes mobilization via direct activation of CD95

The CD95L used in this study is a fusion protein of CD95L trimer connected with the T4-Foldon motif from the fibritin of the bacteriophage T4 (CD95L-T4) and was purified from transfected HEK293T cells (Kleber et al., 2008; Apogenix GmbH). According to the report from Apogenix, the endotoxin level in the purified CD95L solution was <0.5 EU/ml. It is been reported that a low dose of Toll like receptor (TLR) ligand in the blood stream drives the CCR2-dependent emigration of monocytes from the bone marrow (Shi et al., 2011). In order to exclude the possibility that CD95L treatment induced Ly6C^{hi} monocytes mobilization is a result from the effect of endotoxin in CD95L solution, we

neutralized CD95L *in vitro* with CD95 fusion protein (APG, Apogenix GmbH) prior to injection and then injected i.v. the mixed solution to mice to check the blood and lymph node monocyte levels. At 6 hours after the injection of the mixed solution, the ratio of Ly6C^{hi} monocytes among CD45+ reduced significantly in the group of mice injected with CD95L:APG at molar ratio of 1:3 compared to the CD95L injected mice (FIgure 29 A). Unexpectedly, the ratio of Ly6C^{lo} monocytes increased significantly after the treatment with APG (Figure 29 B). Morever, the results showed a trend for the effect of APG in blocking the CD95L-induced recruitment of Ly6C^{hi} monocytes in lymph nodes, and also the ratio of Ly6C^{lo} monocytes was increased in lymph nodes (Figure 29 C, D).

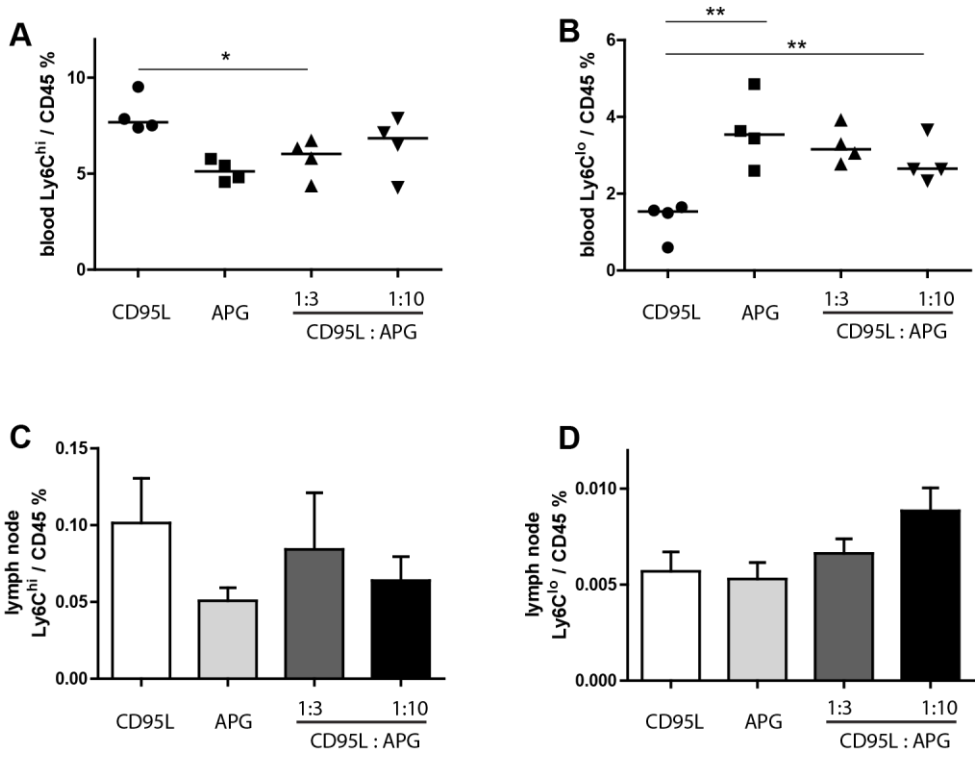

Figure 29. CD95L stimulation drives Ly6C^{hi} monocyte mobilization via activating CD95. CD95L (10µg/ml) was pre-incubated with APG at molar ratio of 1:3 or 1:10 on ice for 30 minutes. After incubation, the mixed solution was i.v. injected to the mice. CD95L or APG i.v. injections were performed as control. The ratio of Ly6C^{hi}/Ly6C^{lo} monocytes in blood **(A, B)** and lymph node cells **(C,D)** were assessed by flow cytometry. Data are presented as dot plot with median or mean ± SEM. n=4, one-way ANOVA, Bonferroni multiple comparison post hoc test, *p<0.05, **p<0.01.

A more direct evidence for the involvement of CD95 in driving Ly6C^{hi} monocytes came out from the study by using *CD95$^{f/f;LysMcre}$* mice. The naïve cre positive and negative mice

showed similar levels of blood Ly6C^{hi} monocytes (Figure 30 A), which indicates that CD95 may not have effect on the turnover of blood Ly6C^{hi} monocytes. However, at 6 hour after CD95L i.v. injection, CD95L-induced Ly6C^{hi} monocytes mobilization was significantly attenuated by the deletion of CD95 in myeloid cells (Figure 30 B).

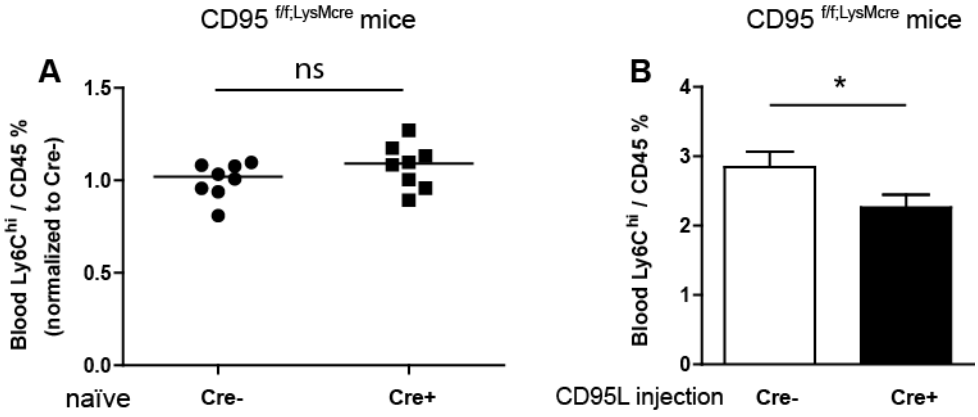

Figure 30. Deletion of CD95 in myeloid cells attenuates CD95L stimulation-induced Ly6C^{hi} monocyte mobilization. Ly6C^{hi} monocyte levels in the blood of naïve *CD95$^{f/f;LysMcre}$* mice **(A)** and *CD95$^{f/f;LysMcre}$* mice 6 hours after CD95L i.v. injection **(B)**. Data are presented as dot plot with median or mean ± SEM. n=8, student's *t* test *p<0.05, ns: not significant.

4.6.5 CD95L treatment increases the CCL2 level in plasma

CCR2 is highly expressed in Ly6C^{hi} monocytes (Geissmann et al, 2003) and is critical for the mobilization of Ly6C^{hi} monocytes from bone marrow and recruitment to inflammatory sites (Tsou et al., 2007). CCL2 is the major ligand for CCR2 and is essential for monocyte recruitment in many inflammatory models (Lu et al., 1998). To clarify whether CCL2 is involved in CD95-induced Ly6C^{hi} monocytes mobilization, mouse plasma CCL2 level was assessed by ELISA of blood samples collected 6 hours after CD95L i.v. injection. In CD95L injected mice, the plasma CCL2 level was significantly higher than the saline injected control mice (Figure 31). Pre-blocking of CD95L with APG at molar ratio of 1:10 reduced the plasma CCL2 level (Figure 31).

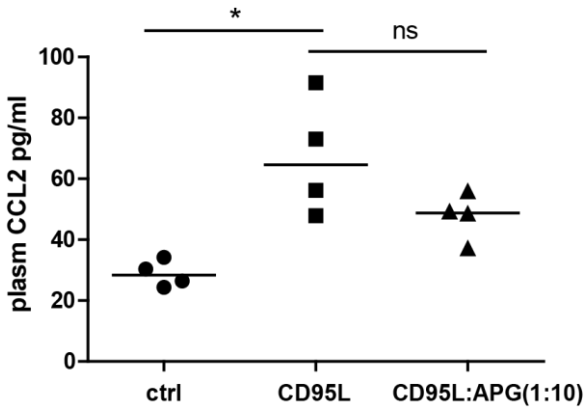

Figure 31. CD95L treatment increases the plasma CCL2 level. Mice were i.v. injected with saline, CD95L (10µg) or APG pre-blocked CD95L (CD95L:APG=1:10). At 6 hours after the injection, blood samples were collected and the plasma CCL2 levels were measure by ELISA. Data are presented as dot plot with median, one-way ANOVA, Bonferroni multiple comparison post hoc test, n=4, *p<0.05, ns= not significant.

To further confirm the involvement of CCL2 in CD95 induced monocytes mobilization and recruitment, neutralization of CCL2 with anti-CCL2 antibody (100µg) was performed after CD95L i.v. injection to mice. However, we did not observe obvious significant reduction of Ly6C^{hi} monocytes after neutralization of CCL2 in CD95L treated mice (Figure 32 A). Surprisingly, the level of Ly6C^{hi} monocytes in lymph nodes was increased after neutralization of CCL2 (Figure 32 C).

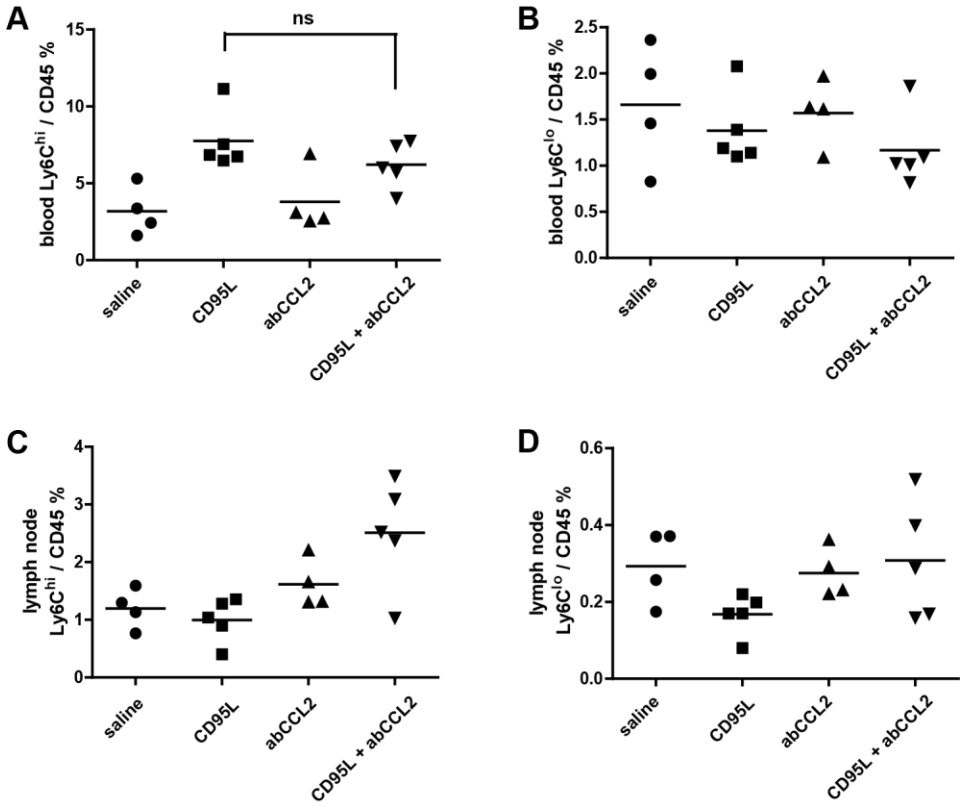

Figure 32. The effect of CCL2 neutralization in CD95L-induced monocyte mobilization and recruitment. CCL2 neutralization was performed by injection of anti-CCL2 antibody (100µg) 10 minutes after CD95L injection. Blood and lymph node samples were collected 6 hours after injection. The ratio of $Ly6C^{hi}$ / $Ly6C^{lo}$ monocytes in blood **(A, B)** and lymph node cells **(C, D)** were assessed by flow cytometry. Data are presented as dot plot with median. n=4-5, one-way ANOVA, Bonferroni multiple comparison post hoc test, ns: not significant.

4.6.6 Involvement of CD95 in the recruitment of $Ly6C^{hi}$ monocytes in a peritonitis model

We employed a thioglycollate induced-peritonitis mouse model to investigate the involvement of CD95 in recruiting $Ly6C^{hi}$ monocytes during inflammation. Thioglycollate induced-peritonitis is a broadly used model to study the recruitment of monocytes. However little is known about the kinetics of $Ly6C^{hi}$ monocyte recruitment in this model. Therefore, we started by investigating the time point of peak recruitment of $Ly6C^{hi}$ monocytes. In naïve mice, 93% of the peritoneal cells were macrophages. At 2 hours after thioglycollate injection, most of the resident macrophages vanished. Afterwards, the peritoneal $Ly6C^{hi}$ monocytes increased steadily and reached the peak at 12 hours after thioglycollate injection. The differentiation of $Ly6C^{hi}$ monocytes to macrophage started from 6 hours after thioglycollate injection; and at 48 hours 83% of the peritoneal cells were differentiated macrophages (Figure 33 A, B).

