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The role of Themis2 for beta2 integrin function and neutrophil trafficking in innate immunity

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Table of Contents

Abstract

Polymorphonuclear neutrophils (PMNs) are critical components of the innate immune system and play key roles in host defence as well as in tissue repair and homeostasis. However, PMNs can also have a negative impact on our health by maintaining inflammation or causing tissue injury as a collateral damage of their function. The recruitment of PMNs from the bloodstream into the tissue is a tightly controlled multistep process, triggered by an inflammatory microenvironment. Key steps of this cascade, including their slow rolling, adhesion, migration along the endothelium, and transmigration, depend on adhesion molecules of the β_2 integrin family.

The two major β_2 integrins for PMN trafficking are lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18) and macrophage-1 antigen (Mac-1, CD11b/CD18). Integrin function and activity, in general, depend on the interaction of cytoplasmic proteins with the integrin´s intracellular domains. Although, several binding partners of CD18 have been identified in the past, the mechanism that regulates β_2 integrins in PMNs is incompletely understood. In a β_2 integrin interactor screen, the protein thymocyte selection associated family member 2 (Themis2) has been identified. Previous studies showed that Themis2 is involved in B cell receptor and T cell receptor signalling. Since integrin signalling shows similarities to B and T cell receptor signalling and the role of Themis2 in PMNs is as yet unknown[1–3], the aim of this thesis was to characterize the role of Themis2 in integrin signalling and regulation and its impact on PMN trafficking.

I could show, that Themis2 co-localizes with CD18 during β_2 integrin-mediated adhesion. Moreover, in a Hoxb8 cell Themis2 knockout model I found increased binding of the reporter antibody mAb24 upon cell stimulation. Furthermore, genetic deletion of Themis2 *in vitro* increased LFA-1 activation and PMN adhesion under flow conditions. Accordingly, increased murine PMN (mPMN) adhesion accompanied by reduced rolling velocity and markedly increased extravasation were observed in tumour necrosis factor α (TNFα)-stimulated cremaster muscle venules of Themis2$^{-/-}$ mice.

Taken together, these data strongly suggest that Themis2 has a negative impact on β_2 integrin and PMN activation and hence plays an important role in regulating PMN function.

Zusammenfassung

Polymorphkernige Neutrophile (PMNs) sind ein wichtiger Bestandteil des angeborenen Immunsystems und spielen eine Schlüsselrolle bei der Wirtsabwehr sowie bei der Gewebereparatur und -Homöostase. Allerdings können PMNs auch negative Auswirkungen auf unsere Gesundheit haben, indem sie Entzündungen aufrechterhalten oder als Kollateralschaden ihrer Funktion Gewebeschäden verursachen. Die Rekrutierung von PMNs aus dem Blutkreislauf in das entzündete Gewebe ist ein kontrollierter, mehrstufiger Prozess, der durch eine entzündliche Mikroumgebung ausgelöst wird. Entscheidende Schritte dieser Kaskade, wie z. B. das langsame Rollen, die Adhäsion, die Migration entlang des Endothels und die Transmigration, hängen von Adhäsionsmolekülen der β_2-Integrinfamilie ab.

Die beiden wichtigsten β_2-Integrine für die Leukozytenmigration sind das Lymphozyten-Funktions-assoziierte Antigen 1 (LFA-1, CD11a/CD18) und das Makrophagen-1-Antigen (Mac-1, CD11b/CD18). Die Regulierung der β_2-Integrine erfolgt vorwiegend über die Interaktion von zytoplasmatischen Proteinen mit den intrazellulären Domänen der Integrine. Obwohl in der Vergangenheit mehrere Bindungspartner von CD18 identifiziert wurden, ist der Mechanismus der Regulierung der β_2-Integrine in PMNs noch nicht vollständig verstanden. In einem Screening für β_2-Integrin-Interaktoren wurde das Protein „Thymocyte Selection Associated Family Member 2“ (Themis2) entdeckt. Frühere Studien haben gezeigt, dass Themis2 an der B-Zell-Rezeptor- und T-Zell-Rezeptor-Signalübertragung beteiligt ist. Da die Integrin-Signalübertragung Ähnlichkeit mit der B- und T-Zell-Rezeptor-Signalübertragung aufweist und die Rolle von Themis2 in PMNs noch unbekannt ist[1–3], ist es das Ziel dieser Arbeit, die Rolle von Themis2 für die Integrin-Signalübertragung und -Regulierung und seine Auswirkungen auf die Leukozytenmigration zu charakterisieren.

Ich konnte zeigen, dass Themis2 während der β_2-Integrin-vermittelten Adhäsion mit CD18 co-lokalisiert. Darüber hinaus fand ich in einem zellulären Hoxb8 Themis2 knockout-Modell eine erhöhte Bindung des Reporter-Antikörpers mAb24 nach Zellstimulation. Des Weiteren verstärkte die genetische Deletion von Themis2 *in vitro* die PMN-Adhäsion unter Flussbedingungen und die LFA-1-Aktivierung. Passend dazu wurde in TNFα-stimulierten Cremaster-Muskelvenolen von Themis2$^{-/-}$ Mäusen eine verstärkte Adhäsion von murinen PMNs (mPMNs) in Verbindung mit einer reduzierten Rollgeschwindigkeit und einer deutlich erhöhten Auswanderung beobachtet.

Insgesamt deuten diese Daten darauf hin, dass Themis2 einen negativen Einfluss auf die β_2-Integrin- und PMN-Aktivierung hat und somit eine wichtige Rolle bei der Regulierung der PMN-Funktion spielt.

Table of Figures and Tables

Abbreviations

ADM	adhesion medium
ALI	acute lung injury
APRIL	proliferation-inducing ligand
ANOVA	analysis of variance
BAFF	B cell activating factor
bp	base pair
BSA	bovine serum albumin
CABIT	cysteine-containing all beta in themis
CD	cluster of differentiation
CXCL1	C-X-C motif chemokine ligand 1
CXCR2	CXC motif chemokine receptor 2
d	days
Dap12	DNAX activation protein 12
DAMPs	damage-associated molecular patterns
DC	dendritic cell
(d)HL-60	(differentiated) human promyelocytic leukemia cells
DFP	diisopropyl phosphofluoridate
dHoxb8 cells	Hoxb8-SCF cell-derived neutrophils
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxid
DNA	deoxyribonucleic acid
EC	endothelial cell
ECM	extracellular matrix
EGFP	enhanced green fluorescent protein
EDTA	ethylenediamine-tetraacetic acid
emPAI	exponentially modified protein abundance index
ER	estrogen-responsive element
ERK	extracellular signal-regulated kinase
ESL-1	E-selectin ligand-1
FACS	fluorescence-activated cell sorting
FC	flow cytometry
FITC	fluorescein isothiocyanate
FOV	field of view
fMLP	N-formyl-L-methionyl-L-leucyl-phenylalanine
FPR	formyl peptide receptor
fwd	forward
GAREM	Grb2-associated regulator of ERK/MAPK
GPCR	G-protein coupled receptor
Grb2	growth factor receptor bound protein 2
GTPase	guanosine triphosphatase
h	hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPK-1	hematopoietic progenitor kinase-1
ICAM-1	intercellular adhesion molecule-1
ICB1	induced on contact with basement membrane 1
IgG	immunoglobulin G
IL-1β	interleukin-1β
i.p.	intraperitoneal
ITAM	immunoreceptor tyrosine-based activation motif
LAD	leukocyte adhesion deficiency
LAT	linker for activation of T cells
LFA-1	lymphocyte function-associated antigen-1
LPS	lipopolysaccharide
LTB_4	leukotriene B4
m	murine
Mac-1	macrophage-1 antigen
mAbp1	mammalian actin binding protein
MAPK	mitogen-activated protein kinase
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minutes
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NLS	nuclear localization sequence
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PIP_3	phosphatidylinositol (3,4,5)-triphosphate
PLCγ2	phospholipase c-gamma2
PMA	phorbol myristate acetate
PRR	proline-rich region
PSGL-1	P-selectin glycoprotein ligand-1
PTP	protein tyrosine-phosphatase
Rap1	Ras-related protein 1
RhoG	Ras homology growth-related
RIAM	Rap1-interacting adapter molecule
rm	recombinant murine
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute 1640
SAM	sterile alpha motif
SEM	standard error of the mean

SFK	Src family kinase
SH2 domain	Src homology 2 domain
SH3 domain	Src homology 3 domain
SHP-1	Src homology region 2 domain-containing phosphatase-1
Syk	spleen tyrosine kinase
TAE	tris base, acetic acid and EDTA
TBS	tris buffered saline
TBST	tris buffered saline with Tween®20
TEM	transendothelial migration
Themis1	Thymocyte-expressed molecule involved in selection
Themis2	thymocyte selection associated family member 2
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factors-α
VCAM-1	vascular cell adhesion molecule-1
WB	western blot

1. INTRODUCTION

1.1 The inflammatory response

Multicellular organisms are exposed to pathogens and injury on a daily basis. In this context, they rely on a functioning immune system, since the tissue´s response to any kind of injury or pathogen is inflammation, which is controlled by the immune system. Already more than 2,000 years ago, the cardinal signs of acute inflammation were described by Celsus as heat (calor), pain (dolor), redness (rubor), and swelling (tumor). At the beginning of the first millennium, Galen of Pergamon identified loss of function (functio laesa) as the fifth cardinal sign of acute inflammation. He was also the first to record in medical literature the fact that injury leads to inflammation (phlogosis). The acute inflammatory response can be induced by biological, chemical, or physical triggers, such as bacteria, acid burn and surgical trauma, or by ischemia[4–6]. In general, a controlled inflammatory response is considered beneficial, because it is a protective response that provides removal of adverse stimuli and a healing process to repair tissue damage. However, in case of dysregulation it can become detrimental as well (e.g. septic shock)[7]. The inflammatory response consists mainly of two parts: On the one hand the microcirculatory response, which leads to increased blood supply by dilatation of arterioles and exudation of plasma proteins by increased permeability of capillaries and on the other hand, the cellular response, which is characterized by the migration of leukocytes from the vascular system into the affected tissue.

One of the first responders during acute inflammation is a subset of myeloid leukocytes, the neutrophils[8]. Neutrophils have characteristic segmented nuclei and their cytoplasm contains secretory vesicles and granules containing proteases and antimicrobial peptides; they are also referred to as polymorphonuclear neutrophils (PMNs)[9]. At the end of the 19th century the zoologist Elie Metchnikoff was the first to describe PMNs as phagocytic cells that play an important role in combating bacteria and other foreign particles, thus laying the foundation for the theory of the acute inflammatory response[5,10].

1.1.1 The role of PMNs

PMNs are crucial cellular components of the innate immune system by playing a key role in host defence against invading pathogens (e.g. bacteria, fungi and protozoa) as well as in tissue repair and tissue homeostasis[11,12]. Once pathogens have invaded the host tissue, resident macrophages respond with phagocytosis and by secreting factors (e.g. CXC motif chemokine receptor 2 (CXCR2)

ligands), which lead to the mobilization of PMNs from the bone marrow, followed by recruitment from the blood stream to the site of inflammation, where PMNs exert their anti-microbial functions[13,14]. Through phagocytosis PMN engulf complement- (iC3b) or immunoglobulin-opsonized particles in a CD11b and Fcγ receptor-dependent manner[15]. Subsequently, the particle is enclosed by the cell membrane and a phagosome is formed. The phagosome fuses with primary and secondary granules to form the phagolysosome. In this process, calcium-dependent nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase becomes activated, and reactive oxygen species (ROS) are produced by oxygen consumption[16]. This process is referred to as respiratory burst[17]. Together with oxidative active hypochlorous acid, ROS are responsible for the elimination of the engulfed material[16,18]. In addition, granules of PMNs contain several proteases such as neutrophil elastase, matrix metalloproteases, cathepsin G and antimicrobial peptides e.g. myeloperoxidase (MPO), lysozymes and defensines. The content of the granules can either be fused with the phagolysosome or secreted to eliminate pathogens[19]. Additionally, PMNs are able to generate neutrophil extracellular traps (NET). To this end, they eject decondensated chromosomal DNA, which is equipped with neutrophil elastase and MPO. After pathogens are immobilized by these extracellular fibers, they are destroyed[20].