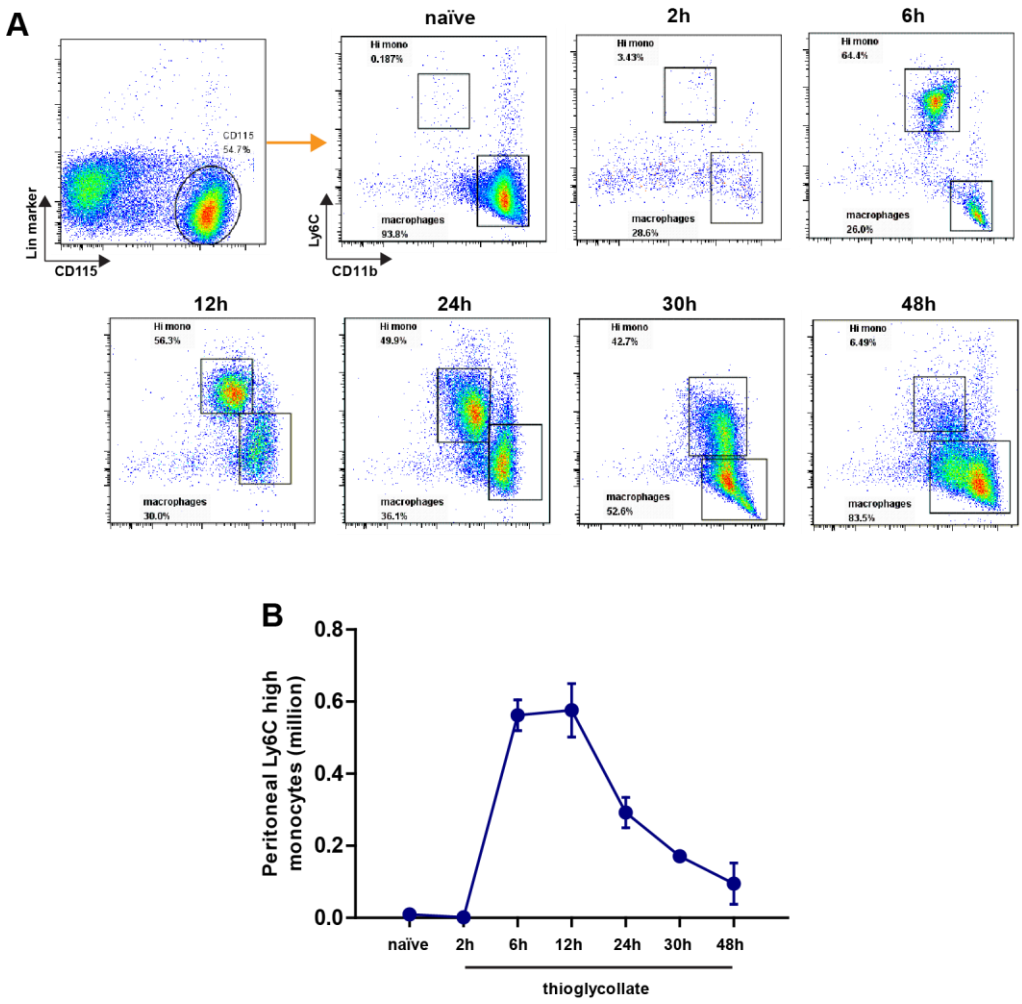

Figure 33. Kinetics of Ly6C^{hi} monocytes recruitment in thioglycollate induced peritonitis. Mice were ip injected with thioglycollate for 2, 6, 12, 24, 30 and 48 hours. Peritoneal cells were stained with monocytes markers and examined by flow cytometry. **(A)** Flow cytometry plot of peritoneal cells after thioglycollate injection. Ly6C^{hi} monocytes were gated according to the profile of FSC/SSC, Lin marker negativity and CD45, CD115, CD11b, Ly6C positivity. Macrophages were gated according to the higher CD11b and lower Ly6C level than Ly6C^{hi} monocytes. **(B)** Absolute numbers of peritoneal Ly6C^{hi} monocytes after thioglycollate injection. Total number of peritoneal cells was assessed by hemocytometer counting and Ly6C^{hi} monocytes ratio was assessed by flow cytometry. Data are presented as mean ± SEM, n=3.

We chose the time point of 12 hours after thioglycollate injection to test the involvement of CD95 in Ly6C^{hi} monocyte recruitment. To neutralize endogenous CD95L, two doses of APG were i.v. injected to mice with a 6 hours interval after thioglycollate injection (Figure 34 A). APG neutralization significantly blocked the infiltration of total immune

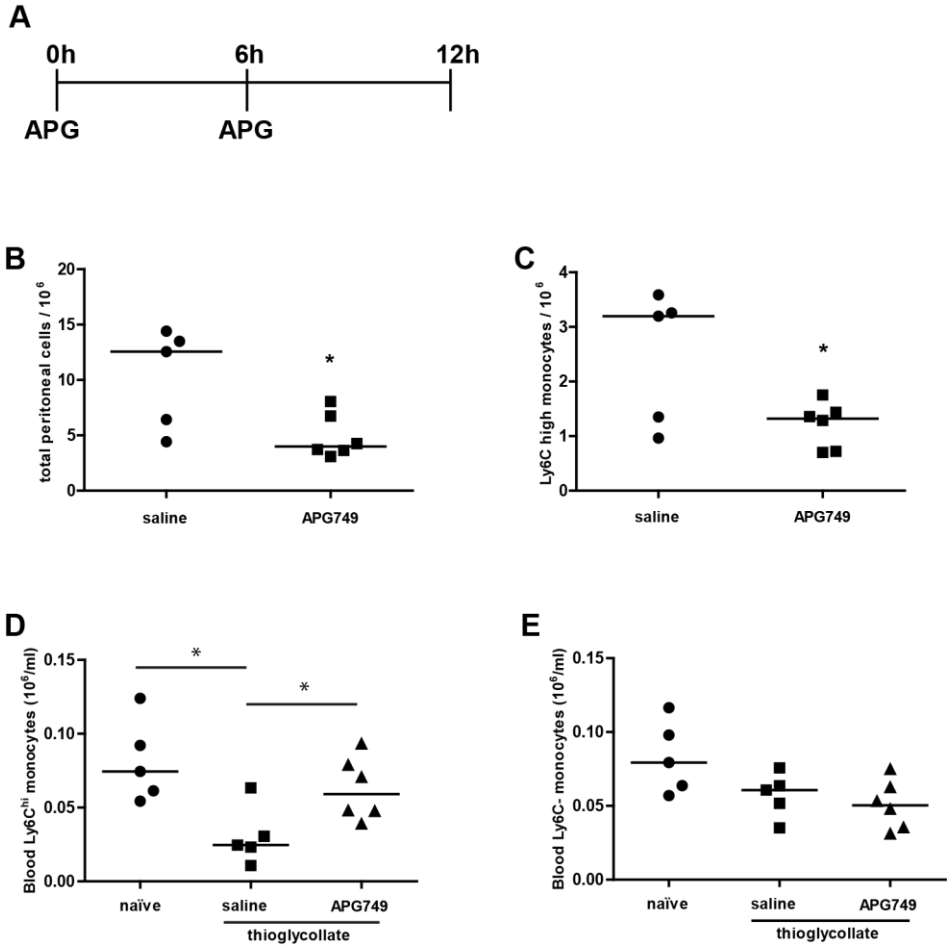

Figure 34. CD95L neutralization blocks Ly6C^{hi} monocyte recruitment in thioglycollate induced peritonitis. (A) Scheme for thioglycollate and APG injeciton. Two doses of APG (50µg) were i.v. injected after thioglycollate injetion with 6 hours interval. Mice were sacrificed 12 hours after thioglycollate injection and peritoneal cells were collected for testing the monocytes by flow cytometry. **(B, C)** The numbers of total peritoneal cells and Ly6C^{hi} monocytes in saline or APG treated mice after thioglycollate injection. **(D, E)** The ratio of blood Ly6C^{hi}/ Ly6C^{lo} monocytes in naïve, saline or APG treated mice after thioglycollate injection. Data are presented as dot plot with median, student's *t* test or one-way ANOVA, Bonferroni multiple comparison post hoc test, n=5-6, *p<0.05.

cells and Ly6C^{hi} monocytes to the peritoneal cavity (Figure 34 B, C). Compared with saline injected control mice, blood Ly6C^{hi} monocyte levels significantly decreased in mice injected with thioglycollate (Figure 34 D). This indicates that most of the blood Ly6C^{hi} monocytes were recruited to the peritoneal cavity after thioglycollate injection and APG neutralization attenuated this recruitment and kept the blood Ly6C^{hi} monocytes at the control level (Figure 34 D). However, neither thioglycollate injection nor APG neutralization impacted the blood Ly6C^{lo} monocytes in a minor way which reflected that

Ly6C^{lo} monocytes were not recruited in thioglycollate induced-peritonitis (Figure 34 E). Taken together, our data demonstrates that CD95 signaling also contributes to the innate response via selective recruitment of Ly6C^{hi} monocytes during inflammation.

5 DISCUSSION

5.1 CD95 as a driver for myeloid cell recruitment

It has been two and a half decades since two research groups found that the monoclonal antibodies anti-APO-1 and anti-Fas induced apoptosis in human cell lines (Trauth et al., 1989; Yonehara et al., 1989). Over these years, the majority of studies on CD95-CD95L interactions have focused on apoptosis as the primary outcome. It is now well-known that engagement of CD95 with CD95L leads to the formation of a death-inducing signaling complex which propagates apoptotic signal through active caspase-8 to downstream type I or type II apoptosis pathways (Figure 5; Peter and Krammer, 2003; Strasser et al., 2009; Hughes et al., 2009). However, these studies on apoptotic function eclipsed the role of CD95 as an important mediator of other cellular processes. As described in section of 2.6.3, accumulating evidence reveal that CD95 also has important non-apoptotic functions, such as mediating cell survival, proliferation and migration.

CD95 belongs to the TNF receptor superfamily. Most of the molecules in this superfamily are expressed by or can target cells of the immune system, and they have multiple functions ranging from promoting cellular differentiation, survival to the production of inflammatory cytokines and chemokines (Croft et al., 2012). In line with this, apart from apoptotic functions in the immune system, CD95 ligation induces production of pro-inflammatory mediators in a variety of cell types (described in section 2.6.2). Cytokines and chemokines of TNF-α, IL-1β, IL-6, CXCL1 and CXCL8 activate endothelial cells or leukocytes which in turn lead to the recruitment of leukocytes to the inflammation site (Ley et al., 2007). CD95-induced leukocyte infiltration was firstly found in early studies which intended to see the apoptotic function of CD95 in CD95-negative tumor cells *in vivo* (Arai et al., 1997; Seino et al., 1997). In these studies, the transplantation of CD95L-transfected CD95-negative tumor cells was rejected by inflammatory response including neutrophils recruitment. Other studies using boyden chamber assay demonstrated that soluble CD95L (sCD95L) induced the transmigration of human neutrophils *in vitro* (Seino et al., 1998; Ottonello et al., 1999; Dupont et al., 2007). In these publications, the activation of neutrophils was not observed upon

sCD95L treatment, and neutrophils from *Fas*$^{lpr/lpr}$ mice showed no response to sCD95L. Although these findings are promising, it is still unclear whether CD95-induced neutrophil recruitment or migration is through direct activation of neutrophils or it is just the secondary effect of CD95-induced pro-inflammatory mediators.

5.1.1 CD95 in mediating slow rolling

Leukocyte recruitment is a cascade of sequential cellular processes including slow rolling, leukocyte activation and firm adhesion, and transendothelial cell migration (Figure 1; Ley et al., 2007). Rolling starts when leukocytes are tethered by the ligation of selectins on activated endothelial cells to their ligands on leukocytes, such as PSGL-1 and CD44, in a shear stress-dependent manner. Subsequently, rolling leukocytes encounter signals from cytokines and chemokines which in turn induce slow rolling and adhesion via the inside-out and outside-in integrin signal pathways.

Parallel plate flow chamber connected to a pump system has been used for many years to study leukocyte rolling adhesion, without many of the complicating factors present *in vivo* (Mclntire et al., 1987; Lawrence et al., 1991). The shortcomings for this system are leukocyte isolation-induced activation and the requirement of large amount of cells. A recently developed *ex vivo* model called-autoperfused mouse flow chamber assay has overcome these disadvantages (described in section 4.2.1).

In the autoperfused flow chamber assay, sCD95L or immobilized CD95L induced slow rolling of neutrophils and increased the number of rolling cells (Figure 13). These effects were abolished in mice with CD95 deficiency in myeloid cells (*CD95*$^{f/f;LysMCre}$). It indicates that CD95 induces slow rolling via direct effect on neutrophils but not via the induction of cytokines and chemokines as published previously.

The upregulation of selectins and ICAMs in endothelial cells and selectin ligand and ICAM ligands in leukocytes plays important roles in rolling and adhesion (Pober and Sessa, 2007). Unlike the effect of TNF-α on endothelial cells, CD95L stimulation or CD95/CD95L depletion in endothelial cells have no impact on the expression level of adhesion molecules (Figure 22, 24, 25). A previous study has shown that crosslinking of CD95 with antibody or CD95L rapidly triggered downmodulation of L-selectin, CD44, LFAα and LFAβ in CD95 sensitive T cell blasts (Kabelitz et al., 1996). However, in our

study, upon CD95L treatment *in vitro* or *in vivo*, or CD95 deletion in myeloid cells, we did not observe any effects on cell surface expression levels of most integrins, the main mediators in rolling and adhesion,. We are only able to detect the upregulation of integrin α_M in CD95 deleted neutrophils (Figure 14, 15, 19). Taken together, these results demonstrate that CD95 induces neutrophil slow rolling independent on the induction of pro-inflammatory mediators and upregulation of adhesion molecules.

5.1.2 CD95 in mediating adhesion and transmigration

Cellular events of adhesion and transmigration start when rolling cells are activated by signaling from chemokines. As described above, some studies reported that sCD95L induced the transmigration of human neutrophils in an *in vitro* boyden chamber assay (Seino et al., 1998; Ottonello et al., 1999; Dupont et al., 2007). Nonetheless, two other reports from the same researchers claimed that CD95 activation reduced neutrophil adhesion to endothelial cells via disturbing the translocation of PKCδ which was necessary for integrin β2-mediated adhesion (Greenstein et al., 2000; Hendey et al., 2002). In these two studies, CD95 was activated by crosslinking with anti-CD95 antibodies. The cross-linked CD95 with antibody had been shown to interact with Fc receptor and promote the apoptosis-inducing activity in target cells (Xu et al., 2003). Furthermore, the fact that CD95 agonistic antibodies were applied for half an hour in these two studies made them less convincing, since neutrophil adhesion happens within minutes in physiological conditions (Ley et al., 2007).

Ending the controversy, previous work of our lab showed that CD95 induced macrophage adhesion on ICAM1 coated chambers in a static adhesion assay and triggered myeloid cell transmigration via activation of MMP9 in *in vivo* inflammatory models (Letellier et al., 2010). In order to draw a complete picture of CD95's role in mediating myeloid cell recruitment, we performed *in vivo* studies using intravital microscopy (Data not shown, collaboration work with Dr. Alexander Zarbock, University of Münster, Germany). The cremaster muscles of mice were injected with IL-1β or TNF-α to induce inflammation, and subsequently the parameters of rolling, adherent and transmigrating cells in the vessels were examined by intravital microscopy. We observed that stimulation of CD95L by i.v. injection showed trends of increased rolling flux fraction (percent rolling cells) and decreased rolling velocity. On the other hand, the rolling flux

fraction and the numbers of adherent and transmigrated cells were significantly decreased in *CD95*$^{f/f;LysMcre}$ bone marrow reconstituted mice compared to the *wt* controls. These findings are consistent with the published work of our lab. Taken together, our studies reveal that, during inflammation, CD95 participates in myeloid cell recruitment via mediation of slow rolling, adhesion and transmigration in a cell autonomous manner.

5.2 Integrin activation, a novel function for CD95 in mediating myeloid cell recruitment

Integrin activation plays a central role in regulating leukocyte rolling, adhesion and transmigration. In the rolling process, selectin engagement with their ligand on leukocyte triggers the activation of integrin via the SFKs-Syk-BTK-PLC-γ2-p38/PI3K pathway (Figure 2, section of 2.2.1). Subsequently, rolling cells encounter more stimuli which in turn fully activate integrin via the GPCRs-PLC-Ca^{2+}/DAG-Rap1 pathway (Figure 3, section of 2.3.1.1). Dose CD95 also activate these pathways in mediating leukocyte recruitment?

5.2.1 CD95 activates Syk

Syk is a non-receptor tyrosine kinase that consists of two tandem SRC-homology 2 (SH2) domains and a carboxy-terminal tyrosine kinase domain (Turner et al., 2000). Syk plays crucial roles in integrin signaling as deficiency of Syk kinase results in complete ablation of β1, β2, and β3 integrin signaling events in neutrophils and macrophages (Mocsai et al., 2002). And Syk is reported to be necessary for E-selectin-induced integrin-mediated rolling (Zarbock et al., 2007). Syk is activated downstream of Src kinase. Upon activation, SFKs phosphorylate ITAM-containing adaptor proteins. Binding of Syk's SH2 domains to the phosphorylated ITAM domain of adaptor proteins induces kinase activation and re-localization of Syk to the downstream molecules (Turner et al., 2000). Activated Syk directly interact with PLC-γ isoforms and PI3K (Mocsai et al., 2010). Does CD95 activate Syk also via SFKs?

The members of SFKs consist of c-Src, Lck, Fyn, Lyn, C-Yes, C-Fgr, Hck, Blk and Yrk. Hck, Fgr, and Lyn are the main Src-family kinases expressed in myeloid leukocytes (Lowell, 2004). Lyn-/- B cells exhibited a reduced susceptibility to CD95-mediated apoptosis (Wang et al., 1996). It has been reported that Fyn and Lck were activated upon CD95L stimulation in Jurkat cells (Schlottmann et al., 1996). This study showed that the recruitment of Lck to CD95 is dependent on the CD95 intracellular domain. In addition, recruitment of Fyn to CD95 was also described in activated T cells (Atkinson et al., 1996). They further identified the presence of a highly conserved tyrosine-containing YXXL motif located in the death domain of CD95 that is similar to the canonical ITAM motif. Later study showed this motif to be phosphorylated upon CD95 activation and served as a docking site for SH2-containing tyrosine phosphatase-1 (SHP-1), SHP-2 and SH2-containing inositol phosphatase (SHIP) in neutrophils (Daigle et al., 2002). Importantly, a screen study using antibodies against SFKs identified that Lyn was the major SFK phosphorylated and recruited to CD95 upon CD95L stimulation in myeloid cells (Letellier et al., 2010). These findings convince us that SFKs activation is involved in CD95-induced rolling signal.

5.2.2 CD95 activates BTK

BTK is a member of Tec family kinases which belong to the second largest family of non-receptor tyrosine kinases. BTK has multidomains interacting with different molecules including PKC isoforms, Syk, Wiskott-Aldrich Syndrome Protein etc. This characteristic endows BTK with multiple functions (Vargas et al., 2009). Btk-deficiency is responsible for X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (Xid) in mice which are associated with reduced integrin-mediated adhesion (Vargas et al., 2009). Other studies revealed a crucial role of BTK in neutrophils activation and recruitment upon stimulation of chemokines and selectins (Lachance et al., 2002; Yago et al., 2010; Mueller et al., 2010).

Following signal initiation, activation of BTK is downstream of Src and Syk family kinases. This interaction brings PLC-γ2 in close vicinity to BTK, which results in its phosphorylation and activation in lymphoma cell lines (Hashimoto et al., 1999). In the scenario of neutrophils rolling, E-selectin engagement leads to the activation of the ITAM-containing adaptor molecules DAP12 and FcRγ by Src-kinase Fgr (Zarbock et al.,

2008), which results in the recruitment and phosphorylation of Syk. Syk in turn activates BTK (Yago et al., 2010; Mueller et al., 2010; Figure 2). Furthermore, follow-up work from Zarbock's lab shows that Btk activates two parallel pathways in seletin-mediated rolling, one is PI3Kγ activation dependent and another one is PLC-γ2-CalDAG-GEFI-Rap1-p38MAPK pathway (Stadtmann et al., 2011).

Upon CD95L stimulation, we observed the up-regulated phosphorylation of Syk, BTK and PLC-γ2 in myeloid cells (Figure 9, 10). However, in *Syk-/-* macrophages, CD95L-induced BTK activation was not impaired as compared to the *wt* cells. Also the phosphorylation level of PLC-γ2 was up-regulated, although basal levels were lower than the in *wt* cells (Figure 10). This result indicates Syk is not involved in CD95-induced BTK activation but partially involved in CD95-induced PLC-γ2 activation.

Interestingly, in B cells BTK was found associated with CD95 via its kinase and pleckstrin homology (PH) domains and prevented the CD95-FADD interaction, which in turn blocked the apoptotic signal transduction (Vassilev et al., 1999). Notably, this association was enhanced further upon CD95 activation and could be abrogated with a BTK inhibitor (Vassilev et al., 1999). In line with this viewpoint, we observed a CD95L stimulation-dependent association of BTK with CD95 in macrophages (Figure 11A). Furthermore, treatment of BTK inhibitor totally abolished CD95-induced PLC-γ2 activation (Figure 11B, D). These data suggest that CD95-induced PLC-γ2 activation is BTK-dependent and it depicts two pathways of CD95-induced PLC-γ2 activation, one is through CD95-Syk-BTK- PLC-γ2 and another is CD95-BTK- PLC-γ2.

5.2.3 CD95 activates Rap1 and induces open conformational change of integrin

Rap GTPases are the major regulators transducing signals of extracellular stimulation to activate integrin in the process of lymphocyte recruitment (described in section 2.3.1.3). It is been described that selectin signaling induces Rap1 activation via the PLC-γ2-CalDAG-GEFI-Rap1 pathway (Stadtmann et al., 2011). In line with this, Rap1 activation upon CD95L stimulation was detected in neutrophils (Figure 16). In addition, integrin activation upon CD95L treatment was observed by performing active integrin reporter antibodies binding assay and soluble ICAM1 binding assay (Figure 17, 18). Our studies

reveal that integrin activation can be triggered by CD95-induced PLC-γ2 activation via Rap1 (Figure 35)

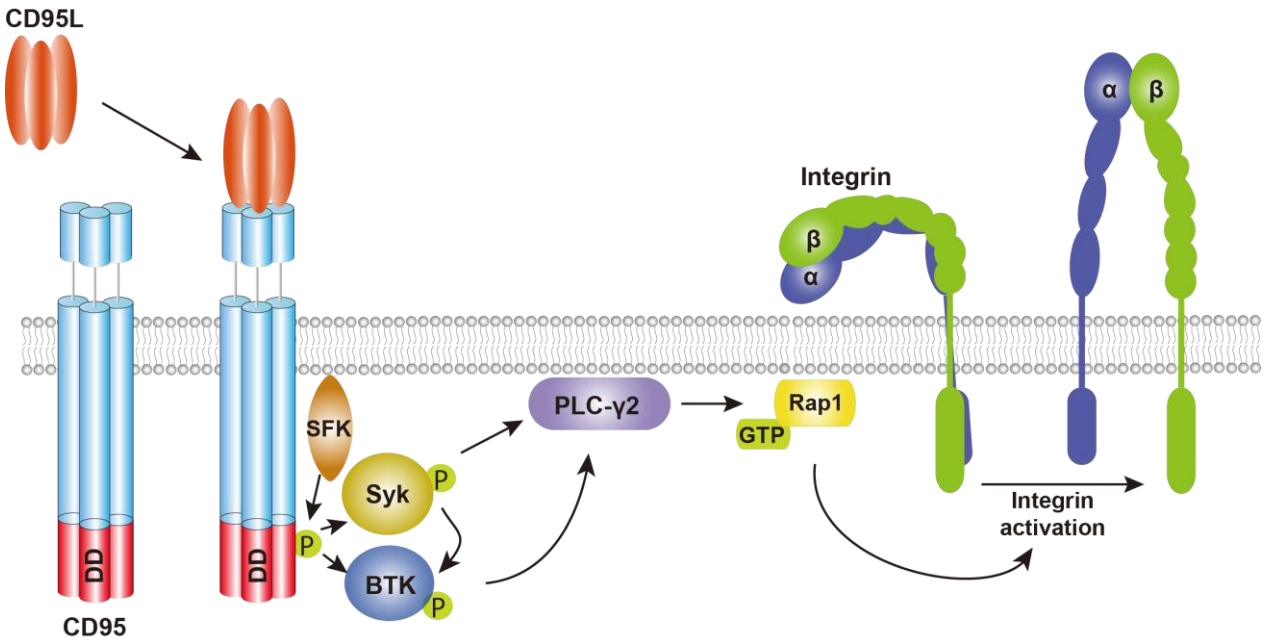

Figure 35. The signal pathway for CD95-induced integrin activation Upon CD95L stimulation, Syk and BTK are recruited to the death domain of CD95, which in turn induce the activation of CD95-SyK-BTK-PLC-γ2 and CD95-BTK-PLC-γ2 pathways. Subsequently, activated PLC-γ2 leads to integrin activation via Rap1GTP.

5.2.4 CD95-induced integrin activation – the insights of CD95 coupling with selectin and integrin signals

Soluble or coated CD95L induce neutrophil slow rolling in the autoperfused mouse flow chamber assay. However, as no rolling cells appeared in the flow chamber coated only with CD95L, it seems that the strength of CD95-CD95L engagement in not strong enough for tethering (capturing) leukocytes. This also indicates that CD95 signaling needs to cooperate with selectin signaling to initiate the slow rolling.

Selectin ligands PSGL-1 and CD44 are enriched in lipid raft (Miner et al., 2008; Neame et al., 1995). The 3 SFKs of neutrophils, Fgr, Hck, Lyn, which are activated upon the engagement of selectin to its ligands (Yago et al., 2010), also associate with cholesterol-dependent membrane rafts (Lowell et al., 2004). Interestingly, neutrophil slow rolling has

been reported to be dependent on intact lipid rafts to signal slow rolling on E-selectin and P-selectin, as raft disruption blocked selectin-mediated activation of all 3 SFKs (Yago et al., 2010). The clustering of lipid rafts is regulated by the actin cytoskeleton (Chichili and Rodgers, 2007). Ezrin/radixin/moesin (ERM) proteins, which are the linkers between cytoskeleton to integral membrane proteins via their FERM domains, associate with PSGL-1 and CD44 through their cytoplasmic domains (Yonemura et al., 1998; Serrador et al., 2002). Moreover, ligation of PSGL-1 to selectin recruits Syk to an atypical ITAM on ERM proteins bound to the cytoplasmic domain of PSGL-1 (Urzainqui et al., 2002), and induces slow rolling via association of the conventional ITAM adaptors DAP12 and FcRγ (Yago et al., 2010).