In addition to these mechanisms, PMNs also contribute to adaptive immunity by supporting the more specific adaptive immune responses[8]. Through interaction with dendritic cells (DCs), T and B cells, they have the ability to modulate adaptive immune responses[21,22]. PMNs are substantial producers of B cell activating factor (BAFF) and proliferation-inducing ligand (APRIL), which are necessary for B cell activation and survival[23]. Besides that, they are involved in T cell regulation at different levels. On the one hand they can act as immunosuppressors and dampen the proliferation of T cells, probably due to arginase 1 in PMN granules and ROS production[24,25]. On the other hand, PMNs can promote T_H1 and T_H17 differentiation of T cells due to upregulation of major histocompatibility complex class (MHC) II levels during interferon stimulation. Moreover, NET formation by PMNs can prime $CD4^+$ T helper cells[26,27]. All in all, this complex interaction drives a seamless immune response[8].

In humans, PMNs account for 50–70% of circulating leukocytes, compared with only 10–25% in mice[28]. After PMNs have been generated from myeloid precursors in the bone marrow they enter the blood stream and circulate with a half-life of a several hours to up to 5.4 days in humans and up to 18 h in mice[29]. Mature PMNs in the circulation have an average diameter of 7–10 µm, a segmented nucleus and contain secretory vesicles and granules in the cytoplasm[9]. 55–60% of the bone marrow are dedicated to PMN production and the production rate can increase 10-fold to

up to 10^{12} cells per day during bacterial infection[11]. PMN turnover rate can also be boosted or delayed in response to ongoing inflammatory processes[8]. The clearance of PMNs occurs via engulfment by macrophages in the bone marrow, liver and spleen as well as by apoptosis[30].
They are the first leukocytes to arrive at the lesion site and initiate angiogenesis by releasing effector molecules[31–34]. However, PMNs play a double-edged role in health and disease, as they may also have adverse effects by maintaining inflammation or causing tissue injury as a collateral damage to their function. Furthermore, they may promote tumor metastasis or thrombosis and contribute to cardiovascular diseases by increasing instability of atherosclerotic plaques[34–37]. Hence, balanced and tightly regulated PMN functions and recruitment processes are fundamental during inflammation[38].

1.1.2 PMN recruitment cascade

The recruitment of PMNs from the bloodstream into the inflamed tissue is a tightly controlled multistep process[39]. DCs and tissue resident macrophages recognize bacterial components or other pathogen-associated molecular patterns (PAMPs) via corresponding innate pattern-recognition receptors (PRR), for instance members of the Toll-like receptor (TLR) family[7]. Likewise tissue damage-induced inflammation is induced upon recognition of damage-associated molecular patterns (DAMPs), which are molecular structures released by damaged cells[4]. The cascade is triggered by an inflammatory microenvironment, created by cytokines, chemokines and lipid mediators[31–33,40]. This cascade includes several steps that ultimately lead to the transmigration of PMNs through the vessel wall[32].
Initially, PMNs are captured to the inflamed endothelium and start rolling along the vessel wall in a selectin-mediated manner (Figure 1)[41]. Selectins are the main receptors, mediating this initial capture of circulating PMNs to ligands that are expressed on the endothelium[42]. The transient leukocyte-endothelial interactions leading to cell rolling last for seconds up to a few minutes, depending on the type of selectins involved. The selectin family consists of three family members: L-, E- and P-selectins. The leukocyte selectin, L-selectin, is present on almost all circulating leukocytes and is the key receptor for inducing leukocyte capture in peripheral sites of inflammation and injury and in secondary lymphoid tissues[43]. P- and E-selectin expression is induced on the endothelium during chronic and acute inflammation and is important to mediate the rolling of PMNs[42,44]. In contrast to P-selectin, E-selectin induces slower rolling velocities and thus facilitates the PMN recruitment in the underlying tissue[45]. However, upon stimulation, P-

selectin is present on the surface of endothelial cells (ECs) within a few minutes, whereas E-selectin exposure occurs later, due to de-novo synthesis in response to stimulation[46,47].

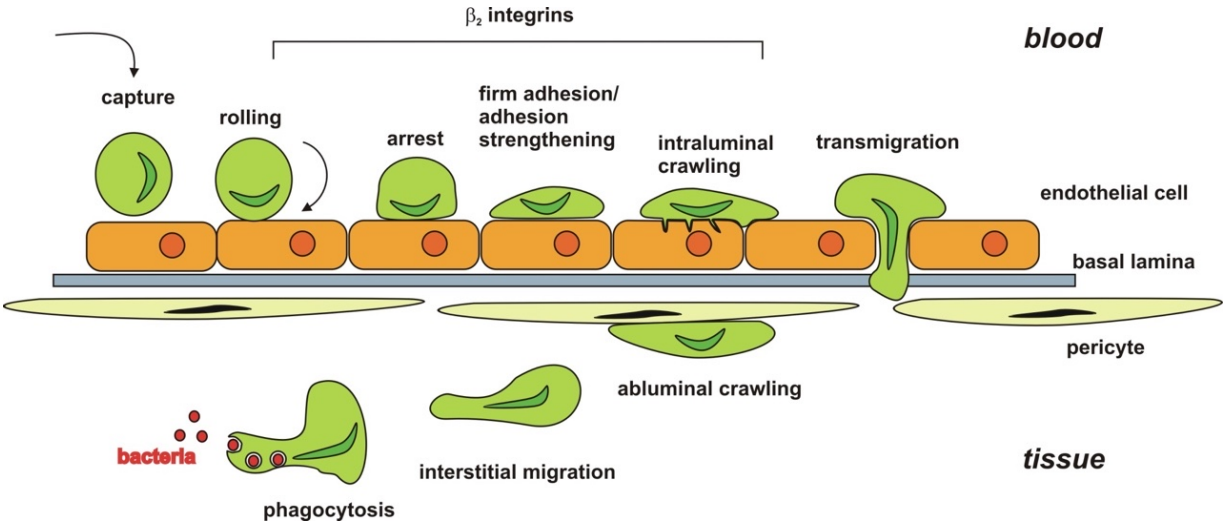


Figure 1. The PMN recruitment cascade.
After the initial capturing of free-flowing PMNs, PMNs start to role on the inflamed endothelium. Subsequently, β_2 integrin-dependent slow rolling, adhesion, adhesion strengthening and intraluminal crawling occur. This is followed by β_2 integrin-mediated transmigration and abluminal crawling. Finally, PMN conduct interstitial migration towards the site of inflammation or tissue injury where they are able to fulfil their functions such as phagocytosis or chemokine release (adapted from Schymeinsky et al., 2011[41]).

The steps following initial capture, including slow rolling, adhesion, adhesion strengthening, spreading, and intraluminal crawling as well as transmigration and abluminal crawling depend on adhesion molecules of the β_2 integrin family[33,48–50]. The β_2 integrins expressed on PMNs interact with specific ligands on ECs[51]. PMN transmigration through the endothelial cell layer is taking place either via the transcellular or via the paracellular route and depends strongly on interactions between PMNs and DCs[51]. Subsequently, PMNs pass the basal membrane of the blood vessel and perform abluminal crawling along pericytes of the vessel[51,52].

Finally, PMNs perform interstitial migration towards the site of tissue injury or infection by sensing and following chemokine gradients[53]. Chemoattractants derived from infecting bacteria, such as N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP), can override chemotactic stimuli originating from intermediate sites (e.g., the endothelium), such as leukotriene B_4 (LTB_4). In this way, chemoattractant stimuli can be functionally divided into intermediate or end-target chemoattractants, with PMNs more responsive to the latter[31,54,55]. Once at the target site, PMNs

fulfil their functions such as phagocytosis, production of ROS, chemokine release and NET formation[16,20,22,56].

1.2 β_2 integrins

Integrins are heterodimeric transmembrane glycoproteins consisting of non-covalently associated α and β chains[50]. They represent the largest family of cell adhesion receptors, which anchor cells within their surrounding extracellular matrix or mediate tight interactions with counter-receptors expressed on other cells. The latter function is primarily executed by integrins expressed on leukocytes. Integrins consist of a large extracellular domain, single-pass transmembrane segments and a short intracellular tail. Integrins have no enzymatic activity, but the short cytoplasmic domains act as scaffolds for signaling and structural proteins, which form a link with the actin cytoskeleton. Thereby integrins connect extracellular ligands with the intracellular cytoskeleton[57]. The ligand-binding affinity of integrins can be quickly regulated by conformational changes[58].

The integrin family can be divided according to the β-subunit: β_1 (CD29), β_2 (CD18) and β_3 (CD61) integrins, with all of them being expressed by human PMNs[59–61]. β_2 integrins are expressed only on leukocytes and are the most abundant integrins on PMNs[60,62]. There are four different β_2 integrins, which comprise a variable α-subunit (CD11a-CD11d) and the conserved β-subunit (CD18). The dimerization of β_2 integrins occurs already within the endoplasmatic reticulum and only integrin dimers are transported to the cell membrane[62]. Due to the fact that CD18 is expressed abundantly, the amount of β_2 integrin on the cell surface depends on the expression level of the particular α-subunit[62,63]. The four β_2 integrins expressed are lymphocyte function-associated antigen 1 (LFA-1, $\alpha L\beta_2$, CD11a/CD18), macrophage-1 antigen (Mac-1, $\alpha M\beta_2$, CD11b/CD18), gp150/95 (CD11c/CD18), $\alpha_D\beta_2$ (CD11d/CD18). The α-subunit determines the functional properties of the β_2 integrin, although all β_2 integrins have some functional overlap[62]. The two important β_2 integrins for PMN trafficking are LFA-1 and Mac-1[32,50]. Upon PMN activation, the expression of Mac-1 is up-regulated and it gets mobilized from intracellular stores, whereas LFA-1 is constitutively expressed on PMNs[64].

The critical role of β_2 integrins in PMN recruitment is clearly demonstrated by a group of autosomal recessive disorders, the leukocyte adhesion deficiency (LAD) syndromes: Patients with LAD type I e.g., suffer from strong recurrent bacterial and fungal infections, due to a genetic defect in β_2 integrin (CD18) expression. The absence of β-subunit CD18 results in the lack of the entire

inflammatory response, due to impaired PMN adhesion and recruitment[65,66]. LAD type III is characterized by a genetic defect in the protein Kindlin-3, which leads to impaired activation of β_2 integrins, emphasizing that acitvation is crucial for β_2 integrin function[67]. Activity and function of β_2 integrins are predominantly controlled by intracellular (inside-out signaling) and extracellular signals (outside-in signaling) via the interaction of cytoplasmic proteins with the intracellular domains of the integrin subunits[68].

1.2.1 Integrins during neutrophil migration

Integrins in general and β_2 integrins in particular exist in different conformations with low, intermediate or high affinity for the ligand. This change in the integrin conformation, also called integrin activation, can either be induced by the ligand (outside-in activation) or triggered by intracellular signaling events (inside-out signaling).