It has been reported that CD95 clustering upon engagement occurred concomitantly with reorganization of the actin cytoskeleton and aggregation of lipid rafts (Söderström et al., 2005). Accordingly, CD95 clustering in sphingolipid-rich membrane rafts is necessary for the induction of CD95 signals (Grassme et al., 2001). Importantly, it has been shown that CD95 is indirectly bound to actin via direct and specific binding to ezrin FERM domain (Lozupone et al., 2004), and that the organization of the microfilaments affects the outcome of CD95 stimulation (Parlato et al., 2000). Furthermore, CD44 has been reported to bind to CD95 and block the apoptotic signal transduction (Mielgo et al., 2006), and the formation of CD44, ezrin signal complex modulate CD95 signal (Mielgo et al., 2007). These findings suggest that CD95 may associate with selectin ligands and form a signal complex with SFKs and cytoskeleton proteins in mediating leukocyte rolling.

As in other signaling cascades, coupling kinases to the integrins, such as Src and Syk kinases, is believed to be triggered by physical clustering of the integrins and induces the outside-in pathway by phosphorylating each other (Abram and Lowell, 2009). It has been reported that Src and Syk kinases directly interact with the cytoplasmic domain of β_2, and β_3 (Arias-Salgado et al., 2003). Moreover, another study suggests that Syk coupling to integrins was mediated by ITAM containing adaptor proteins DAP12 and FcRγ (Mócsai et al., 2006).

Similar to the canonical ITAM motif, the YXXL motif in CD95 serves as a docking site for SH2 containing proteins. This motif is involved in the CD95 -induced Lyn-Syk-mediated

signaling cascade in myeloid cell recruitment (Letellier et al., 2010). Interestingly, our studies demonstrate a CD95L stimulation-dependent association of CD95 with integrin $\alpha_L\beta_2$ in macrophages (Figure 20). This association is also confirmed by increased colocalization of CD95 and integrin upon CD95L stimulation in U937 cells, a human leukemic monocyte lymphoma cell line (Figure 21). These findings give us a strong hint that CD95-induced rolling and adhesion signaling involves the formation of a signaling complex containing selectin ligands, SFKs, integrins and cytoskeleton proteins. Nonetheless, it still remains interesting and important to find the protein components involved in this signaling complex for further study. These findings will give us a better understanding on the decision between CD95-induced apoptotic or non-apoptotic signaling.

5.3 Decision of apoptosis or survival – from the view of CD95-induced integrin activation

CD95 is a confusing but fascinating molecule as it can trigger either death or survival. CD95 has been viewed mainly as a death-inducing receptor (Peter et al., 2007). On the other hand, an increasing amount of publications reveal the non-apoptotic functions of CD95, such as inducing cell survival, proliferation and migration, which are mediated mostly through the activation of MAPKs, NF- κB and PI3K pathways (Wajant et al., 2003; Peter et al., 2007; Martin-Villalba et al., 2013). Our studies present a new non-apoptotic function of CD95, which is the induction of myeloid cell rolling and adhesion via the pathway of CD95-Syk/BTK- PLC-γ2-Rap1-integrin activation (Figure 35).

BTK has dual-functions in regulating apoptosis (Uckun, 1998). It prevents the activation of anti-apoptotic transcription factor STAT3 and promotes oxidative stress-induced apoptosis in irradiated B-lineage cells (Uckun et al., 1996; Uckun et al., 2007). On the contrary, following the BCR activation, BTK regulates apoptosis in B cells via the induction of Bcl-$_{XL}$, which belongs to the Bcl-2 family and prevents the release of cytochrome c in apoptosis induction, (Anderson et al., 1996). Moreover, an alternate form of BTK is found to be highly expressed in breast cancer cells and down regulation

or inhibition of this protein causes apoptosis, which shows BTK also serves as a survival signal in cancer cells (Eifert et al., 2013). Interestingly, the same researchers, who found BTK-induced B cell apoptosis, reported that BTK associates with CD95 in B cells via its kinase and PH domains and prevented the apoptotic signal transduction by blocking CD95-FADD interaction (Vassilev et al., 1999). As the functions of BTK in apoptosis are controversial, another group shows that BTK deficiency does not affect mouse macrophage apoptosis induced by DNA damage or CD95 engagement (Khare et al., 2011). However, it has been shown that human Btk-deficient neutrophils produced more ROS after engagement of TLRs or TNFRs, which was associated with more apoptosis and could be reversed by transduction of recombinant Btk (Honda et al., 2012). These findings indicate that the role of BTK in apoptosis is dependent on either the cell types or signaling context in the cells. In our studies, CD95-induced BTK activation did not lead to any apoptosis outcome.

Integrins play an important role in the survival of leukocytes (Abram and Lowell, 2009). Integrins-activated ERK and AKT induce the upregulation of IAPs (Inhibitors of Apoptosis), c-Flip and anti-apoptotic homologs of BCL-2 (e.g., Bcl-2, Bcl-XL, Mcl-1), and downregulation of pro-apoptotic homologs of BCL-2 (e.g. Bim, Bid, Bmf) (reviewed by Vachon, 2011). Integrin outside-in signaling-activated Src can phosphorylate pro-caspase 8 to suppress its activation (Frisch, 2008). Focal adhesion kinase (FAK), another important kinase activated by outside-in signaling, binds to the death domain kinase receptor-interacting protein 1(RIP1) and prevents its recruitment of FADD to the DISC formation (Kurenova et al., al 2004). In line with this, the DISC formation was not detected in myeloid cells upon CD95L stimulation (unpublished data from our lab). This might be contributed by the survival signal of CD95 signaling-activated integrins (Figure 35) and the binding of SFK to the death domain of CD95. In addition, integrin $\alpha_L\beta_2$ is associated with CD95 upon CD95L stimulation (Figure 20, 21). This association might lead to the formation of a signal complex containing selectin ligands, SFKs, integrins and cytoskeleton proteins, which block the FADD recruitment to CD95 and thus the formation of the DISC.

5.4 Other cells involved in CD95-mediated innate response

5.4.1 Endothelial cells present CD95L in the recruitment of myeloid cells

During inflammation, endothelial cells participate in leukocyte recruitment by presenting selectins and chemokines upon the stimulation of pro-inflammatory cytokines, which are released by activated tissue resident innate immune cells (described in section 2.4.1).

Early studies show that CD95 engagement causes rapid, extensive and disseminated endothelial cells apoptosis throughout the body (Cardier et al., 1999; Janin et al., 2002). However, in these studies CD95 was activated by conjugating with agonistic CD95-specific antibody, which was suggested to induce CD95-mediated apoptosis via co-stimulation of FcγRIIB (Xu et al., 2003). In line with this, we did not detect any apoptotic effect of CD95L in *in vitro* cultured endothelial cells or endothelial cells in CD95L i.v. injected mice.

Myeloid cells are the major source for CD95L during inflammation (Letellier et al., 2010). CD95L was also shown to be expressed by endothelial cells (Sata and Walsh, 1998). To find out whether endothelial cells present CD95L during inflammation, we performed the tamoxifen-induced deletion of CD95L in endothelial cells of *CD95L*$^{f/f;Ve\text{-}CadherinERT2/4cre}$ mouse line (Figure 23). And we observed an impaired neutrophil recruitment in mice with CD95L deletion in endothelial cells as compared to the *wt* control. It indicates that endothelial cells also contribute to the CD95-induced myeloid cell recruitment by presenting CD95L. Nonetheless, how CD95L expression is regulated in endothelial cells during myeloid cell recruitment deserves further investigation.

Adhesion molecules expression can be triggered in endothelial cells upon the stimulation of TNF-α. However, unlike TNF-α, CD95L stimulation has no effect on the expression of adhesion molecules in *in vitro* cultured endothelial cells (Figure 22). In addition, deletion of CD95 in endothelial cells (*CD95*$^{f/f;Ve\text{-}CadherinERT2/4cre}$) also has no impacts on the expression levels of adhesion molecules and neutrophil recruitment (Figure 23E, 25).

Interestingly, an early study showed that CD95L expression in endothelial cells was downregulated upon TNFα treatment (Sata and Walsh, 1998). And over expression of CD95L by adenovirus transfection in endothelial cells markedly attenuated TNFα-

induced T cells and macrophages infiltration and adherent mononuclear cells underwent apoptosis (Sata and Walsh, 1998). Nevertheless, as they also observed, the infiltrated cells upon TNFα treatment might be killed by CD95-induced apoptosis since it has been suggested that the decision of CD95-induced death or survival signaling depends on the stimulation threshold (Lavrik et al., 2007). In line with this, tumor endothelial cells selectively and highly express CD95L, which serves as a barrier to prevent the infiltration of CD8 cells via induction of apoptosis in the establishment of immune tolerance (Motz et al., 2014).

5.4.2 CD95 selectively drives inflammatory monocytes recruitment

Monocytes comprise the second wave of immune cells recruitment during the innate immune response. Monocytes consist of different subtypes which have distinct functions and respond differently during inflammation (described in section 2.5).

As other immune cells, CD95 in monocytes are well documented with apoptotic functions (Um et al., 1996; Kiener et al., 1997; Blomberg et al., 2009). The CD95 signaling is also likely to play an *in vivo* role in governing monocyte/macrophage homeostasis. Compared with congenic control C57BL/6 mice, CD95-deficient mice (*Fas*^{lpr}) display increased numbers of circulating monocytes in the steady state and in a model of systemic inflammatory arthritis (Brown et al., 2004). Contrary to this, circulating monocytes are increased upon CD95 activation by i.v. injection of CD95L, especially for the subtype of Ly6C^{hi} subtype (Figure 26). CD95L triggered Ly6C^{hi} monocyte mobilization, with a peak at 6 hours after CD95L injection. At 36 hours after CD95L injection, it decreased and returned to control levels (Figure 26). The turnover time of Ly6C^{hi} monocytes is reported very short by chasing the monocytes with BrdU labeling. In mice injected with MC21 antibody which ablated the blood Ly6C^{hi} monocytes, the replenishment of Ly6C^{hi} monocytes was observed 6 hours after MC21 treatment (Yona et al., 2012). So we believe that CD95L stimulation-mobilizated Ly6C^{hi} monocytes are from the monocytes reservoir but not from newly generated monocytes in the bone marrow. We also observed the increased-recruitment of Ly6C^{hi} monocytes in the lymph nodes upon CD95L injection. In consistent with our findings, another apoptosis-inducing ligand, the TNF-related apoptosis-inducing ligand (TRAIL) is also reported to induce

chemotactic migration of monocytes *in vivo* via a death receptor 4-mediated RhoGTPase pathway (Wei et al., 2010).

The different response of Ly6C^{hi} and Ly6C^{lo} monocytes to CD95L stimulation might be related to their difference in CD95 expression (Figure 28). However, deletion of CD95 only shows a minor effect on attenuating the CD95L-induced Ly6C^{hi} monocyte mobilization (Figure 30). It also might be dependent on the cytokine response upon CD95L treatment, as increased plasma CCL2 level was detected (Figure 31). Nonetheless, the source of CCL2 after CD95L treatment remains unclear. Interestingly, it has been reported that bone marrow mesenchymal stem cells and their progeny, including CCL12-abundant reticular cells, rapidly expressed CCL2 in response to circulating TLR ligands and induced Ly6C^{hi} monocyte mobilization to the bloodstream (Shi et al., 2011). More importantly, a recent investigation shows that CD95-induced apoptosis on target cells is associated with the production of cytokines and chemokines, such as IL-6, IL-8, CXCL1, CCL2 and GMCSF, which serve as “find-me” signals for the phagocytes in clearance of dead cells (Cullen et al., 2013).

Taken together, out studies reveal a CD95-induced integrin activation pathway, the CD95-Syk/BTK-PLC-γ2-Rap1-integrin, that mediates myeloid cells rolling and adhesion. CD95L is presented by endothelial cells in the process. Moreover, CD95 selectively induces the mobilization and recruitment of inflammatory monocytes in a CCL2-dependent manner. As a conclusion, our studies identify a pathway of CD95 chemotactic axis for innate immune cell recruitment.

6 REFERENCES

List of references

Abram, C.L., and Lowell, C. a (2009). The ins and outs of leukocyte integrin signaling. Annu. Rev. Immunol. *27*, 339–362.

Alberts, B., Johnson,A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2007). Molecular Biology of the Cell. New York: Garland Science.

Alderson, M.R., Armitage, R.J., Maraskovsky, E., Tough, T.W., Roux, E., Schooley, K., Ramsdell, F., and Lynch, D.H. (1993). Fas transduces activation signals in normal human T lymphocytes. J. Exp. Med. *178*, 2231–2235.

Alderson, M.R., Tough, T.W., Davis-Smith, T., et al. (1995). Fas ligand mediates activation-induced cell death in human T lymphocytes. J. Exp. Med. *181*, 71–77.

Alexander Zarbock, Clifford A. Lowell, and K.L. (2007). Spleen Tyrosine Kinase Syk Is Necessary for E-Selectin-Induced αLβ2 Integrin-Mediated Rolling on Intercellular Adhesion Molecule. Immunity *26*, 773-783.

Alon, R., Aker, M., Feigelson, S., Sokolovsky-Eisenberg, M., Staunton, D. E., Cinamon, G., Grabovsky, V., Shamri, R. and Etzioni, A. (2003). A novel genetic leukocyte adhesion deficiency in subsecond triggering of integrin avidity by endothelial chemokines results in impaired leukocyte arrest on vascular endothelium under shear flow. Blood *101*, 4437- 4445.

Altemeier, W. a, Zhu, X., Berrington, W.R., Harlan, J.M., and Liles, W.C. (2007). Fas (CD95) induces macrophage proinflammatory chemokine production via a MyD88-dependent, caspase-independent pathway. J. Leukoc. Biol. *82*, 721–728.

Anderson, J., and Teutsch, M. (1996). An essential role for Bruton's tyrosine kinase in the regulation of B-cell apoptosis. Proc. … *93*, 10691–10696.

Annemieke A. DE MELKER, Duco KRAMER, I.K. and A.S. (1997). The two phenylalanines in the GFFKR motif of the integrin a6A subunit are essential for heterodimerization. Biochem. J. *537*, 529-537.

Arai,H., Gordon,D., Nabel,E.G., and Nabel,G.J. (1997). Gene transfer of Fas ligand induces tumor regression in vivo. Proc. Natl. Acad. Sci. U. S. A *94*, 13862-13867.

Arias-Salgado, E.G., Lizano, S., Sarkar, S., Brugge, J.S., Ginsberg, M.H., and Shattil, S.J. (2003). Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. Proc. Natl. Acad. Sci. U. S. A. *100*, 13298–13302.

Ashkenazi, a., Dixit, V.M. (1998). Death Receptors: Signaling and Modulation. Science (80-.). *281*, 1305–1308.

Atkinson,E.A., Ostergaard,H., Kane,K., Pinkoski,M.J., Caputo,A., Olszowy,M.W., and Bleackley,R.C. (1996). A physical interaction between the cell death protein Fas and the tyrosine kinase p59fynT. J. Biol. Chem. *271*, 5968-5971.

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., and Geissmann, F. (2007). Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science *317*, 666–670.

Auffray, C., Sieweke, M.H., and Geissmann, F. (2009). Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu. Rev. Immunol. *27*, 669–692.

Badorff, C., Ruetten, H., Mueller, S., Stahmer, M., Gehring, D., Jung, F., Ihling, C., Zeiher, A.M., and Dimmeler, S. (2002). Fas receptor signaling inhibits glycogen synthase kinase 3 beta and induces cardiac hypertrophy following pressure overload. J. Clin. Invest. *109*, 373–381.

Balachandran, S., Thomas, E., and Barber, G.N. (2004). A FADD-dependent innate immune mechanism in mammalian cells. Nature *432*, 401–405.

Bamboat, Z.M., Ocuin, L.M., Balachandran, V.P., Obaid, H., Plitas, G., and DeMatteo, R.P. (2010). Conventional DCs reduce liver ischemia/reperfusion injury in mice via IL-10 secretion. J. Clin. Invest. *120*, 559–569.

Barnhart, B.C., Legembre, P., Pietras, E., Bubici, C., Franzoso, G., and Peter, M.E. (2004). CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells. EMBO J. *23*, 3175–3185.

Beglova, N., Blacklow, S.C., Takagi, J., and Springer, T. a (2002). Cysteine-rich module structure reveals a fulcrum for integrin rearrangement upon activation. Nat. Struct. Biol. *9*, 282–287.

Bellgrau, D., Gold, D., Selawry, H., Moore, J., Franzusoff, A., and Duke, R.C. (1995). A role for CD95 ligand in preventing graft rejection. Nature *377*, 630–632.

Berlin, C., Bargatze, R.F., Campbell, J.J., von Andrian, U.H., Szabo, M.C., Hasslen, S.R., Nelson, R.D., Berg, E.L., Erlandsen, S.L., and Butcher, E.C. (1995). Alpha 4 Integrins Mediate Lymphocyte Attachment and Rolling Under Physiologic Flow. Cell *80*, 413–422.

Bezman, N., and Koretzky, G. a (2007). Compartmentalization of ITAM and integrin signaling by adapter molecules. Immunol. Rev. *218*, 9–28.

Birch, K. A., Pober, J. S., Zavoico, G. B., Means, A. R. & Ewenstein, B. M. (1992). Calcium/calmodulin transduces thrombin-stimulated secretion: studies in intact and minimally permeabilized human umbilical vein endothelial cells. J. Cell Biol. *118*, 1501–1510.

Blomberg, J., Ruuth, K., Jacobsson, M., Höglund, A., Nilsson, J. a, and Lundgren, E. (2009). Reduced FAS transcription in clones of U937 cells that have acquired resistance to Fas-induced apoptosis. FEBS J. *276*, 497–508.

Bossaller, L., Chiang, P.-I., Schmidt-Lauber, C., Ganesan, S., Kaiser, W.J., Rathinam, V. a K., Mocarski, E.S., Subramanian, D., Green, D.R., Silverman, N., et al. (2012). Cutting edge: FAS (CD95) mediates noncanonical IL-1β and IL-18 maturation via caspase-8 in an RIP3-independent manner. J. Immunol. *189*, 5508–5512.

Brown, N.J., Hutcheson, J., Bickel, E., Scatizzi, J.C., Albee, L.D., Haines, G.K., Eslick, J., Bradley, K., Taricone, E., and Perlman, H. (2004). Fas Death Receptor Signaling Represses Monocyte Numbers and Macrophage Activation In Vivo. J. Immunol. *173*, 7584–7593.

Burbach, B.J., Medeiros, R.B., Mueller, K.L., and Shimizu, Y. (2007). T-cell receptor signaling to integrins. Immunol. Rev. *218*, 65–81.

Cambi, A., Joosten, B., Koopman, M., de Lange, F., Beeren, I., Torensma, R., Fransen, J. A., Garcia-Parajo, M., van Leeuwen, F. N. and Figdor, C. G. (2006). Organization of the integrin LFA-1 in nanoclusters regulates its activity. Mol. Biol. Cell *17*, 4270-4281.

Campbell, J.J., Hedrick, J., Zlotnik,A., Siani, M. A., Thompson, D. A. and Butcher, E.C. (1998). Chemokines and the Arrest of Lymphocytes Rolling Under Flow Conditions. Science *279*, 381–384.

Cardier, J.E., Schulte, T., Kammer, H., Kwak, J., and Cardier, M. (1999). Fas (CD95, APO-1) antigen expression and function in murine liver endothelial cells: implications for the regulation of apoptosis in liver endothelial cells. FASEB J. *13*, 1950–1960.

Carlin, L.M., Stamatiades, E.G., Auffray, C., Hanna, R.N., Glover, L., Vizcay-Barrena, G., Hedrick, C.C., Cook, H.T., Diebold, S., and Geissmann, F. (2013). Nr4a1-Dependent Ly6C(low) Monocytes Monitor Endothelial Cells and Orchestrate Their Disposal. Cell *153*, 362–375.

Carman, C. V, and Springer, T. a (2004). A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. J. Cell Biol. *167*, 377–388.

Chao Shi, Ting Jia, Simon Mendez-Ferrer, Tobias M. Hohl, Natalya V. Serbina, Lauren Lipuma, Ingrid Leiner, Ming O. Li, Paul S. Frenette, and E.G.P. (2011). Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. Immunity *34*, 590–601.

Chen, G.Y., and Nuñez, G. (2010). Sterile inflammation: sensing and reacting to damage. Nat. Rev. Immunol. *10*, 826–837.

Chen, L., Park, S.-M., Tumanov, A. V, Hau, A., Sawada, K., Feig, C., Turner, J.R., Fu, Y.-X., Romero, I.L., Lengyel, E., et al. (2010). CD95 promotes tumour growth. Nature *465*, 492–496.

Chesnutt, B.C., Smith, D.F., Raffler, N. a, Smith, M.L., White, E.J., and Ley, K. (2006). Induction of LFA-1-dependent neutrophil rolling on ICAM-1 by engagement of E-selectin. Microcirculation *13*, 99–109.

Chichili, G.R., and Rodgers, W. (2007). Clustering of membrane raft proteins by the actin cytoskeleton. J. Biol. Chem. *282*, 36682–36691.

Cifuni, S. M., Wagner, D. D. and Bergmeier, W. (2008). CalDAG-GEFI and protein kinase C (PKC) represent alternative pathways leading to activation of integrin αIIbβ3 in platelets. Blood *112*, 1696-1703.

Constantin, G., Majeed, M., Giagulli, C., Piccio, L., Kim, J.Y., Butcher, E.C., and Laudanna, C. (2000). Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. Immunity *13*, 759–769.

Cooper, D., Norling, L. V, and Perretti, M. (2008). Novel insights into the inhibitory effects of Galectin-1 on neutrophil recruitment under flow. J. Leukoc. Biol. *83*, 1459–1466.

Cooper, M.D., and Alder, M.N. (2006). The evolution of adaptive immune systems. Cell 124, 815–822.

Corsini, N.S., Sancho-Martinez, I., Laudenklos, S., Glagow, D., Kumar, S., Letellier, E., Koch, P., Teodorczyk, M., Kleber, S., Klussmann, S., et al. (2009). The death receptor CD95 activates adult neural stem cells for working memory formation and brain repair. Cell Stem Cell *5*, 178–190.

Crittenden JR, Bergmeier W, Zhang Y, Piffath CL, Liang Y, Wagner DD, Housman DE, Graybiel AM (2004) CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. Nat. Med. *10*,982-986.

Croft, M., Duan, W., Choi, H., Eun, S.-Y., Madireddi, S., and Mehta, A. (2012). TNF superfamily in inflammatory disease: translating basic insights. Trends Immunol. *33*, 144–152.

Cros, J., Cagnard, N., Woollard, K., Patey, N., Zhang, S.-Y., Senechal, B., Puel, A., Biswas, S.K., Moshous, D., Picard, C., et al. (2010). Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. Immunity *33*, 375–386.

Cullen, S.P., Henry, C.M., Kearney, C.J., Logue, S.E., Feoktistova, M., Tynan, G. a, Lavelle, E.C., Leverkus, M., and Martin, S.J. (2013). Fas/CD95-Induced Chemokines Can Serve as "Find-Me" Signals for Apoptotic Cells. Mol. Cell *49*, 1034–1048.

Daigle,I., Yousefi,S., Colonna,M., Green,D.R., and Simon,H.U. (2002). Death receptors bind SHP-1 and block cytokine-induced anti-apoptotic signaling in neutrophils. Nat. Med. *8*, 61-67.

Dangerfield,J., Larbi,K.Y., Huang,M.T., Dewar,A., and Nourshargh,S. (2002). PECAM-1 (CD31) homophilic interaction up-regulates alpha6beta1 on transmigrated neutrophils in vivo and plays a functional role in the ability of alpha6 integrins to mediate leukocyte migration through the perivascular basement membrane. J. Exp. Med. *196*, 1201-1211.

De Caterina, R., Libby, P., Peng, H.B., Thannickal, V.J., Rajavashisth, T.B., Gimbrone, M. a, Shin, W.S., and Liao, J.K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. J. Clin. Invest. *96*, 60–68.

Deak, J.C., Cross, J. V, Lewis, M., Qian, Y., Parrott, L. a, Distelhorst, C.W., and Templeton, D.J. (1998). Fas-induced proteolytic activation and intracellular redistribution of the stress-signaling kinase MEKK1. Proc. Natl. Acad. Sci. U. S. A. *95*, 5595–5600.

Deban, L., Russo, R.C., Sironi, M., Moalli, F., Scanziani, M., Zambelli, V., Cuccovillo, I., Bastone, A., Gobbi, M., Valentino, S., et al. (2010). Regulation of leukocyte recruitment by the long pentraxin PTX3. Nat. Immunol. *11*, 328–334.

Desbarats, J., and Newell, M.K. (2000). Fas engagement accelerates liver regeneration after partial hepatectomy. Nat. Med. *6*, 920–923.

Desbarats, J., Birge, R.B., Mimouni-Rongy, M., Weinstein, D.E., Palerme, J.-S., and Newell, M.K. (2003). Fas engagement induces neurite growth through ERK activation and p35 upregulation. Nat. Cell Biol. *5*, 118–125.

Dupont, P.J., and Warrens, A.N. (2007). Fas ligand exerts its pro-inflammatory effects via neutrophil recruitment but not activation. Immunology *120*, 133–139.

Eifert, C., Wang, X., Kokabee, L., Kourtidis, A., Jain, R., Gerdes, M.J., and Conklin, D.S. (2013). A novel isoform of the B cell tyrosine kinase BTK protects breast cancer cells from apoptosis. Genes. Chromosomes Cancer *52*, 961–975.

Eskan, M. a, Jotwani, R., Abe, T., Chmelar, J., Lim, J.-H., Liang, S., Ciero, P. a, Krauss, J.L., Li, F., Rauner, M., et al. (2012). The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss. Nat. Immunol. *13*, 465–473.

Evans, R., Patzak, I., Svensson, L., De Filippo, K., Jones, K., McDowall, A., and Hogg, N. (2009). Integrins in immunity. J. Cell Sci. *122*, 215–225.

Farley, S.M., Dotson, A.D., Purdy, D.E., Sundholm, A.J., Schneider, P., Magun, B.E., and Iordanov, M.S. (2006). Fas ligand elicits a caspase-independent proinflammatory response in human keratinocytes: implications for dermatitis. J. Invest. Dermatol. *126*, 2438–2451.