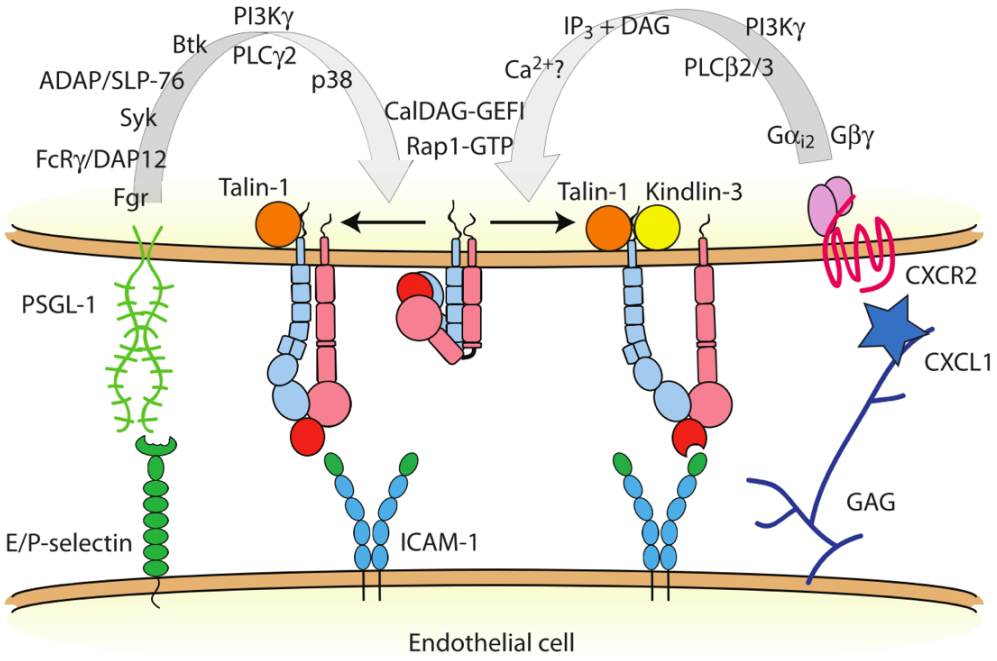


Figure 2. β_2 integrin activation.
β_2 integrins can change their conformation in response to external cues. Under normal physiological conditions, β_2 integrins on circulating PMNs are in a bent conformation with low ligand affinity. Initial capture and rolling of PMNs to the endothelium are mediated primarily by the binding of E-/P-selectin to PSGL-1. PSGL-1 transduces an intracellular signal that leads to the partial activation of the integrin and a shift from the bent towards an extended conformation with intermediate affinity to its ligand ICAM-1. By forming transient interactions, this interaction is highly important to reduce the rolling velocity of PMNs. For full activation of the integrin with high affinity, chemokine receptor signaling for example via CXCR2 is necessary. Talin-1 and Kindlin-3 are critical integrin co-activators and have distinct roles in the induction of conformational rearrangements. (adapted from Lefort & Ley, 2012[69]).

Under normal physiological conditions, when PMNs circulate in non-inflamed vessels, integrins are in a bent conformation with low ligand affinity[32,70]. The initial capture and rolling of PMNs is mediated primarily by the binding of E- or P-selectin on activated ECs to the selectin ligand P-selectin glycoprotein ligand 1 (PSGL-1) (Figure 2) or CD44 on PMNs.

PSGL-1 transduces intracellular signals that lead to the partial activation of the integrin and a shift from the bent to an extended conformation with intermediate affinity to its ligand ICAM-1, which is expressed on ECs[69]. These transient interactions are crucial for further reducing the rolling velocity down to slow rolling of PMNs[32]. However, besides the extension of the β_2 integrin the induction of fully activated integrins with high affinity for the ligand includes the opening of the alpha domain, the so-called headpiece. This step requires additional chemokine receptor signaling (Figure 3). Those G-protein-coupled receptors (GPCRs), for example CXCR2 or formyl peptide receptor 1 (FPR1), trigger signaling cascades that lead to the open-headpiece (high affinity) conformation of β_2 integrins and result eventually in PMN arrest[52]. The adaptor proteins Talin-1 and Kindlin-3 are critical integrin co-activators and have distinct roles in the induction of conformational rearrangements. Both proteins bind to the cytoplasmic domain of CD18 and both are required for the induction of the high-affinity state of the β_2 integrin[62,71,72]. However, Talin-1 binding alone might be sufficient for the unbending of the β_2 integrin and the induction of the intermediate affinity state[73,74]. Recruitment of Talin-1 to the plasma membrane to activate β_2 integrins is accomplished synergistically by two mechanisms, through a Ras-related protein 1 (Rap1)/Rap1-interacting adapter molecule (RIAM)/Talin-1 complex as well as through direct membrane-bound Rap1/Talin-1 interactions[75–77]. Kindlin-3 is suggested to be especially required for opening of the headpiece, but not for the extension of the β_2 integrin, since Kindlin-3 knockout mice exhibit defective PMN arrest but normal slow rolling of PMNs[71,73].

Taken together, integrin inside-out signaling leads to a conformational change of the β_2 integrin, thereby inducing a high affinity open state and strong binding to ligands[78]. The engagement of ligands in turn triggers outside-in signaling. In this way, the cell senses and responds to signals from the extracellular environment and translates it into intracellular signals that affect a multitude of cellular responses. Subsequently, adhesion strengthening and cytoskeletal rearrangements occur, which are prerequisites for subsequent cell spreading and migration[62].

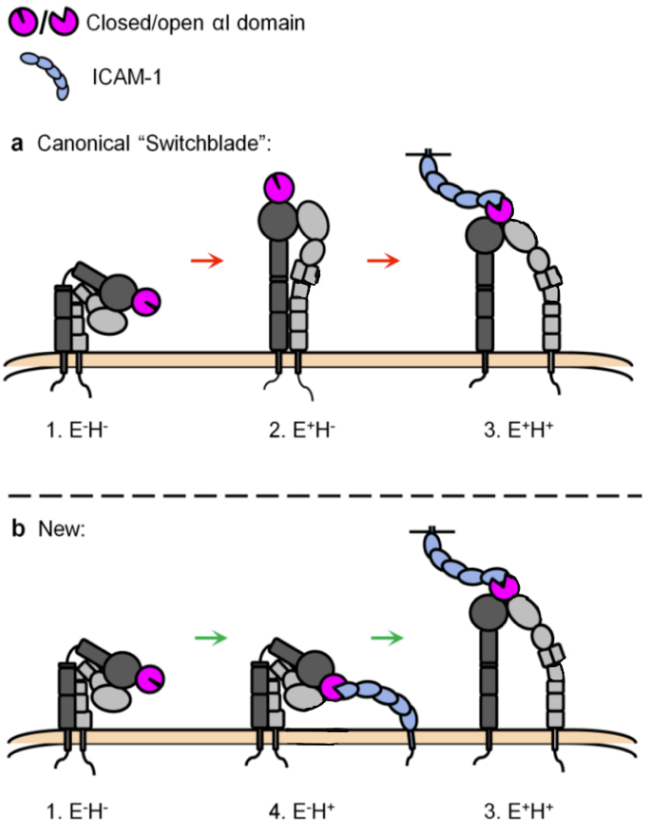


Figure 3. The canonical "switchblade" activation pathway and a newer proposed pathway of β_2 integrins.
Upper panel: canonical switchblade pathway. In their low affinity state (left), β_2 integrins are in a bent conformation, which keeps the ligand binding head domain close to the plasma membrane. In this conformation the β_2 integrin is in an inactive state. The intermediate activity state (middle) is associated with the extension of the extracellular domain but the headpiece (purple) is still closed (E^+H^-). Full β_2 integrin activation is characterized by the high affinity conformation (right), which enables ligand binding with high affinity due to conformational changes and swing out of the hybrid domain and an open headpiece (E^+H^+). Lower panel: Proposed new pathway. The fourth conformation (middle) binds ligands in *cis*. The headpiece is open but the integrin is not extended (E^-H^+). Integrin extension: E^+; headpiece-opening: H^+ (adapted from Fan et al., 2016[79]).

As mentioned above, unstimulated circulating PMNs exhibit β_2 integrins in an inactive conformation, with non-extended ectodomain (E^-) and closed headpiece (H^-). In this conformation integrins have a low affinity for the ligand. When β_2 integrins are fully activated, they have an extended conformation (E^+) with open headpiece (H^+) and are able to bind ligands in *trans* (on another cell), leading potentially to PMN arrest. However, two different states of intermediate activation between the fully inactive and fully active state of β_2 integrins exist: On the one hand, the so-called "canonical switchblade" model with a closed headpiece domain (H^-) and integrin extension (E^+). In this conformation the integrin is able to bind ligands in *trans* with low affinity and high off-rate[64,80]. On the other hand, the so-called "deadbolt" model (Figure 3b), with open

headpiece and (H^+) and bent integrin (E^-). It assumes a hairpin loop in the β tail domain, that may act as a deadbolt to restrict the displacement of the β6-α7 loop of the β-A/I domain and keeps the headpiece closed[80,81]. Due to the bent conformation, this intermediate activation state is able to bind ligands such as ICAM-1 *in cis* (on the same cell)[79]. Thereby, integrins cannot bind ICAM-1 in *trans* resulting in impaired PMN adhesion and aggregation[79]. Binding of selectin ligands and chemokine receptors activates intracellular signaling cascades and can lead to different integrin activation states[73,82]. Recent data shows, that low-affinity states of β_1 integrins may bind ligands substantially faster and thus forming rapid initial connections[83]. However, since integrins with a closed headpiece domain have a much higher off-rate, ligand binding is less stable. In contrast, high-affinity states have slower ligand-binding kinetics, but create more tight bonds.

1.2.2 The CD18 interactome

Although certain binding partners of CD18, such as Talin-1 and Kindlin-3 are known, the underlying mechanism regulating β_2 integrins in PMNs is incompletely understood and requires further investigations[84,85]. To this end, the lab of Prof. Walzog screened for novel interactors of β_2 integrins. For this purpose, the recombinant cytoplasmic tail of CD18 and whole cell lysates of freshly isolated human primary PMNs were used. Pull-down assays and subsequent mass

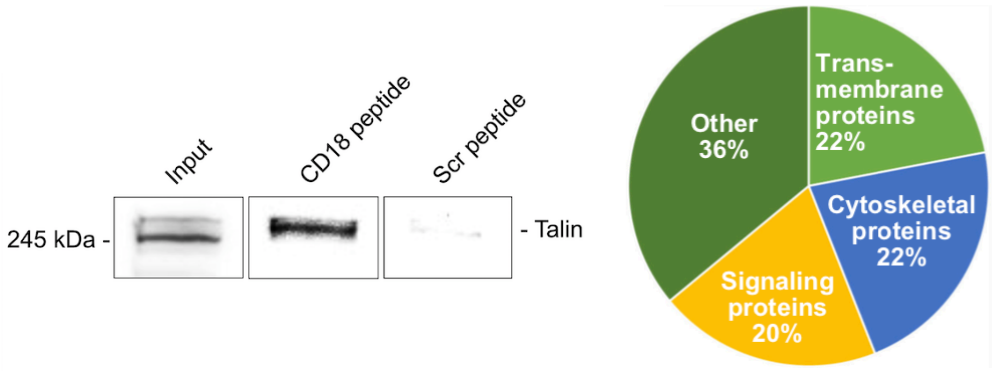


Figure 4. Identification of CD18 interaction partners by CD18 pull-down assay and subsequent mass spectrometry.
Left: Pull down experiments with a CD18 intracellular peptide and a scrambled (scr) peptide as control show specific Talin-1 binding to CD18 by western blot analysis, n = 3. Right: A total of 59 CD18 interacting proteins were identified and classified into four groups. Statistical analysis of the dataset: Two-tailed, paired Student's t-test with Benjamini-Hochberg's multiple testing correction, adjusted p-value < 0.2, n = 4. (Screening was planned by Barbara Walzog and Daniela Begandt and conducted by Sarah Thome, BMC, and statistical analysis was performed by Tobias Straub, Bioinformatic Core Facility, BMC)

spectrometry in cooperation with the core facility for protein analysis at the BMC was performed. The binding of Talin to the CD18 peptide confirmed the success of the pull-down assays (Figure 4, left). In this way, 59 putative interactors were identified, which were grouped into four different protein classes based on their function (Figure 4, right). Amongst known CD18 interactors, such as Talin, the protein thymocyte selection associated family member 2 (Themis2) was identified. The exponentially modified protein abundance index (emPAI) calculates the absolute amount of protein in a sample by the number of sequenced peptides per protein[86]. By means of emPAI the absolute amount of protein in a sample was calculated, based on protein coverage by peptide matches in a database search result. In this way, the emPAI fold change compared the CD18 samples with the scrambled sample (scr), and a high number of emPAI fold change indicated more interaction, since many peptides of the protein in the CD18 sample compared to scr sample means less non-specific binding. Based on this calculation, the proteins with the highest emPAI were chosen for a more detailed analysis. Finally, the choice to characterize a previously unknown but strong CD18 interaction partner fell on Themis2.