Finger, E.B., Puri, K.D., Alon, R., Lawrence, M.B., von Andrian, U.H., and Springer, T. a (1996). Adhesion through L-selectin requires a threshold hydrodynamic shear. Nature *379*, 266–269.

Fisher, G.H., Rosenberg, F.J., Straus, S.E., Dale, J.K., Middelton, L.A., Lin, A.Y., Strober, W., Lenardo, M.J., and Puck, J.M. (1995). Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell *81*, 935–946.

Forsyth, K.D., and Levinsky, R.J. (1990). Preparative procedures of cooling and re-warming increase leukocyte integrin expression and function on neutrophils. J. Immunol. Methods *128*, 159–163.

Frisch, S.M. (2008). Caspase-8: fly or die. Cancer Res. *68*, 4491–4493.

Garland, J.M., Aldridge, a, Wagstaffe, J., and Dexter, T.M. (1983). Studies on the in vivo production of a lymphokine activity, interleukin 3 (IL-3) elaborated by lymphocytes and a myeloid leukaemic line in vitro and the fate of IL-3 dependent cell lines. Br. J. Cancer *48*, 247–259.

Garlanda, C., Bottazzi, B., Bastone, A., and Mantovani, A. (2005). Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. Annu. Rev. Immunol. *23*, 337–366.

Geissmann, F., Jung, S., and Littman, D.R. (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity *19*, 71–82.

Geissmann, F., Manz, M.G., Jung, S., Sieweke, M.H., Merad, M. (2010). Development of Monocytes, Macrophages, and Dendritic Cells. Science *327*, 656–661.

Gil, C.D., Gullo, C.E., and Oliani, S.M. (2010). Effect of exogenous galectin-1 on leukocyte migration: modulation of cytokine levels and adhesion molecules. Int. J. Clin. Exp. Pathol. *4*, 74–84.

Glasser L, F.R. (1990). The effect of various cell separation procedures on assays of neutrophil function -A critical appraisal. Am J Clin Pathol. *93*, 662-669

Grassme, H., Jekle, a, Riehle, a, Schwarz, H., Berger, J., Sandhoff, K., Kolesnick, R., and Gulbins, E. (2001). CD95 signaling via ceramide-rich membrane rafts. J. Biol. Chem. *276*, 20589–20596.

Green, D.R., and Ferguson, T. a (2001). The role of Fas ligand in immune privilege. Nat. Rev. Mol. Cell Biol. *2*, 917–924.

Green, D.R., Droin, N., and Pinkoski, M. (2003). Activation-induced cell death in T cells. Immunol. Rev. *193*, 70–81.

Greenstein, S., and Barnard, J. (2000). Fas activation reduces neutrophil adhesion to endothelial cells. J. Leukoc. *68*, 715–22.

Griffith, T.S., Brunner, T., Fletcher, S.M., Green, D.R., and Ferguson, T.A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. Science *270*, 1189–1192.

Hajishengallis, G., and Chavakis, T. (2013). Endogenous modulators of inflammatory cell recruitment. Trends Immunol. *34*, 1–6.

Hallmann,R., Horn,N., Selg,M., Wendler,O., Pausch,F., and Sorokin,L.M. (2005). Expression and function of laminins in the embryonic and mature vasculature. Physiol Rev. *85*, 979-1000.

Hammond, M.D., Taylor, R. a, Mullen, M.T., Ai, Y., Aguila, H.L., Mack, M., Kasner, S.E., McCullough, L.D., and Sansing, L.H. (2014). CCR2+Ly6Chi Inflammatory Monocyte Recruitment Exacerbates Acute Disability Following Intracerebral Hemorrhage. J. Neurosci. *34*, 3901–3909.

Hashimoto, B.S., Iwamatsu, A., Ishiai, M., Okawa, K., Yamadori, T., Matsushita, M., Baba, Y., Kishimoto, T., Kurosaki, T., and Tsukada, S. (1999). Identification of the SH2 Domain Binding Protein of Bruton's Tyrosine Kinase as BLNK—Functional Significance of Btk-SH2 Domain in B-Cell Antigen Receptor-Coupled Calcium Signaling. Blood *94*, 2357–2364.

Hendey, B. (2002). Fas activation opposes PMA-stimulated changes in the localization of PKCδ: a mechanism for reducing neutrophil adhesion to endothelial cells. J. Leukoc. *71*, 863–70.

Hettinger, J., Richards, D.M., Hansson, J., Barra, M.M., Joschko, A.-C., Krijgsveld, J., and Feuerer, M. (2013). Origin of monocytes and macrophages in a committed progenitor. Nat. Immunol. *14*, 821–830.

Honda, F., Kano, H., Kanegane, H., Nonoyama, S., Kim, E.-S., Lee, S.-K., Takagi, M., Mizutani, S., and Morio, T. (2012). The kinase Btk negatively regulates the production of reactive oxygen species and stimulation-induced apoptosis in human neutrophils. Nat. Immunol. *13*, 369–378.

Hughes, M. a, Harper, N., Butterworth, M., Cain, K., Cohen, G.M., and MacFarlane, M. (2009). Reconstitution of the death-inducing signaling complex reveals a substrate switch that determines CD95-mediated death or survival. Mol. Cell *35*, 265–279.

Humphries, J.D., and Humphries, M.J. (2007). CD14 is a ligand for the integrin alpha4beta1. FEBS Lett. *581*, 757–763.

Ingersoll, M. a, Spanbroek, R., Lottaz, C., Gautier, E.L., Frankenberger, M., Hoffmann, R., Lang, R., Haniffa, M., Collin, M., Tacke, F., et al. (2010). Comparison of gene expression profiles between human and mouse monocyte subsets. Blood *115*, e10–9.

ir-Kirk,T.L., Atkinson,J.J., Broekelmann,T.J., Doi,M., Tryggvason,K., Miner,J.H., Mecham,R.P., and Senior,R.M. (2003). A site on laminin alpha 5, AQARSAASKVKVSMKF, induces inflammatory cell production of matrix metalloproteinase-9 and chemotaxis. J. Immunol. *171*, 398-406.

Ivetic, A., and Ridley, A.J. (2004). Ezrin/radixin/moesin proteins and Rho GTPase signalling in leucocytes. Immunology *112*, 165–176.

Janin A, Deschaumes C, Daneshpouy M, Estaquier J, Micic-Polianski J, Rajagopalan-Levasseur P, Akarid K, Mounier N, Gluckman E, Socié G, A.J. (2002). CD95 engagement induces disseminated endothelial cell apoptosis in vivo: immunopathologic implications. Blood *99*, 2940–2947.

Johnson, Z., Proudfoot, a E., and Handel, T.M. (2005). Interaction of chemokines and glycosaminoglycans: a new twist in the regulation of chemokine function with opportunities for therapeutic intervention. Cytokine Growth Factor Rev. *16*, 625–636.

Jung, U., and Ley, K. (1999). Mice lacking two or all three selectins demonstrate overlapping and distinct functions for each selectin. J. Immunol. *162*, 6755–6762.

Kabelitz, D., Marx, S., Robertson, M.J., and Janssen, O. (1996). Rapid modulation of T lymphocyte surface antigens induced by Fas (CD95, APO-1) ligation. Cell. Immunol. *173*, 108–115.

Kadono, T., Venturi, G.M., Steeber, D. a., and Tedder, T.F. (2002). Leukocyte Rolling Velocities and Migration Are Optimized by Cooperative L-Selectin and Intercellular Adhesion Molecule-1 Functions. J. Immunol. *169*, 4542–4550.

Kaindl, T., Rieger, H., Kaschel, L. M., Engel, U., Schmaus, A., Sleeman, J., and Tanaka, M. (2012). Spatio-temporal patterns of pancreatic cancer cells expressing CD44 isoforms on supported membranes displaying hyaluronic acid oligomers arrays. PLoS One *7*, e42991.

Kasirer-Friede, A., Kahn, M.L., and Shattil, S.J. (2007). Platelet integrins and immunoreceptors. Immunol. Rev. *218*, 247–264.

Katagiri, K., Hattori, M., Minato, N., Irie, S., Takatsu, K. and Kinashi, T. (2000). Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and phosphatidylinositol-3-OH kinase. Mol. Cell. Biol. *20*, 1956-1969.

Katagiri, K., Shimonaka, M., and Kinashi, T. (2004). Rap1-mediated lymphocyte function-associated antigen-1 activation by the T cell antigen receptor is dependent on phospholipase C-gamma1. J. Biol. Chem. *279*, 11875–11881.

Katayama, Y., Hidalgo, A., Chang, J., Peired, A., and Frenette, P.S. (2005). CD44 is a physiological E-selectin ligand on neutrophils. J. Exp. Med. *201*, 1183–1189.

Kennedy, N.J., Kataoka, T., Tschopp, J., and Budd, R.C. (1999). Caspase activation is required for T cell proliferation. J. Exp. Med. *190*, 1891–1896.

Khare, A., Viswanathan, B., Gund, R., Jain, N., Ravindran, B., George, A., Rath, S., and Bal, V. (2011). Role of Bruton's tyrosine kinase in macrophage apoptosis. Apoptosis *16*, 334–346.

Kiener, P., Davis, P., and Starling, G. (1997). Differential induction of apoptosis by Fas–Fas ligand interactions in human monocytes and macrophages. J. … *185*.

Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, et al. (2009). Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. J. Neurosci. *29*,13435–13444.

Kim, M., Carman, C. V., Yang, W., Salas, A. and Springer, T. A. (2004). The primacy of affinity over clustering in regulation of adhesiveness of the integrin {alpha}L{beta}2. J. Cell Biol. *167*, 1241-1253.

Kimbrell, D. a, and Beutler, B. (2001). The evolution and genetics of innate immunity. Nat. Rev. Genet. *2*, 256–267.

Kinashi, T., Aker, M., Sokolovsky-Eisenberg, M., Grabovsky, V., Tanaka, C., Shamri, R., Feigelson, S., Etzioni, A. and Alon, R. (2004). LAD-III, a leukocyte adhesion deficiency syndrome associated with defective Rap1 activation and impaired stabilization of integrin bonds. Blood *103*, 1033-1036.

Kleber, S., Sancho-Martinez, I., Wiestler, B., Beisel, A., Gieffers, C., Hill, O., Thiemann, M., Mueller, W., Sykora, J., Kuhn, A., et al. (2008). Yes and PI3K bind CD95 to signal invasion of glioblastoma. Cancer Cell *13*, 235–248.

Kohan, D.E. (2008). Progress in gene targeting: using mutant mice to study renal function and disease. Kidney Int. *74*, 427–437.

Krammer, P.H. (2000). CD95's deadly mission in the immune system. Nature *407*, 789–795.

Kurenova, E., Li-Hui Xu, Xihui Yang, Albert S. Baldwin Jr., Rolf J. Craven, Steven K. Hanks, Z.L. and W., and Cance, G. (2004). Focal adhesion kinase suppresses apoptosis by binding to the death domain of receptor-interacting protein. Mol. Cell. Biol *24*, 4361.

Lachance, G., Levasseur, S., and Naccache, P.H. (2002). Chemotactic factor-induced recruitment and activation of Tec family kinases in human neutrophils. Implication of phosphatidynositol 3-kinases. J. Biol. Chem. *277*, 21537–21541.

Lavrik, I.N., Golks, A., Riess, D., Bentele, M., Eils, R., and Krammer, P.H. (2007). Analysis of CD95 threshold signaling: triggering of CD95 (FAS/APO-1) at low concentrations primarily results in survival signaling. J. Biol. Chem. *282*, 13664–13671.

Lawrence, M.B., and Springer, T. a (1991). Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell *65*, 859–873.

Lawrence, T., and Natoli, G. (2011). Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nat. Rev. Immunol. *11*, 750–761.

Lee, J.C., Hapel, a J., and Ihle, J.N. (1982). Constitutive production of a unique lymphokine (IL 3) by the WEHI-3 cell line. J. Immunol. *128*, 2393–2398.

Lee, S.-M., Kim, E.-J., Suk, K., and Lee, W.-H. (2011). Stimulation of Fas (CD95) induces production of pro-inflammatory mediators through ERK/JNK-dependent activation of NF-κB in THP-1 cells. Cell. Immunol. *271*, 157–162.

Lefort, C.T., Rossaint, J., Moser, M., Petrich, B.G., Zarbock, A., Monkley, S.J., Critchley, D.R., Ginsberg, M.H., Fa, R., and Ley, K. (2012). Distinct roles for talin-1 and kindlin-3 in LFA-1 extension and affinity regulation. Blood *119*, 4275–4282.

Legembre, P., Barnhart, B.C., Zheng, L., Vijayan, S., Straus, S.E., Puck, J., Dale, J.K., Lenardo, M., and Peter, M.E. (2004). Induction of apoptosis and activation of NF-kappaB by CD95 require different signalling thresholds. EMBO Rep. *5*, 1084–1089.

Letellier, E., Kumar, S., Sancho-Martinez, I., Krauth, S., Funke-Kaiser, A., Laudenklos, S., Konecki, K., Klussmann, S., Corsini, N.S., Kleber, S., et al. (2010). CD95-ligand on peripheral myeloid cells activates Syk kinase to trigger their recruitment to the inflammatory site. Immunity *32*, 240–252.

Léveillé, C., Bouillon, M., Guo, W., Bolduc, J., Sharif-Askari, E., El-Fakhry, Y., Reyes-Moreno, C., Lapointe, R., Merhi, Y., Wilkins, J. a, et al. (2007). CD40 ligand binds to alpha5beta1 integrin and triggers cell signaling. J. Biol. Chem. *282*, 5143-5151.

Ley K, Reutershan J. (2006). Leucocyte-endothelial interactions in health and disease. Handb. Exp. Pharmacol. *176*, 97–133.

Ley, K., Laudanna, C., Cybulsky, M.I., and Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat. Rev. Immunol. *7*, 678–689.

Lim, Y.-C., and Luscinskas, F.W. (2006). Isolation and culture of murine heart and lung endothelial cells for in vitro model systems. Methods Mol. Biol. *341*, 141–154.

Lim, Y.-C., Garcia-Cardena, G., Allport, J.R., Zervoglos, M., Connolly, A.J., Gimbrone, M. a, and Luscinskas, F.W. (2003). Heterogeneity of endothelial cells from different organ sites in T-cell subset recruitment. Am. J. Pathol. *162*, 1591–1601.

Low, W., Smith, a, Ashworth, a, and Collins, M. (1999). JNK activation is not required for Fas-mediated apoptosis. Oncogene *18*, 3737–3741.

Lowell, C. a (2004). Src-family kinases: rheostats of immune cell signaling. Mol. Immunol. *41*, 631–643.

Lozupone, F., Lugini, L., Matarrese, P., Luciani, F., Federici, C., Iessi, E., Margutti, P., Stassi, G., Malorni, W., and Fais, S. (2004). Identification and relevance of the CD95-binding domain in the N-terminal region of ezrin. J. Biol. Chem. *279*, 9199–9207.

Lu, C., Shimaoka, M., Ferzly, M., Oxvig, C., Takagi, J., and Springer, T. a (2001). An isolated, surface-expressed I domain of the integrin alphaLbeta2 is sufficient for strong adhesive function when locked in the open conformation with a disulfide bond. Proc. Natl. Acad. Sci. U. S. A. *98*, 2387–2392.

Luo, B.-H., Carman, C. V, and Springer, T. a (2007). Structural basis of integrin regulation and signaling. Annu. Rev. Immunol. *25*, 619–647.

Marshall, B.T., Long, M., Piper, J.W., Yago, T., McEver, R.P., and Zhu, C. (2003). Direct observation of catch bonds involving cell-adhesion molecules. Nature *423*, 190–193.

Martin-Villalba, A., Llorens-Bobadilla, E., and Wollny, D. (2013). CD95 in cancer: tool or target? Trends Mol. Med. *19*, 329–335.

Matsukawa,A., Kudo,S., Maeda,T., Numata,K., Watanabe,H., Takeda,K., Akira,S., and Ito,T. (2005). Stat3 in resident macrophages as a repressor protein of inflammatory response. J. Immunol. 175, 3354-3359.

Matsumoto, N., Imamura, R., and Suda, T. (2007). Caspase-8- and JNK-dependent AP-1 activation is required for Fas ligand-induced IL-8 production. FEBS J. *274*, 2376–2384.

McDowall, A., Inwald, D., Leitinger, B., Jones, A., Liesner, R., Klein, N. and Hogg, N. (2003). A novel form of integrin dysfunction involving beta1, beta2, and beta3 integrins. J. Clin. Invest. *111*, 51-60.

McLeod, S.J., Shum, A.J., Lee, R.L., Takei, F., and Gold, M.R. (2004). The Rap GTPases regulate integrin-mediated adhesion, cell spreading, actin polymerization, and Pyk2 tyrosine phosphorylation in B lymphocytes. J. Biol. Chem. *279*, 12009–12019.

McIntire, V., and Lawrence, B. (1987). Effect of flow on polymorphonuclear leukocyte/endothelial cell adhesion. Blood *70*, 1284–1290.

Medeiros, R. B., Dickey, D. M., Chung, H., Quale, A. C., Nagarajan, L. R., Billadeau, D. D. and Shimizu, Y. (2005). Protein kinase D1 and the beta 1 integrin cytoplasmic domain control beta 1 integrin function via regulation of Rap1 activation. Immunity *23*, 213-226.

Menasche, G., Kliche, S., Chen, E. J., Stradal, T. E., Schraven, B. and Koretzky, G. (2007). RIAM links the ADAP/SKAP-55 signaling module to Rap1, facilitating T-cell- receptor-mediated integrin activation. Mol. Cell. Biol. *27*, 4070-4081.

Mielgo, a, van Driel, M., Bloem, a, Landmann, L., and Günthert, U. (2006). A novel antiapoptotic mechanism based on interference of Fas signaling by CD44 variant isoforms. Cell Death Differ. *13*, 465–477.

Mielgo, A., Brondani, V., Landmann, L., Glaser-Ruhm, A., Erb, P., Stupack, D., and Günthert, U. (2007). The CD44 standard/ezrin complex regulates Fas-mediated apoptosis in Jurkat cells. Apoptosis *12*, 2051–2061.

Millán, J., and Ridley, A.J. (2005). Rho GTPases and leucocyte-induced endothelial remodelling. Biochem. J. *385*, 329–337.

Millán, J., Hewlett, L., Glyn, M., Toomre, D., Clark, P., and Ridley, A.J. (2006). Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. Nat. Cell Biol. *8*, 113–123.

Miner, J.J., Xia, L., Yago, T., Kappelmayer, J., Liu, Z., Klopocki, A.G., Shao, B., McDaniel, J.M., Setiadi, H., Schmidtke, D.W., et al. (2008). Separable requirements for cytoplasmic domain of PSGL-1 in leukocyte rolling and signaling under flow. Blood *112*, 2035–2045.

Mócsai, A., Abram, C.L., Jakus, Z., Hu, Y., Lanier, L.L., and Lowell, C. a (2006). Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. Nat. Immunol. *7*, 1326–1333.

Mócsai, A., Ruland, J., and Tybulewicz, V.L.J. (2010). The SYK tyrosine kinase: a crucial player in diverse biological functions. Nat. Rev. Immunol. *10*, 387–402.

Mócsai, A., Zhou, M., Meng, F., Tybulewicz, V.L., and Lowell, C. a (2002). Syk is required for integrin signaling in neutrophils. Immunity *16*, 547–558.

Motz, G.T., Santoro, S.P., Wang, L.-P., Garrabrant, T., Lastra, R.R., Hagemann, I.S., Lal, P., Feldman, M.D., Benencia, F., and Coukos, G. (2014). Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. Nat. Med. *20*, 607–615.

Mueller, H., Stadtmann, A., Aken, H. Van, Hirsch, E., Wang, D., Ley, K., and Zarbock, A. (2010). Tyrosine kinase Btk regulates E-selectin – mediated integrin activation and neutrophil recruitment by controlling phospholipase C (PLC) γ 2 and PI3Kγ pathways. Blood *115*, 3118-3127.

Muller, W. a. (2003). Leukocyte–endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol. *24*, 326–333.

Murphy, K., Travers, P., Walport, M., Janeway, C. (2012) Janeway's immunobiology. London u.a.: Garland Science.

Nagata, S., and Golstein, P. (1995). The Fas Death Factor. Science *267*, 1449–1456.

Nagata,S. (1999). Fas ligand-induced apoptosis. Annu. Rev. Genet. *33*, 29-55.

Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, et al. (2007). The healing my- ocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J. Exp. Med. *204*, 3037–47

Nahrendorf, M., Pittet, M. J. & Swirski, F. K. (2010). Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. Circulation *121*, 2437–2445

Neame, S.J., Uff, C.R., Sheikh, H., Wheatley, S.C., and Isacke, C.M. (1995). CD44 exhibits a cell type dependent interaction with triton X-100 insoluble, lipid rich, plasma membrane domains. J. Cell Sci. *108 (Pt 9)*, 3127–3135.

Newton, K., Harris, a W., and Strasser, a (2000). FADD/MORT1 regulates the pre-TCR checkpoint and can function as a tumour suppressor. EMBO J. *19*, 931–941.

Newton, K., Harris, a W., Bath, M.L., Smith, K.G., and Strasser, a (1998). A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. EMBO J. *17*, 706–718.

Notarangelo LD, Miao CH, Ochs HD. (2008).Wiskott-Aldrich syndrome. Curr. Opin. Hematol. *15*,30-36.

Ottonello, L., Tortolina, G., Amelotti, M., and Dallegri, F. (1999). Soluble Fas ligand is chemotactic for human neutrophilic polymorphonuclear leukocytes. J. Immunol. *162*, 3601–3606.

Park, D.R., Thomsen, a. R., Frevert, C.W., Pham, U., Skerrett, S.J., Kiener, P. a., and Liles, W.C. (2003). Fas (CD95) Induces Proinflammatory Cytokine Responses by Human Monocytes and Monocyte-Derived Macrophages. J. Immunol. *170*, 6209–6216.

Parlato, S., Giammarioli, a M., Logozzi, M., Lozupone, F., Matarrese, P., Luciani, F., Falchi, M., Malorni, W., and Fais, S. (2000). CD95 (APO-1/Fas) linkage to the actin cytoskeleton through ezrin in human T lymphocytes: a novel regulatory mechanism of the CD95 apoptotic pathway. EMBO J. *19*, 5123–5134.

Pasvolsky R, Feigelson SW, Kilic SS, Simon AJ, Tal-Lapidot G, Grabovsky V, Crittenden JR, Amariglio N, Safran M, Graybiel AM et al (2007) A LAD-III syndrome is associated with defective expression of the Rap-1 activator CalDAG-GEFI in lymphocytes, neutrophils, and platelets. J. Exp. Med. 204, 1571-1582.

Peter, M.E., and Krammer, P.H. (2003). The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ. *10*, 26–35.

Peter, M.E., Budd, R.C., Desbarats, J., Hedrick, S.M., Hueber, A.-O., Newell, M.K., Owen, L.B., Pope, R.M., Tschopp, J., Wajant, H., et al. (2007). The CD95 receptor: apoptosis revisited. Cell *129*, 447–450.

Petrache, I., Birukova, A., Ramirez, S.I., Garcia, J.G.N., and Verin, A.D. (2003). The role of the microtubules in tumor necrosis factor-alpha-induced endothelial cell permeability. Am. J. Respir. Cell Mol. Biol. *28*, 574–581.

Pober, J.S., and Sessa, W.C. (2007). Evolving functions of endothelial cells in inflammation. Nat. Rev. Immunol. *7*, 803–815.

Randolph, G.J., Jakubzick, C., and Qu, C. (2008). Antigen presentation by monocytes and monocyte-derived cells. Curr. Opin. Immunol. *20*, 52–60.

Rodríguez, R.M., López-Vázquez, A., López-Larrea, C. (2012). Immune systems evolution. Adv. Exp. Med. Biol. 739,237–51.

Rondaij, M.G., Bierings, R., Kragt, A., van Mourik, J. a, and Voorberg, J. (2006). Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. Arterioscler. Thromb. Vasc. Biol. *26*, 1002–1007.

Rushworth, S. a, Murray, M.Y., Zaitseva, L., Bowles, K.M., and Macewan, D.J. (2014). Identification of Bruton's tyrosine kinase as a therapeutic target in acute myeloid leukemia. Blood *123*, 1229–1238.

Salas, A., Shimaoka, M., Kogan, A.N., Harwood, C., von Andrian, U.H., and Springer, T. a (2004). Rolling adhesion through an extended conformation of integrin alphaLbeta2 and relation to alpha I and beta I-like domain interaction. Immunity *20*, 393–406.

Sata M, Walsh .K. (1998). TNFalpha regulation of Fas ligand expression on the vascular endothelium modulates leukocyte extravasation. Nat Med.

Schlottmann, K., and Gulbins, E. (1996). Activation of Src-family tyrosine kinases during Fas-induced apoptosis. J. Leukoc. … *60*.

Schymeinsky, J., Mócsai, A., and Walzog, B. (2007). Molecular mechanisms and clinical implications. Thromb Haemost *98*, 262–273.

Scott, F.L., Stec, B., Pop, C., Dobaczewska, M.K., Lee, J.J., Monosov, E., Robinson, H., Salvesen, G.S., Schwarzenbacher, R., and Riedl, S.J. (2009). The Fas-FADD death domain complex structure unravels signalling by receptor clustering. Nature *457*, 1019–1022.

Sebzda, E., Bracke, M., Tugal, T., Hogg, N. and Cantrell, D. A. (2002). Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling. Nat. Immunol. Immunol. *3*, 251-258

Seino, Ken-ichiro Kazuhisa Iwabuchi, Nobuhiko Kayagaki, Ryukou Miyata, Isao Nagaoka, Akio Matsuzawa, K., and Fukao, H.Y. and K.O. (1998). Cutting edge: chemotactic activity of soluble Fas ligand against phagocytes. J Immunol 4484–4488.

Seino,K., Kayagaki,N., Okumura,K., and Yagita,H. (1997). Antitumor effect of locally produced CD95 ligand. Nat. Med. *3*, 165-170.

Seong, S., and Matzinger, P. (2004). initiates innate immune responses. Nat Rev Immunol *4*, 469–478.

Serbina NV, Jia T, Hohl TM, Pamer EG. (2008). Monocyte-mediated defense against microbial pathogens. Annu. Rev. Immunol. 26,421–52.

Serbina NV, Pamer EG. (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. Nat. Immunol. *7*,311–17.