1.3 The CABIT domain family

The cysteine-containing all-beta in Themis (CABIT) domain defines a protein family consisting of five members in mammals and orthologous ancestors in almost all animal lineages[87]. The mammalian members are Themis1 (thymocyte-expressed molecule involved in selection), Themis2, Themis3, Garem1 (FAM59A) and Garem2 (FAM59B). The family name refers to its founding member, Themis1, which is expressed in T lymphocytes and plays a crucial role for their normal development[87–89]. All members are structurally related proteins defined by containing at least one copy of a CABIT module. CABIT domains are conserved protein domains, which can only be found in metazoans and are likely to be involved in protein-protein interactions[90]. They consists of a highly conserved core motif with a hydrophobic cysteine residue, which is likely to be central to its function, and have a median length of 261 amino acids[87,91]. The presence of the highly conserved core sequences and their requirement for the activity of Themis1 *in vivo* raised early the assumption, that CABIT modules possess a pivotal biological role[92]. Moreover, the fact that they are distinct from other described protein domains suggests that their cellular function might be unique as well[87].

The two GAREM protein subtypes, GAREM1 and GAREM2 (Grb2-associated regulator of ERK/MAPK, subtype 1/2) consist only of one CABIT domain, a proline-rich region (PRR), and a

sterile alpha motif (SAM) domain[87,93,94]. They are adaptor proteins, that function downstream of the EGF receptor signaling pathway. Upon EGF stimulation they get tyrosine phosphorylated and associate with the SH3 domain of the adaptor protein Grb2 via their PRR[94]. In mammals the two variants show distinct expression patterns: whereas GAREM1 is ubiquitously expressed, GAREM2 is predominantly expressed in the brain tissue[93]. GAREM1 plays a role in cell proliferation and carcinogenesis[94–96]. Furthermore, it is also believed to be required for growth and maintenance of normal body size in mice and humans[97]. GAREM2 is suggested to play a role in protein aggregation in cells of neurodegenerative diseases such as Alzheimer's and Huntington's disease[98]. Moreover, behavioral studies with knockout mice revealed, that GAREM2 is involved in altering emotions like anxiety[99].

The three Themis proteins contain two tandem CABIT domains and a highly conserved C-terminal PRR. However, they lack a catalytic domain[87]. Furthermore, Themis1 and Themis2 harbor a centrally located putative nuclear localization sequence (NLS) and a conserved tyrosine residue at the C-terminus, which act as SH2-binding sites upon phosphorylation[1,88–90,100]. The highly conserved and structural similar Themis1 and Themis2 proteins are present in specific cells in all mammals, whereas the more distantly related Themis3 is not present in primates and its expression is limited to the small and large intestine[100].

Themis1 is the first and best characterized protein of the CABIT family and was initially described by several groups in 2009[87,89,100–102] as a T cell specific protein. Its expression is restricted to lymphoid tissues, particularly the thymus and to a lesser extent in the spleen. Themis1 is highly expressed in $CD4^+CD8^+$ thymocytes and has an important role in normal T cell development[87,89,100,102]. During the double positive (DP) stage of thymocyte development, a selection process occurs based on the affinity of their T cell receptor (TCR) for self-peptide ligands bound to major histocompatibility complex (self-pMHC)[103]. This process tests TCR functionality and ensures self-tolerance. Thymocytes with TCRs that do not bind to self-pMHC or bind too strongly are eliminated through apoptosis, known as negative selection. Thymocytes with TCRs that bind weakly to self-pMHC are positively selected and progress to the single positive stage[103]. The result of thymocyte selection is the generation of a mature TCR repertoire that is self-tolerant (due to negative selection) but minimally self-reactive and bind self-pMHC (due to positive selection) to increase the likelihood that the TCR will be able to bind to foreign peptides with sufficient affinity to transmit activating signals. In the absence of Themis1, thymocyte development is partly inhibited at the transition from $CD4^+CD8^+$ thymocytes to single-positive thymocytes, the final stage of thymocyte development. This leads to significant reduction in

numbers of mature $CD4^+CD8^-$ thymocytes, $CD4^-CD8^+$ thymocytes and peripheral T cells, although $CD4^-CD8^+$ thymocyte number are less severely affected compared to $CD4^+CD8^-$ and peripheral T cells[87,89,100,101]. Although various reasons for this phenotype have been proposed, it is most likely that Themis1 enhances TCR signaling, which is required to receive survival signals during positive selection of T cells in the thymus[90,91]. In the absence of Themis1 the threshold for positive selection is not reached, leading to cell death by non-selection or so called "death by neglect" (Figure 5). Themis1 shows constitutive association to the adaptor protein Grb2 and gets tyrosine phosphorylated upon T cell activation by the tyrosine kinases Lck and ZAP70 and is recruited to the transmembrane adaptor linker for activation of T cells (LAT) by Grb2[1,104–106].

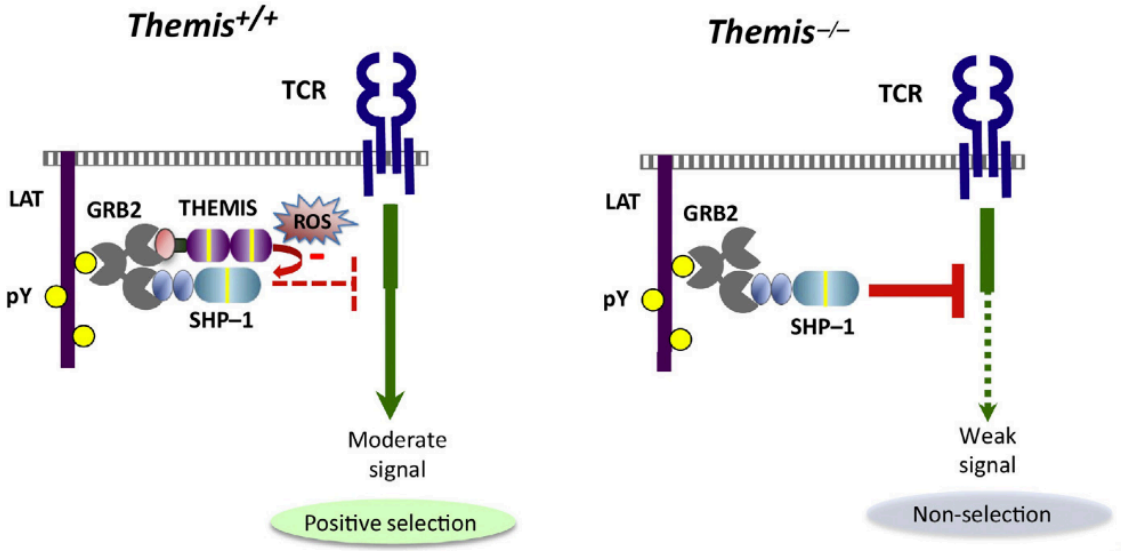


Figure 5. Potential model of Themis1 function in thymocytes.
Themis1 expression is high in $CD4^+CD8^+$ thymocytes when positive selection takes place. Themis1 and SHP-1 bind to the cytosolic adaptor Grb2 and are recruited by Grb2 to the scaffolding adaptor LAT upon TCR stimulation. Left: The CABIT domains of Themis1 bind to the SHP-1 phosphatase domain, which blocks access to the substrate and promotes or stabilizes oxidation of the catalytic cysteine of SHP-1 by ROS, thus inhibiting SHP-1 phosphatase activity. Inhibition of SHP-1 allows TCR interactions with low-affinity to generate signals sufficient for positive selection. Right: In $Themis1^{-/-}$ thymocytes, SHP-1 PTP activity is not downregulated, and TCR interactions with low-affinity, normally sufficient for positive selection, do not result in positive selection, hence leading to cell death by non-selection. The displayed SHP-1 binding to the Grb2 SH3 domain is speculative, since the binding site has not yet been identified; pY, phosphotyrosine (adapted from Choi et al., Trends in Immunol., 2017[90]).

Studies on genetic reconstitution revealed a necessity of the CABIT domains, the PRR and NLS for the *in vivo* activity of Themis1[1,92,104,107]. The CABIT modules of Themis1 have been shown to

directly bind to the protein tyrosine phosphatase (PTP) domain of Src homology region 2 domain-containing phosphatase-1 (SHP-1), thereby sustaining or promoting the oxidation of SHP-1's catalytic cysteine residue. Through this mechanism Themis1 impedes the tyrosine-phosphatase activity of SHP-1 and facilitates thymocyte positive selection via enhancing the signaling response of the T cell antigen receptor[90]. Consistently, the defects observed in Themis1$^{-/-}$ mice are attenuated when SHP-1 is deleted as well[91].

1.4 Themis2

Themis2, also known as Induced on contact with basement membrane 1 (ICB1), contains two CABIT domains, a NLS and a PRR, which can also mediate protein-protein interactions (Figure 6)[87,90,108]. It has a protein size of 663 amino acids and a molecular weight of 74 kDa. Themis2 lacks enzymatic activity, but contains a number of phosphorylation sites, including Y660, which is a putative substrate of Src family kinases (SFKs) (Figure 6). It is ubiquitously expressed with highest levels in the bone marrow, appendix, spleen, lymph node, lung and gall bladder.

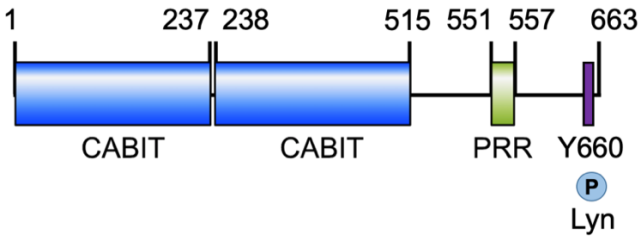


Figure 6. Domain structure of murine Themis2.
Themis2 consists of two CABIT domains (blue), a PRR (green) and phosphorylation site, Y660 (purple). The exact position of the NLS is unknown. The Y660 is the putative substrate of SFKs and proposed binding site of their SH2 domains. The PRR represents the potential binding site for Vav1 and Grb2 (modified from Choi et al., Trends Immunol., 2017[90]).

The protein was first identified and described by Peirce et al. in 2010 in a phospho-proteome screen of RAW264.7 cells, which is a murine macrophage cell line[88]. In this study they showed, that it functions as a signaling scaffold in macrophages, which selectively regulates TLR responses and cytokine production. Moreover they found, that the expression of Themis2 is induced during the development from murine bone marrow precursors to macrophages and in response to inflammatory stimuli both *in vivo* as well as *in vitro*[88]. Upon LPS challenge Themis2 gets tyrosine

phosphorylated and interacts with the SFK Lyn. Y660F mutation of Themis2 inhibits this LPS-induced phosphorylation and interaction with Lyn. Moreover, Themis2 interacts with Vav1 and Grb2. This interaction is interrupted when either Y660 or the PRR is mutated and thereby also reduces around 50% of Themis2 capacity to promote LPS-induced TNF production. This finding suggests that both the PRR motif and Y660 are involved in the interaction of Grb2 with Themis2, similar to Themis1. Besides its function in macrophages, Themis2 is known to be predominantly expressed in myeloid cells and B cells[88,90,109]. However, activated B cells and macrophages involved in inflammation, exhibit reduced Themis2 expression[88,110]. The role of Themis2 in PMNs has not yet been explored.

Previous studies showed Themis2 to be involved in B cell receptor (BCR) and T cell receptor (TCR) signalling[1,2,91]. However, B cell development appears to be normal in the bone marrow of Themis2$^{-/-}$ mice, since Themis2 knockout mice show similar numbers of B cell subsets in the spleen and lymph nodes compared to wild-type mice[2]. However, Themis2 impacts a key point of adaptive immunity and self-tolerance – the positive and negative selection of lymphocytes by antigens. Positive and negative selection of B cells is necessary to ensure optimized B cell responses to foreign antigens and to prevent autoimmune reactions towards self-antigens. Positive selection requires binding of the pre-BCR to its ligand and negative selection leads to apoptosis of self-reactive B cells[111,112].

Themis2 is involved in the process of positive selection to self and foreign antigens, whereas B cell activation with high-avidity antigens is unaffected[110]. Mice lacking Themis2 show impaired positive selection of developing B cells to self and foreign antigens after immunization with low-avidity antigens. However, negative selection is not affected[2]. Thus, via modulation of the intracellular response after BCR stimulation Themis2 seem to lower the threshold for B cell activation for low-avidity antigens, which can be overcome when BCRs are stimulated by high-avidity antigens. This modulation is most likely mediated by the constitutive interaction of Themis2 to Grb2, Lyn and the signal transducer phospholipase c-γ2 (PLC-γ2). Upon BCR stimulation by low-avidity antigens Themis2 increases the activation of PLC-γ2 and its downstream pathways.

At the amino acid level Themis2 shares 29% identity and 65% homology with the T cell specific Themis1[87]. Due to this high similarity, ectopic expression of Themis2 in T cells, which primarily express Themis1, rescues the development of thymocytes and TCR-mediated signaling in Themis1-deficient mice. This indicates that the conserved domains of these two proteins are responsible for their biological activity and that they are functionally interchangeable in T-cell development[1]. In the Themis1 knockout situation, Themis2 gets tyrosine-phosphorylated by SFKs and recruited

upon TCR engagement to the scaffolding adaptor LAT within Grb2/Vav1 signaling complexes[1]. Thus, Themis2 binds to Grb2, although its PRR motif (PxxPxK) differs from the PRR motif of Themis1 (RxPxxP)[88]. The finding that Themis2 facilitates positive selection of thymocytes by enhancing TCR signaling in the absence of Themis1 is strongly reminiscent of the role of Themis2 in BCR signaling.

Themis2 has been shown to be involved in TLR4 mediated signaling in macrophages and interacts with Lyn, which is also known to interact with CD18[88,113]. Furthermore, it interacts with Vav1, which is also an important integrin signaling protein. Interaction between Themis2 with Vav1 and Grb2 has been shown in co-immunoprecipitation experiments in B cells and thymocytes of Themis1$^{-/-}$ mice with transgenic Themis2 expression[1,88]. Based on these previous findings, a role of Themis2 in β_2 integrin regulation and signaling seems very well possible.

2. AIM OF THE THESIS

Themis2 has been shown to be predominantly expressed in B cells and myeloid cells and its expression is upregulated during the differentiation of neutrophil-like HL-60 cells. Furthermore, it is known to play a role in BCR signaling. However, its role in PMNs is unknown. In the Walzog lab Themis2 has been identified as a potential β_2 integrin interaction partner specifically enriched in human PMN lysates in pull down experiments using the cytoplasmic tail of the β_2 integrin (CD18). Since the classic immunoreceptor signaling of BCR and TCR owes high similarities to β_2 integrin signalling[1–3] Themis2 might play a role in β_2 integrin regulation and signaling, which is critical for PMN biology. Therefore, the aim of my PhD project was to elucidate the potential role of Themis2 in β_2 integrin regulation and signaling as well as for PMN trafficking.

In the first part of this thesis, the role of Themis2 for PMN trafficking *in vitro* by different flow chamber assays was analyzed to study β_2 integrin-dependent steps of the recruitment cascade, such as PMN rolling, adhesion and migration. Following, PMN extravasation during acute inflammation in the absence of Themis2 was studied, which was conducted by inflammation models in Themis2$^{+/+}$ and Themis2$^{-/-}$ mice. To investigate the role of Themis2 for PMN function and intracellular signaling, ICAM-1 binding assays and reporter-antibody assays were conducted. This helped to delineate the role of Themis2 in integrin regulation and thereby possibly provided a better understanding of PMN trafficking and neutrophil biology in general.

3. MATERIALS

3.1 Mouse strains

Cryopreserved sperm from Themis2$^{-/-}$ mice (C57BL/6J-Themis2tm1d(KOMP)Wtsi/Nimr mice) were kindly provided by Victor Tybulewicz (Imperial College London, UK) and used for *in vitro* fertilization of C57BL/6J oocytes by the Core facility for Animal Models (CAM) of the Biomedical center (BMC). Mice were housed under specific pathogen-free conditions and bred in heterozygous matings to obtain wildtype littermate controls. Both male and female mice from the age of 10 weeks onwards were used for the experiments. Mouse experiments were performed in accordance with the German federal animal protection laws and approved by the Bavarian Government (Regierung von Oberbayern, Munich, Germany).

3.2 Chemicals, Kits and Plasticware

Name	Supplier
2-Mercaptoethanol	Sigma Aldrich, Germany
acetic acid	AppliChem, Germany
agarose	Genaxxon, Germany
ammoniumperoxodisulfate (APS)	AppliChem, Germany
ampicillin	AppliChem, Germany
BlueStar Plus™ prestained protein marker	Nippon, Japan
BD FACS™ lysing solution	BD Bioscience
bovine serum albumin (BSA)	Sigma Aldrich, Germany
bromphenole blue	AppliChem, Germany
β-estradiol	Sigma Aldrich
calcium chloride ($CaCl_2$)	AppliChem, Germany
casein	Sigma Aldrich, Germany
dimethyl sulfoxide (DMSO)	AppliChem, Germany
dithiothreitol (DTT)	AppliChem, Germany
Dulbecco's modified eagle medium (DMEM)	Biochrom, Germany
Escherichia coli BioParticles™ Texas Red™ conjugate	Thermo Fisher Scientific, Germany
ethanol absolute	Th. Geyer, Germany
ethylenediamine-tetraacetic acid (EDTA)	AppliChem, Germany
Eukitt quick-hardening mounting medium	Sigma Aldrich, Germany
fetal calf serum (FCS)	Biochrom, Germany

fluorescence microbeads	Polysciences, Germany
GeneRuler™ 1 kb DNA ladder	Nippon, Japan
GeneRuler™ 100 bp DNA ladder	Nippon, Japan
Giemsa's azur eosin methylene blue	Sigma Aldrich, Germany
glucose	AppliChem, Germany
glutaraldehyde solution	Sigma Aldrich, Germany
glycine	AppliChem, Germany
Hank's balanced salt solution	Biochrom, Germany
HEPES	AppliChem, Germany
hydrochloric acid, 37% (HCl)	AppliChem, Germany
isopropanol	Merck KGaA, Germany
lipofectamin 2000	Thermo Fisher Scientific, Germany
lipopolysaccharide (LPS) from *Salmonella eneritidis*	Sigma Aldrich, Germany
live cell imaging solution (LCISol)	Thermo Fisher Scientific, Germany
manganese(II) chloride ($MnCl_2$)	AppliChem, Germany
magnesium chloride ($MgCl_2$)	AppliChem, Germany
May-Grünwald solution	AppliChem, Germany
methanol	Th. Geyer, Germany
midori green	Nippon, Japan
Mini-PROTEAN® TGX Stain-free™ protein gels	Bio-Rad, Germany
mouse serum	Abcam, Great Britain
Mammalian protein extraction buffer (M-PER)	Thermo Fisher Scientific, Germany
MycoSPY® Master Mix	Biontex, Germany
NucleoSpin gel and PCR clean-up	Macherey-Nagel, Germany
PageRuler™ prestained protein ladder	Thermo Fisher Scientific, Germany
paraformaldehyde (PFA) 37%	Sigma Aldrich, Germany
phenylmethylsulfonyl fluoride (PMSF)	Sigma Aldrich, Germany
phorbol myristate acetate (PMA)	Merck, Germany
PCRBio Rapid Extract PCR Kit	Nippon, Japan
penicillin / streptomycin (P/S)	Biochrom, Germany
percoll	Sigma Aldrich, Germany
phenol red	Biochrom, Germany
phosphate buffered saline (PBS)	Biochrom, Germany
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, Germany
Poly-L-Lysin	Merck KGaA, Germany
Ponceau solution	AppliChem, Germany
potassium bicarbonate ($KHCO_3$)	Sigma Aldrich, Germany

ProLong™ Gold antifade reagent	Thermo Fisher Scientific, Germany
protease inhibitor Mix B	Sigma Aldrich, Germany
PureYield™ Plasmid Miniprep System	Promega, Germany
Qubit™ Protein Assay Kit	Thermo Fisher Scientific, Germany
Roswell Park Memorial Institute 1640 (RPMI) medium	Biochrom, Germany
SiR-actin	Spirochrom, Switzerland
sodium chloride (NaCl)	AppliChem, Germany
sodium dihydrogen carbonate (NaH_2PO_4)	Sigma Aldrich, Germany
sodium dodecyl sulfate (SDS)	AppliChem, Germany
sodium fluoride (NaF)	Sigma Aldrich, Germany
sodium hydrogen carbonate (Na_2HCO_3)	AppliChem, Germany
sodium orthovanadate (Na_3VO_4)	Sigma Aldrich, Germany
SYTOX™ Red dead cell stain	Thermo Fisher Scientific, Germany
TritonX-100	Sigma Aldrich, Germany
trypan blue	Sigma Aldrich, Germany
trypsin/EDTA	Biochrom, Germany
Tween 20	Sigma Aldrich, Germany
xylol	AppliChem, Germany
µ–Slide membrane ibiPore flow chamber	IBIDI, Germany
µ-Slide VI0.1 flow chamber	IBIDI, Germany

3.3 Buffers and Solutions

Name	Ingredients
4x Lämmli-buffer	250 mM Tris-HCl pH 6.8 8% SDS 40% glycerol 20% 2-Mercaptoethanol 0.002% bromophenol blue in H_2O_{dest}
Immunofluorescence (IF) antibody staining and permeabilization solution	10% (w/v) BSA 0.1% TritonX-100 in PBS
IF blocking solution	10% (w/v) BSA in PBS
10x running buffer	2 M glycine 250 mM Tris 1% (w/v) SDS in H_2O_{dest}
10x TBS buffer	250 mM tris-HCl 1.5 M NaCl in H_2O_{dest}
50x TAE buffer	2 M tris 1 M sodium acetate 62.5 mM EDTA pH 8.5 in H_2O_{dest}

adhesion medium (ADM)	1.2 mM Ca^{2+} 1 mM Mg^{2+} 0.25% BSA 0.1% glucose 20 mM Hepes pH 7.4 in Hank's balanced salt solution
agarose gel solution	1 x TAE buffer 1–2% (w/v) agarose 1 µg/mL midori green
BD FACS™ lysing solution	Dilution 1:10 in H_2O_{dest}
cell lysis buffer	M-PER 1 mM DFP 100 mM sodium orthovanadate 100 mM sodium fluoride protease inhibitor cocktail
TBST	1 x TBS 0.1% Tween 20
WB blocking solution	1 x TBS 5% (w/v) skim milk powder 0.1% Tween 20

3.4 Recombinant proteins

Name	Supplier
recombinant (r) murine (m) CXCL1	PeproTech, USA
rmE-selectin/Fc	R&D Systems, USA
murine fibrinogen	Innovative Research, USA
rmG-CSF	PeproTech, USA
rmICAM1 w/o Fc	STEMCELL, Germany
rmICAM-1/Fc	R&D Systems, USA
leukotriene B_4	Sigma Aldrich, Germany
rmP-selectin/Fc	R&D Systems, USA
rmTNF-α	R&D Systems, USA
human fibrinogen	R&D Systems, USA
human fibrinogen Alexa-647	Thermo Fisher Scientific, Germany
hICAM-1	R&D Systems, USA
Interleukin-8 (IL-8)	R&D Systems, USA
N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP)	Sigma Aldrich, Germany