Serrador, J.M., Urzainqui, A., Alonso-Lebrero, J.L., Cabrero, J.R., Montoya, M.C., Vicente-Manzanares, M., Yáñez-Mó, M., and Sánchez-Madrid, F. (2002). A juxta-membrane amino acid sequence of P-selectin glycoprotein ligand-1 is involved in moesin binding and ezrin/radixin/moesin-directed targeting at the trailing edge of migrating lymphocytes. Eur. J. Immunol. *32*, 1560–1566.

Shattil, S.J., Kim, C., and Ginsberg, M.H. (2010). The final steps of integrin activation: the end game. Nat. Rev. Mol. Cell Biol. *11*, 288-300.

Shechter, R., London, A., Varol, C., Raposo, C., Cusimano, M., Yovel, G., Rolls, A., Mack, M., Pluchino, S., Martino, G., et al. (2009). Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. PLoS Med. *6*, e1000113.

Shechter, R., Miller, O., Yovel, G., Rosenzweig, N., London, A., Ruckh, J., Kim, K.-W., Klein, E., Kalchenko, V., Bendel, P., et al. (2013). Recruitment of Beneficial M2 Macrophages to Injured Spinal Cord Is Orchestrated by Remote Brain Choroid Plexus. Immunity *21*, 555–569.

Shechter, R., Raposo, C., London, A., Sagi, I., and Schwartz, M. (2011). The glial scar-monocyte interplay: a pivotal resolution phase in spinal cord repair. PLoS One *6*, e27969.

Shen, B., Delaney, M.K., and Du, X. (2012). Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. Curr. Opin. Cell Biol. *24*, 600-606.

Shi, C., and Pamer, E.G. (2011). Monocyte recruitment during infection and inflammation. Nat. Rev. Immunol. *11*, 762–774.

Shi, C., Jia, T., Mendez-Ferrer, S., Hohl, T.M., Serbina, N. V, Lipuma, L., Leiner, I., Li, M.O., Frenette, P.S., and Pamer, E.G. (2011). Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. Immunity *34*, 590–601.

Siemsen, D.W., Malachowa, N., Schepetkin, I. a, Whitney, A.R., Kirpotina, L.N., Lei, B., Deleo, F.R., and Quinn, M.T. (2007). Neutrophil isolation from nonhuman species. Methods Mol. Biol. *1124*, 19–37.

Sigal, a., Bleijs, D. a., Grabovsky, V., van Vliet, S.J., Dwir, O., Figdor, C.G., van Kooyk, Y., and Alon, R. (2000). The LFA-1 Integrin Supports Rolling Adhesions on ICAM-1 Under Physiological Shear Flow in a Permissive Cellular Environment. J. Immunol. *165*, 442–452.

Sixt,M., Hallmann,R., Wendler,O., Scharffetter-Kochanek,K., and Sorokin,L.M. (2001). Cell adhesion and migration properties of beta 2-integrin negative polymorphonuclear granulocytes on defined extracellular matrix molecules. Relevance for leukocyte extravasation. J. Biol. Chem. *276*, 18878-18887.

Söderström, T.S., Nyberg, S.D., and Eriksson, J.E. (2005). CD95 capping is ROCK-dependent and dispensable for apoptosis. J. Cell Sci. *118*, 2211–2223.

Spahn, J.H., and Kreisel, D. (2014). Monocytes in sterile inflammation: recruitment and functional consequences. Arch. Immunol. Ther. Exp. (Warsz). *62*, 187–194.

Stadtmann, A., Brinkhaus, L., Mueller, H., Rossaint, J., Bolomini-Vittori, M., Bergmeier, W., Van Aken, H., Wagner, D.D., Laudanna, C., Ley, K., et al. (2011). Rap1a activation by CalDAG-GEFI and p38 MAPK is involved in E-selectin-dependent slow leukocyte rolling. Eur. J. Immunol. *41*, 2074–2085.

Stevens, T., Garcia, J. G., Shasby, D. M., Bhattacharya, J. & Malik, A. B. (2000). Mechanisms regulating endothelial cell barrier function. Am. J. Physiol., Lung Cell Mol. Physiol. *279*, 419–422.

Strasser, A., Jost, P.J., and Nagata, S. (2009). The many roles of FAS receptor signaling in the immune system. Immunity *30*, 180–192.

Swirski, F.K., Libby, P., Aikawa, E., Alcaide, P., Luscinskas, F.W., Weissleder, R., and Pittet, M.J. (2007). Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. J. Clin. Invest. *117*, 195–205.

Szmydynger-Chodobska, J., Strazielle, N., Gandy, J.R., Keefe, T.H., Zink, B.J., Ghersi-Egea, J.-F., and Chodobski, A. (2012). Posttraumatic invasion of monocytes across the blood-cerebrospinal fluid barrier. J. Cereb. Blood Flow Metab. *32*, 93–104.

Tadokoro, S., Shattil, S.J., Eto, K., Tai, V., Liddington, R.C., de Pereda, J.M., Ginsberg, M.H., and Calderwood, D. a (2003). Talin binding to integrin beta tails: a final common step in integrin activation. Science *302*, 103–106.

Takahashi, T., Tanaka, M., Brannan, C.I., Jenkins, N.A., Copeland, N.G., Suda, T., and Nagata, S. (1994). Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell *76*, 969–976.

Tamm, C., Robertson, J.D., Sleeper, E., Enoksson, M., Emgård, M., Orrenius, S., and Ceccatelli, S. (2004). Differential regulation of the mitochondrial and death receptor pathways in neural stem cells. Eur. J. Neurosci. *19*, 2613–2621.

Trauth,B.C., Klas,C., Peters,A.M., Matzku,S., Moller,P., Falk,W., Debatin,K.M., and Krammer,P.H. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 245, 301-305.

Tsou CL, Peters W, Si Y, Slaymaker S, Aslanian AM, et al. (2007). Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. J. Clin. Invest.*117*,902–9.

Tsujioka, H., Imanishi, T., Ikejima, H., Kuroi, A., Takarada, S., Tanimoto, T., Kitabata, H., Okochi, K., Arita, Y., Ishibashi, K., et al. (2009). Impact of heterogeneity of human peripheral blood monocyte subsets on myocardial salvage in patients with primary acute myocardial infarction. J. Am. Coll. Cardiol. *54*, 130–138.

Turner, M., Mee, P.J., Costello, P.S., Williams, O., Price, A.A., Duddy, L.P., Furlong, M.T., Geahlen, R.L., and Tybulewicz, V.L. (1995). Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature 378, 298–302.

Turner, M., Schweighoffer, E., and Colucci, F. (2000). Tyrosine kinase SYK: essential functions for immunoreceptor signalling. Immunol. Today *5699*, 148–154.

Uckun, F., Ozer, Z., and Vassilev, A. (2007). Bruton's tyrosine kinase prevents activation of the anti-apoptotic transcription factor STAT3 and promotes apoptosis in neoplastic B-cells and B-cell precursors exposed to oxidative stress. Br. J. Haematol. *136*, 574–589.

Uckun, F.M. (1998). Bruton's tyrosine kinase (BTK) as a dual-function regulator of apoptosis. Biochem. Pharmacol. *56*, 683–691.

Uckun, F.M., Waddick, K.G., Mahajan, S., Jun, X., Takata, M., Bolen, J., and Kurosaki, T. (1996). BTK as a mediator of radiation-induced apoptosis in DT-40 lymphoma B cells. Science (80-.). *273*, 1096–1100.

Um, H., Orenstein, J., and Wahl, S. (1996). Fas mediates apoptosis in human monocytes by a reactive oxygen intermediate dependent pathway. J. Immunol.

Urzainqui, A., Serrador, J.M., Viedma, F., Yáñez-Mó, M., Rodríguez, A., Corbí, A.L., Alonso-Lebrero, J.L., Luque, A., Deckert, M., Vázquez, J., et al. (2002). ITAM-based interaction of ERM proteins with Syk mediates signaling by the leukocyte adhesion receptor PSGL-1. Immunity *17*, 401–412.

Vachon, P.H. (2011). Integrin signaling, cell survival, and anoikis: distinctions, differences, and differentiation. J. Signal Transduct. *2011*, 738137.

Vargas, L., Berglo, A., and Smith, C.I.E. (2009). Bruton ' s tyrosine kinase (Btk): function , regulation , and transformation with special emphasis on the PH domain. 58–73.

Vassilev, A., Ozer, Z., Navara, C., Mahajan, S., and Uckun, F.M. (1999). Bruton ' s Tyrosine Kinase as an Inhibitor of the Fas / CD95 Death-inducing Signaling Complex *. J. Biol. Chem. *274*, 1646–1656.

Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003). Non-apoptotic Fas signaling. Cytokine Growth Factor Rev. *14*, 53–66.

Wang, F., Lu, Z., Hawkes, M., Yang, H., Kain, K.C., and Liles, W.C. (2010). Fas (CD95) induces rapid, TLR4/IRAK4-dependent release of pro-inflammatory HMGB1 from macrophages. J. Inflamm. (Lond). *7*, 30.

Wang, J., Koizumi, T., and Watanabe, T. (1996). Altered antigen receptor signaling and impaired Fas-mediated apoptosis of B cells in Lyn-deficient mice. J. Exp. Med. *184*, 831–838.

Wang,S., Voisin,M.B., Larbi,K.Y., Dangerfield,J., Scheiermann,C., Tran,M., Maxwell,P.H., Sorokin,L., and Nourshargh,S. (2006). Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating neutrophils. J. Exp. Med. *203*, 1519-1532.

Watanabe N, Bodin L, Pandey M, Krause M, Coughlin S. (2008). Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin αIIbβ3. J. Cell Biol. *181*,1211-22.

Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A., and Nagata, S. (1992). Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature *356*, 314–317.

Weber, C.H., and Vincenz, C. (2001). The death domain superfamily: a tale of two interfaces? Trends Biochem. Sci. *26*, 475–481.

Wegener, K.L., Partridge, A.W., Han, J., Pickford, A.R., Liddington, R.C., Ginsberg, M.H., and Campbell, I.D. (2007). Structural basis of integrin activation by talin. Cell *128*, 171–182.

Wei, W., Wang, D., Shi, J., Xiang, Y., Zhang, Y., Liu, S., Liu, Y., and Zheng, D. (2010). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induces chemotactic migration of monocytes via a death receptor 4-mediated RhoGTPase pathway. Mol. Immunol. *47*, 2475–2484.

Williams, N., Eger, R.R., Moore, M. a. S., and Mendelsohn, N. (1978). Differentiation of Mouse Bone Marrow Precursor Cells into Neutrophil Granulocytes by an Activity Separation from WEHI-3 Cell-Conditioned Medium. Differentiation *11*, 59–63.

Wollert, K.C., Heineke, J., Westermann, J., Ludde, M., Fiedler, B., Zierhut, W., Laurent, D., Bauer, M.K. a., Schulze-Osthoff, K., and Drexler, H. (2000). The Cardiac Fas (APO-1/CD95) Receptor/Fas Ligand System : Relation to Diastolic Wall Stress in Volume-Overload Hypertrophy in Vivo and Activation of the Transcription Factor AP-1 in Cardiac Myocytes. Circulation *101*, 1172–1178.

Xia, L., Sperandio, M., Yago, T., Mcdaniel, J.M., Cummings, R.D., Pearson-white, S., Ley, K., and Mcever, R.P. (2002). P-selectin glycoprotein ligand-1 – deficient mice have impaired leukocyte tethering to E-selectin under flow. J. Clin. Invest. *109*, 939–950.

Xu, Y., Szalai, A.J., Zhou, T., Zinn, K.R., Chaudhuri, T.R., Li, X., Koopman, W.J., and Kimberly, R.P. (2003). Fc gamma Rs modulate cytotoxicity of anti-Fas antibodies: implications for agonistic antibody-based therapeutics. J. Immunol. *171*, 562–568.

Yago, T., Shao, B., Miner, J.J., Yao, L., Klopocki, A.G., Maeda, K., Coggeshall, K.M., and McEver, R.P. (2010). E-selectin engages PSGL-1 and CD44 through a common signaling pathway to induce integrin alphaLbeta2-mediated slow leukocyte rolling. Blood *116*, 485–494.

Yona, S., Kim, K.-W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guilliams, M., Misharin, A., et al. (2012). Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. Immunity *1*, 1–13.

Yonehara,S., Ishii,A., and Yonehara,M. (1989). A cell-killing monoclonal antibody (anti- Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J. Exp. Med. 169, 1747-1756.

Yonemura, S., Hirao, M., Doi, Y., Takahashi, N., Kondo, T., Tsukita, S., and Tsukita, S. (1998). Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. J. Cell Biol. *140*, 885–895.

Zarbock, A., Abram, C.L., Hundt, M., Altman, A., Lowell, C. a, and Ley, K. (2008). PSGL-1 engagement by E-selectin signals through Src kinase Fgr and ITAM adapters DAP12 and FcR gamma to induce slow leukocyte rolling. J. Exp. Med. *205*, 2339–2347.

Zhang, J., Cado, D., Chen, A., Kabra, N., and Winoto, A. (1998). Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. Nature *1430*, 1426–1430.

Ziegler-Heitbrock, L. (2007). The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J. Leukoc. Biol. *81*, 584–592.

Zuliani, C., Kleber, S., Klussmann, S., Wenger, T., Kenzelmann, M., Schreglmann, N., Martinez, a, del Rio, J. a, Soriano, E., Vodrazka, P., et al. (2006). Control of neuronal branching by the death receptor CD95 (Fas/Apo-1). Cell Death Differ. *13*, 31–40.