3.5 Antibodies and fluorescent dyes

Target	Label	Reactivity	Clone or catalogue number	Company
β actin	-	mouse anti-human	C4	Santa Cruz Biotechnology, USA
CXCR2	Alexa Fluor 647	rat anti-mouse	TG11	BioLegend, USA
CD11a	Alexa Fluor 594	rat anti-mouse	2D7	BioLegend, USA
CD11a	PE	rat anti-mouse	2D7	BD Biosciences, USA
CD11a	-	rat anti-mouse	M17/4	eBioscience, USA
CD11a/CD18	Alexa Fluor 488	mouse anti-human	m24	BioLegend, USA
CD11a/CD18	-	mouse anti-human	KIM127	Kindly provided by M. Sperandio (BMC Munich, Germany)
CD11b	PE	rat anti-mouse	M1/70	eBioscience, USA
CD11b	-	rat anti-mouse	M1/70	eBioscience, USA
CD16/CD32		rat anti-mouse	2.4G2	BD Biosciences, USA
CD18	PE, FITC	rat anti-mouse	C71/16	BD Biosciences, USA

CD18	-	mouse anti-human	CTB104	Santa Cruz Biotechnology, USA
CD34	eF660	rat anti-mouse	RAM34	eBioscience, USA
CD117 (c-Kit)	PerCP-eF710	rat anti-mouse	2B8	eBioscience, USA
GAPDH		mouse	6C5	Merck Millipore, USA
GFP	-	rabbit anti-human	FL	Santa Cruz Biotechnology, USA
Gr-1	FITC	rat anti-mouse	RB6-8C5	BD Biosciences, USA
Hoechst 33342	-	-	-	Thermo Scientific, USA
ICB1 pAb (Themis2)	-	rabbit anti-human	AP9910b	Abgent, USA
IgG1	PE	mouse anti-human	H2	Southern Biotech, USA
Integrin beta-2	APC	mouse anti-human	1B4/CD18	BioLegend, USA
isotype control	PE	rat anti-mouse	IgG2a, κ	BD Biosciences, USA
isotype control	PE	rat anti-mouse	IgG2b, κ	eBioscience, USA
Ly6-G	FITC	rat anti-mouse	1A8	BioLegend, USA
Phalloidin	Alexa Fluor 546	-	A22283	Thermo Fisher Scientific, Germany
anti-rabbit Ig	Alexa Fluor 647	donkey anti-rabbit	-	Thermo Fisher Scientific, Germany
anti-rabbit Ig	680 RD	donkey anti-rabbit	925-68073	Li-Cor Biotechnology, USA
anti-mouse Ig	800 CW	donkey anti-mouse	926-32212	Li-Cor Biotechnology, USA
anti-rabbit Ig	800 CW	donkey anti-rabbit	925-32213	Li-Cor Biotechnology, USA
Themis2		rabbit anti-mouse		Kindly provided by R. Cornall (NDM Oxford, U.K.)

3.6 Cell lines

Name	Description	Source
CHO-MGF	immortalised Chinese hamster ovary cell line	Hans Häcker; St. Jude Children's Research Hospital, Memphis, USA
HL-60	human promyelocytic leukemia cell line	ATCC®CRL-240™, Manassas, VA
Hoxb8-SCF	immortalized murine progenitor cell line	self-made
WEHI-3B	murine myelomonocytic leukemia cell line	DSMZ ACC 26, Germany

3.7 Cell culture media

Applied for	Composition
Cryo-conservation	FCS 10% DMSO
Hoxb8-SCF cells	RPMI 1640 10% FCS 100 U/mL penicillin 100 µg/mL streptomycin 4% SCF containing CHO supernatant 30 µM beta 2-mercaptoethanol 10 µM β-Estradiol
Hoxb8-SCF differentiation towards PMN	RPMI 1640 10% FCS 100 U/mL penicillin 100 µg/mL streptomycin 4% SCF containing CHO supernatant 20 ng/mL G-CSF
Murine bone marrow-derived PMN	RPMI 1640 10% FCS 100 U/mL penicillin 100 µg/mL streptomycin 3% IL-3 containing WEHI-3B supernatant
WEHI-3B, CHO-MGF, HL-60 cells	RPMI 1640 + 10% (v/v) FCS 100 U/mL penicillin 100 µg/mL streptomycin

3.8 Equipment

Name	Company
AxioCam Hsm camera	Zeiss, Germany
Axiotech Vario intravital microscope	Zeiss, Germany
Axiovert 200M microscope	Zeiss, Germany
Confocal scanner unit CSU-X1	Yokogawa Electric Corporation, Japan
Coulter A C T counter	Coulter Corporation, USA
CytoFLEX S	Beckman Coulter, Germany
EM CCD camera	Photometrics, USA
Examiner spinning disk confocal microscope	Zeiss, Germany
NanoDrop 2000	Life Technologies, Germany
Odyssey® CLx imaging system	Li-Cor Biotechnology, USA
ProCyte Dx hematology analyzer	IDEXX Laboratories, Germany
PowerWave HT microplate reader	Biotek, USA
SP8X WLL microscope	Leica Biosystems, Germany

3.9 Software

Name	Company
Adobe Photoshop and Illustrator	Adobe, USA
Chemotaxis and Migration Tool	IBIDI, Germany
EndNote X7.4	Clarivate Analytics, USA,
FACS Diva	BD Biosciences, USA
FlowJo V10	Treestar, USA
Fiji/ImageJ	NIH, USA
Leica Application Suites	Leica, Germany
Microsoft Office	Microsoft Corporation, USA
Prism 9	GraphPad Software, USA
SnapGene Viewer	SnapGene, USA

4. METHODS

4.1 Genomic PCR

To identify the genotype of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice, DNA was isolated from mouse ear biopsies and analysed by Polymerase Chain Reaction (PCR) using the PCRBIO Rapid Extract PCR kit (PCRBiosystems, United Kingdom) according to the manufacturer's protocol. PCR was carried out using 2x PCRBIO HS Taq Mix Red (Table 1) and specifically designed primers (Table 2) to distinguish Themis2$^{+/+}$ and Themis2$^{-/-}$ mice. Themis2$^{+/+}$ locus was amplified with a forward (fwd) primer binding in exon 4 and a reverse (rev) primer binding in intron 4, generating a PCR product of 807 base pairs (bp). Themis2$^{-/-}$ locus, missing exon 4, was amplified with the same rev primer and a fwd primer binding to an inserted flippase recognition target site generating a PCR product of 364 bp.

The PCR protocol started with a primary denaturation step at 95 °C for 1 min, followed by 40 cycles of denaturation for 15 s at 95 °C, hybridization of 15 s at the annealing temperature of the primers and 30 s extension at 72 °C. The PCR protocol ended with a final extension step of 5 min at 72 °C. For the PCR of Themis2$^{+/+}$ and Themis2$^{-/-}$ alleles an annealing temperature of 63 °C, respectively 65 °C, was used. DNA fragments were separated according to their size by agarose gel electrophoresis with 2% agarose in TAE buffer. DNA fragments were stained by adding 1 µg/mL Midori Green before casting the gel and visualized by UV light (260 nm). DNA ladder was run on the same gel as size standard to determine the actual band size.

Table 1. PCR components

Components	Concentration
2x PCRBIO HS Taq Mix Red	1 x
Forward primer (10 µM)	0.5 µM
Reverse primer (10 µm)	0.5 µM
Template DNA	2 µL of isolated DNA
H_2O	x µL

Table 2. Primers used for genotyping

Name	5' – 3' sequence	Tm [°C]	Supplier
Themis2 targ fwd	GGTCTGAGCTCGCCATCAGTTC	66	Metabion, Germany
Themis2 WT fwd Exon4	GACCTGACTGTGGTGGAGGC	65	Metabion, Germany
Themis2 WT rev	CAATCAGCGTAGGGCAACTG	60	Metabion, Germany

4.2 Cell culture and differentiation

To ensure optimal growth conditions all cell lines were cultured in their appropriate media at 37 °C, in 5% CO_2 and 95% air humidity. Hoxb8-SCF cells and human promyelocytic leukaemia cell line HL-60 were grown in suspension and sub-cultured every 2–3 days by withdrawal of the bulk of the suspension and keeping 0.5–2 mL in the culture flask and adding fresh media. WEHI-3B and CHO-MGF cells grow as adherent cells and were sub-cultured at 90% confluence by adding trypsin/EDTA to detach the cells. A small amount of cells (about 100–500 µL) was then transferred into a new culture dish containing fresh media. To obtain IL-3 containing supernatant from WEHI-3B cells or SCF containing supernatant from CHO-MGF cells, the cells were allowed to grow to 100% confluence for 2 d and the supernatant was collected, filtered with a pore size of 0.45 µm and frozen at -80 °C.

To induce differentiation towards neutrophil-like cells (dHL-60), HL-60 cells (1 x 10^6/plate) were incubated for 6 d with culture media supplemented with 1.3% DMSO[114,115]. For differentiation of Hoxb8-SCF cells towards neutrophils, Hoxb8-SCF cells (2.5 x 10^5/plate) were washed in PBS and subsequently cultured in differentiation media for 4 d[116].

4.3 Cryopreservation and revival of frozen cells

For long term storage, cells were pelleted at 300 x g for 5 min, re-suspended at 2 x 10^6 cells per mL in freezing medium and aliquoted into cryotubes. Subsequently cryotubes were put in a NALGENE™ Cryo 1 °C freezing container (Thermo Fisher Scientific, Germany) allowing freezing at constant rate of about -1 °C per minute and kept at -80 °C overnight before long term storage at -196 °C in liquid nitrogen. To re-cultivate frozen cell stocks, cryotubes were briefly thawed in a water bath at 37 °C and washed once with culture medium in order to get rid of the remaining DMSO before transferring the cells to a new culture flask containing fresh culture medium. For all

in vitro assays the exact number of live cells was determined using a Neubauer counting chamber (Th. Geyer, Germany) in combination with trypan blue staining to indicate dead cells.

4.4 Isolation of murine and human PMN

Murine bone marrow PMNs were obtained by flushing bone marrow cells from femur and tibia of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice with PBS and loading the cells onto a discontinuous Percoll gradient (52%/ 64%/ 72%) with subsequent centrifugation at 1000 x g for 30 min without brake. Murine PMNs enriched in the Percoll gradient interphase between 72% and 64% due to their specific density and were collected, washed once with PBS and cultured for 24 h in RPMI 1640 supplemented with 20% WEHI-3B-conditioned medium[116,117]. Human PMNs were isolated from the blood of healthy donors by venepuncture. After collection of 20 mL blood, the blood was anticoagulated with 50 µL heparin. Two thirds of the blood were transferred in two different tubes. One third of the collected blood was briefly centrifuged at low speed and the plasma on top was transferred onto the other tubes in order to gain more leukocytes. In the presence of 40% (v/v) autologous plasma, erythrocytes were allowed to sediment. Subsequently, the leukocyte-rich plasma was transferred onto a discontinuous Percoll gradient (55%/ 74%) and centrifuged at 600 x g for 20 min. PMNs were isolated as previously described[59].

4.5 Western Blot analysis

In order to determine protein expression by means of immunoblotting technique cell lysates of mPMNs and hPMNs were prepared at a concentration of 1 x 10^6 cells/30 µL cell lysis buffer, respectively 1 x 10^6 cells/10 µL cell lysis buffer in case of Hoxb8 cells. Cell lysates were prepared in M-PER buffer supplemented with 1 mM DFP, 100 mM sodium orthovanadate, 100 mM sodium fluoride, and protease inhibitor cocktail and incubated on a spinning wheel at 4 °C for 30 min. Subsequently the lysates were drawn up and down through a syringe. Cell debris was removed by centrifugation at 13.000 x g for 1 min and total protein concentration of the supernatant was determined employing Pierce™ BCA protein assay kit or the Qubit system (Thermo Fisher Scientific, USA). Proteins were denatured and reduced by mixing with the appropriate amount of 4 x Laemmli-buffer and incubated at 95 °C for 7 min. To separate proteins according to their molecular weight, total protein lysates were applied to SDS-PAGE. Protein lysates and BlueStar

Plus™ Prestained Protein Marker were loaded on 4–20% Mini-PROTEAN® TGX Stain-free™ protein gels and electrophoresis was done in 1 x running buffer at 180–240 V. After separation of the proteins they were transferred onto a nitrocellulose membrane by semi-dry blotting technique for immunodetection at 1.3 A for 10 min. Subsequently the membrane was blocked for 1 h in blocking solution while gently shaken on a platform shaker at room temperature. Membranes were incubated with primary antibody on a shaker at 4 °C overnight. Afterwards, they were incubated with appropriate near-infrared labelled secondary antibodies for 1 h and detection was performed using the Odyssey® CLx Imaging system and analysed using Fiji software (NIH, USA).

4.6 Immunostaining and confocal microscopy

To identify the subcellular localization of Themis2 as well as CD18 and F-Actin in confocal super-resolution microscopy (CSRM) was applied. dHL-60 cells were seeded onto fibrinogen-coated dishes and cells were stimulated by addition of 100 nM fMLP for 12 min at 37 °C. mPMNs were seeded onto mICAM-1-coated dishes (øFc, 3 µg/mL) and stimulated with 100 ng/mL CXCL1 for 10 min at 37 °C. Afterwards the cells were fixed with 4% paraformaldehyde (PFA). Following permeabilization and blocking, immunostaining was performed overnight at 4 °C using rabbit anti-human Themis2 pAb and mouse anti-human β_2 integrin mAb followed by the application of appropriate secondary Abs for 45 min. Afterwards the cell nuclei were stained with the nuclear dye Hoechst 33342 for 20 min followed by F-Actin staining with Phalloidin Alexa Fluor 546 for 35 min. Subsequently cells were mounted with ProLong™ Gold antifade reagent and CSRM was performed using Leica TCS SP8 STED microscope equipped with 63 x/1.40 oil immersion objective and four lasers with an excitation wavelength of 405 nm, 488 nm, 561 nm and 647 nm. Images were analyzed using Fiji software.

4.7 Rolling velocity

Rolling velocity of isolated mPMNs from Themis2$^{+/+}$ and Themis2$^{-/-}$ mice was calculated *in vitro* by using Ibidi µ-slides VI$^{0.1}$ coated with 20 µg/mL rmE-selectin with or without 15 µg/mL rmICAM-1, at 4 °C overnight. 1 x 10^6 cells were resuspended in 1 mL adhesion medium and perfused through the channels for 6 min at constant shear stress (1 dyne/cm^2). Using the software Fiji, the videos were analysed and the rolling velocity of the cells was calculated with the plugin MtrackJ.

4.8 Induction of adhesion under flow conditions

Induction of adhesion of primary mPMNs from Themis2$^{+/+}$ and Themis2$^{-/-}$ mice and Themis2$^{+/+}$ and Themis2$^{-/-}$ Hoxb8 cell-derived PMNs (dHoxb8 cells) under flow conditions *in vitro* was analysed using IBIDI µ-Slide VI$^{0.1}$ flow chambers. Flow chambers were coated with 10 µg/mL rmP-selectin (isolated mPMN) or 5 µg/mL rmP-selectin (dHoxb8 cells), 3 µg/mL rmICAM-1, and 5 µg/mL rmCXCL1 at 4 °C overnight. Cells (7.5 x 10^5/mL) in adhesion medium were perfused through the channels for 9 min at a constant shear stress of 1 dyne/cm^2. Using an Axiovert 200M microscope equipped with a Plan-Apochromat 20 x/0.75 NA objective, AxioCam HR digital camera, and a temperature-controlled environmental chamber (Zeiss, Germany), time-lapse videos were recorded from 18 different points of view. The number of rolling and adherent cells after 1, 3, 5, 7, and 9 min was counted offline using Fiji software as described previously[118]. Cells were defined as adherent when they remained at the same spot for longer than 15 s.

4.9 2D Migration Assay

Mechanotactic crawling of Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMN was analysed using IBIDI µ-Slide VI$^{0.1}$ flow chambers coated with 10 µg/mL rmP-selectin-Fc, 12.5 µg/mL rmICAM-1 and 5 µg/mL rmCXCL1 at 4 °C overnight. mPMN in ADM were loaded into channels (4 x 10^5 cells/channel) and allowed to adhere for 10 min at 37 °C. Subsequently, flow of 1 dyne/cm^2 was applied and crawling of mPMN was recorded by time-lapse video at 5 s time intervals. The microscope set-up was the same as described in chapter 4.8. Euclidian distance, crawling velocity and percentage of crawling cells was calculated offline using Fiji software and the MtrackJ plugin.

4.10 Adhesion strengthening under flow conditions

Adhesion strengthening of isolated mPMNs of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice was measured as described previously by calculating the number of remaining cells in relation to the number of initially adherent cells (100%) in response to gradual increase in shear stress from 0.5 to 8.0 dyne/cm^2 at 90 s intervals[115]. mPMNs (4 x 10^5/sample in ADM) were allowed to adhere on

rmICAM-1 (3 µg/mL) and rmCXCL1 (5 µg/mL) for 10 min and subsequently shear stress was applied. Analysis were done using Fiji software.

4.11 Analysis of the expression of surface proteins on Hoxb8 and dHoxb8 cells

To characterize Themis2$^{+/+}$ and Themis2$^{-/-}$ Hoxb8 cells as well as humanised CD18 (hCD18) Themis2$^{+/+}$ and Themis2$^{-/-}$ Hoxb8 cells during differentiation to PMN-like cells, expression of the surface proteins CD11a, CD11b, CD18, CXCR2, CD34, c-Kit, Ly6-G and Gr-1 on day 0 (d0) and day 4 (d4) was analysed by flow cytometry. Cells (4 x 10^5/sample) were incubated with 50 µL staining solution containing the appropriate antibodies or the corresponding isotype controls in PBS supplemented with 1% FCS for 30 min on ice. Afterwards cells were kept on ice, washed and immediately analysed by a LSRFortessa flow cytometer. Fluorescence intensities were measured and mean fluorescence intensities (MFI) and histograms were analysed using FlowJo V10 software (Treestar, USA).

4.12 ICAM-1 binding assay

LFA-1-specific rmICAM-1/Fc binding was analysed by flow cytometry as described previously[73,115]. Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs or Hoxb8 cell-derived PMNs (2 x 10^5/sample) were incubated with a function blocking rat anti-mouse CD11b mAb (30 µg/mL, clone M1/70) to block Mac-1 activity. Afterwards rmICAM-1/Fc (20 µg/mL) and PE-labelled mouse anti-human IgG_1 gamma chain specific mAb (10 µg/mL, clone H2) were added and cells were stimulated with rmCXCL1 (100 ng/mL), fMLP (10 µM), Mn^{2+} (3 mM) or left untreated at 37 °C for 3 min. For blocking LFA-1-specific binding to rmICAM-1-Fc, cells were incubated with a function blocking rat anti-mouse CD11a mAb (30 µg/mL, clone M17/4) prior to stimulation in addition to CD11b mAb incubation. After stimulation ice-cold FACS Lysing solution was added (1:10 diluted in H_2O) immediately and put on ice for 10 min to fix the cells. Subsequently cells were washed twice and analysed with LSRFortessa flow cytometer. In order to calculate the LFA-1-specific rmICAM-1/Fc binding, the anti-CD11a antibody-treated control was used defining 95% of PMNs as negative for LFA-1 binding to rmICAM-1/Fc. This threshold was applied to all samples, and the percentage of PMNs with positive LFA-1-specific rmICAM-1/Fc binding was calculated accordingly. Data were analysed using FlowJo v10 software.

4.13 Affinity regulation

In order to investigate LFA-1 affinity regulation with the help pf the conformation specific reporter antibodies KIM127 and mAb24, d4 differentiated hCD18 Themis2$^{+/+}$ and Themis2$^{-/-}$ Hoxb8 cells, which were generated by Markus Moser and Thomas Bromberger (Technical University Munich, Germany), (5 x 10^5cells/sample) were pre-incubated with RPMI adhesion medium containing rat anti-mouse CD16/CD32 (Fc-block, BD Biosciences, USA; 1.25 µg/mL) for 10 min at room temperature. Afterwards conformation-specific anti-human CD11a/CD18 (clone m24, AF-488-labelled, Biolegend, USA, 1 µg/mL) or KIM127 antibodies were added, and cells were stimulated with 100 ng/mL rmCXCL1, 10 µM fMLP, 1 µg/mL PMA, 100 nM LTB_4, 10 mM EDTA or left untreated at 37 °C for 3 min. For KIM127 staining addition of an anti-mouse AF-488 (Invitrogen, USA, 6.6 µg/mL) secondary antibody was required. The percentage of Hoxb8 cells with KIM127 and mAb24 binding was calculated, by defining a threshold of the fluorescence intensity where 95% of Hoxb8 cells in the EDTA-treated control were considered as negative. Fluorescence intensity was measured using CytoFLEX cytometer (Beckman Coulter, USA) and data were analysed using FlowJo v10 software.

4.14 Phagocytosis Assay

To analyse phagocytosis Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells were differentiated for 4 d. Beforehand the Texas Red™ labelled *E.coli* BioParticles® were prepared at 1 mg/mL in $H_2O_{dest,}$ mixed and sonicated for 5 min. Afterwards the particles were opsonized with mouse serum for 1 h at 37 °C with 500 rpm shaking and washed with Life cell imaging solution (LCISol). Cells were washed with LCISol and transferred into an ice-cold 96-well plate (1 x 10^5 cells/well). 25 µL of Bioparticles® solution was added and cells were incubated for 15 min at 4 °C (as control) and 37 °C, respectively. After the incubation time, the reaction was immediately stopped by adding ice-cold PBS to the samples followed by centrifugation for 5 min at 300 g and 4 °C. Cells were kept on ice and two washing steps were conducted with LCISol. Subsequently cells were stained with SYTOX™ Red dead cell stain (0.1% in 1% FCS) and immediately analysed with a LSRFortessa flow cytometer. Right before acquisition quenching technique was applied, by adding 5 µL of 5% trypan blue solution to the sample in order to discriminate between ingested and adherent particles.

4.15 Intravital microscopy of TNF-α-stimulated mouse cremaster muscle venules

In order to investigate PMN recruitment *in vivo*, intravital microscopy of mouse cremaster muscle venules was performed as described previously[119]. 2.5 h after intrascrotal injection of 500 ng rmTNF-α, mice were anaesthetized and the cremaster muscle was exteriorized and moisturized with PBS (37 °C). Postcapillary venules of a range from 20–40 µm in diameter and a mean blood flow between 2000–3000 µm/s, were recorded using an Olympus BX51WI microscope with a 40 x/0.80 NA water immersion objective and a CCD camera (model CF8/1, Kappa). Rheological parameters and microvascular parameters such as venular diameter and venular vessel segment length as well as number of rolling and adherent PMNs per mm, and mean rolling velocities were calculated from the recorded movies using Fiji software. PMNs that remained attached to the vessel wall at the same spot for more than 30 s were defined as adherent cells. Systemic leukocyte counts were analysed from whole blood samples with ProCyte Dx haematology analyser.

4.16 Histological analysis of TNF-α-stimulated mouse cremaster whole mounts

For quantification of perivascular leukocytes, dissected cremaster muscles were fixed with 4% PFA and stained with Giemsa's azur eosin methylene blue as described before[118]. The number of perivascular PMNs and other leukocyte subtypes of at least 5 venules of each cremaster muscle whole mount was counted. Analysis was performed using a Zeiss Axioscop 40 microscope equipped with a Zeiss 100 x/1.25 NA oil immersion objective.

4.17 Peritonitis model of acute inflammation

Themis2$^{+/+}$ and Themis2$^{-/-}$ mice were injected intraperitoneally (i.p.) with TNF-α (500 ng/animal) or CXCL1 (600 ng/animal). The mice were sacrificed 2 h, respectively 4 h after injection and the peritoneal cavity was flushed with 5 mL of ice-cooled PBS. The total number of extravasated PMNs in the peritoneal lavage was determined using ProCyte Dx haematology analyser or quantified via flow cytometry with a PE-labelled rat anti-mouse Ly6-G antibody.

4.18 Statistical analysis

All data were analyzed and plotted using GraphPad Prism 9 software (GraphPad Software Inc.) and shown as means ± SEM. Statistical significance for pairwise comparison of experimental groups was determined using an unpaired Student's t test. For multiple comparisons, a 2-way ANOVA with Sidak's multiple comparisons test (comparison of all experimental groups against each other) or Bonferroni's multiple comparisons test (to compare two mean values at each row) was used. P values < 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***) or ≤ 0.0001 (****) were considered as statistically significant.

5. RESULTS

5.1 Themis2 in myeloid cells

5.1.1 Expression and localization of Themis2

First, we investigated whether Themis2 is expressed in human and murine PMNs at both mRNA and protein levels. By PCR analysis, Themis2 was shown to be expressed in murine Hoxb8 cells and Hoxb8 cell-derived PMNs (dHoxb8 cells) as well as in primary mPMNs. Furthermore, Themis2 was expressed in the neutrophil-like human promyelocytic leukemia cell line HL-60, and in dHL-60 as well as in freshly isolated hPMNs (Figure 7A).

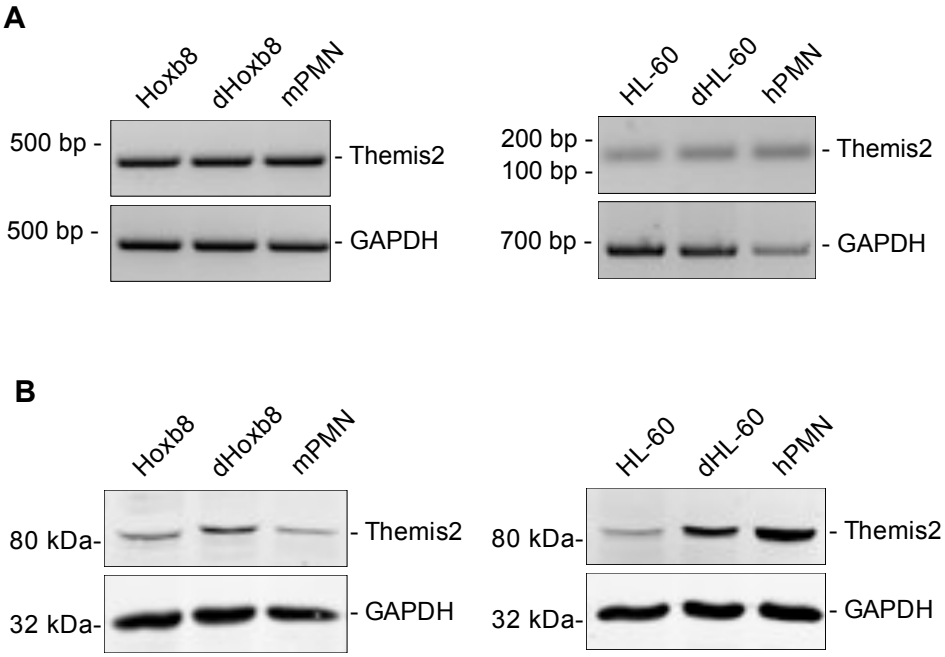


Figure 7. Expression of Themis2 in murine and human myeloid cells.
Representative gel electrophoresis of PCR products (A) and of western blots of protein lysates (B) showing Themis2 expression in murine and human myeloid cells at the mRNA and protein level. GAPDH served as loading control. n =3.

Western blot analyses also showed Themis2 protein expression in these cell lines (Figure 7b). These results clearly confirmed our group´s earlier findings from mass spectrometry data and demonstrated Themis2 expression in murine and human neutrophils at the mRNA and on protein levels.

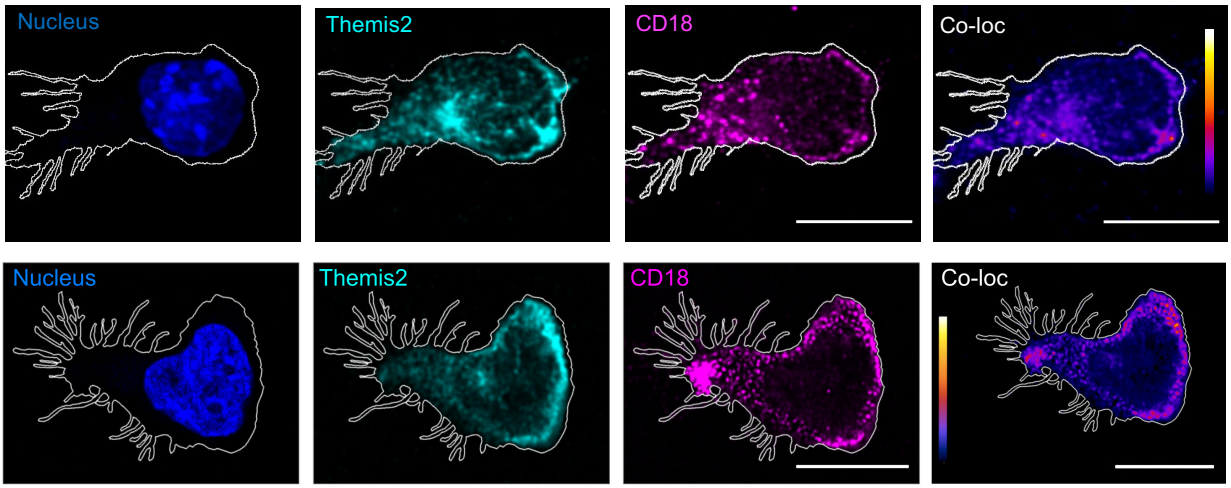


Figure 8. Localization of Themis2 and CD18 in CXCL1-stimulated mPMNs and fMLP-stimulated dHL-60 cells.
CXCL1-induced adhesion of mPMNs to rmICAM-1 (upper panel) and fMLP-induced adhesion of dHL-60 cells to hFibrinogen (lower panel). Nucleus (blue), Themis2 (cyan), CD18 (magenta). Scale bar 10 µM. n = 3 for mPMNs and dHL-60 cells. Co-loc colocalization.

As Themis2 was identified in an interactor screen with the β_2 integrin cytoplasmic domain, we investigated the cellular localization of both proteins using confocal super-resolution microscopy (CSRM) to show their potential co-localization in PMNs (Figure 8). Here, Themis2 co-localized with CD18 during β_2 integrin-mediated adhesion on immobilized fibrinogen of fMLP-stimulated dHL-60 cells (lower panel) as well as in CXCL1-stimulated mPMNs on immobilized rmICAM-1 (upper panel). The co-localization was mainly found in the cell front and less in the uropod of the cells. This finding further supports the hypothesis of an interaction between Themis2 and CD18.

5.1.2 Characterization of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice

In order to analyze Themis2 function in PMN trafficking and β_2 integrin function *in vivo,* we received sperm of Themis2 knockout mice from the laboratory of Victor Tybulewicz (Imperial College London). Cryopreserved sperm of Themis2$^{-/-}$ mice were used for the *in vitro* fertilization of C57BL/6J oocytes, which were then transferred into recipient foster mothers by the Core facility for Animal Models (CAM) of the Biomedical Center (BMC). In order to obtain littermate controls heterozygous mice were mated. Themis2 has a protein size of 663 amino acids and a molecular weight of 74 kDa in the murine system. The Themis2$^{+/+}$ locus was amplified with a fwd primer binding in exon 4 and a rev primer binding in intron 4, generating a PCR product of 807 bp.

Themis2$^{-/-}$ locus, missing exon 4, was amplified with the same rev primer and a fwd primer binding to an inserted flippase recognition target site generating a PCR product of 364 bp (Figure 9).

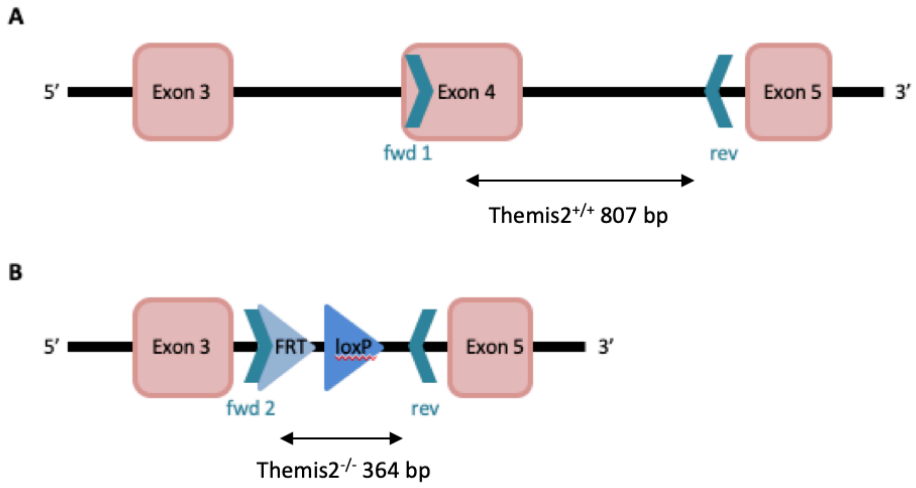


Figure 9. Schematic representation of the genotyping strategy.
Red boxes represent exon 3, 4 and 5 of the Themis2 gene and the blue triangles indicate the position of the remaining FRT and loxP sites. The green arrows indicate the positions of the primers used for genotyping Themis2$^{+/+}$ and Themis2$^{-/-}$ mice. The expected lengths of the PCR products are indicated below.

Thus, in the Themis2$^{-/-}$ mice the largest of six exons of the Themis2 gene, exon 4 is deleted. Exon 4 has a size of 1,088 bp and encodes for the C-terminus of the first CABIT domain and the complete second CABIT domain as well as for the NLS and PRR. The deletion of exon 4 leads to a frameshift between the exons 3 and 5[110]. DNA was isolated from mouse tail biopsies and genotypes were determined by genomic PCR analyses (Figure 10).

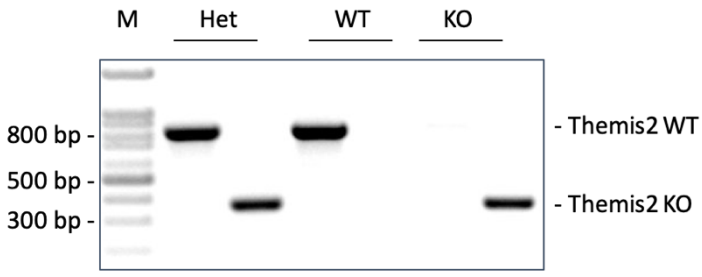


Figure 10. Genotyping PCR of the Themis2$^{+/+}$ and Themis2$^{-/-}$ locus.
Representative gel image shows the PCR products of Themis$^{+/+}$ (WT), Themis2$^{-/-}$ (KO) and heterozygous (HET) mice. M marker.

5.2 The role of Themis2 for PMN trafficking *in vitro*

5.2.1 mPMN rolling and adhesion under flow conditions

In order to decipher the role of Themis2 during β_2 integrin-mediated PMN trafficking, one of the first steps of the recruitment cascade, the induction of adhesion under physiological flow conditions, was analysed. Therefore, PMNs from the bone marrow of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice were isolated and perfused through flow chambers coated with CXCL1, rmICAM-1 and rmP-selectin, at a shear stress of 1 dyne/cm^2. This experiment revealed that the number of rolling PMNs was not altered between Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs. In both Themis2$^{+/+}$ and Themis2$^{-/-}$ PMNs the number of rolling cells increased over time with the highest numbers of rolling cells measured after 7 and 9 min (Figure 11a).

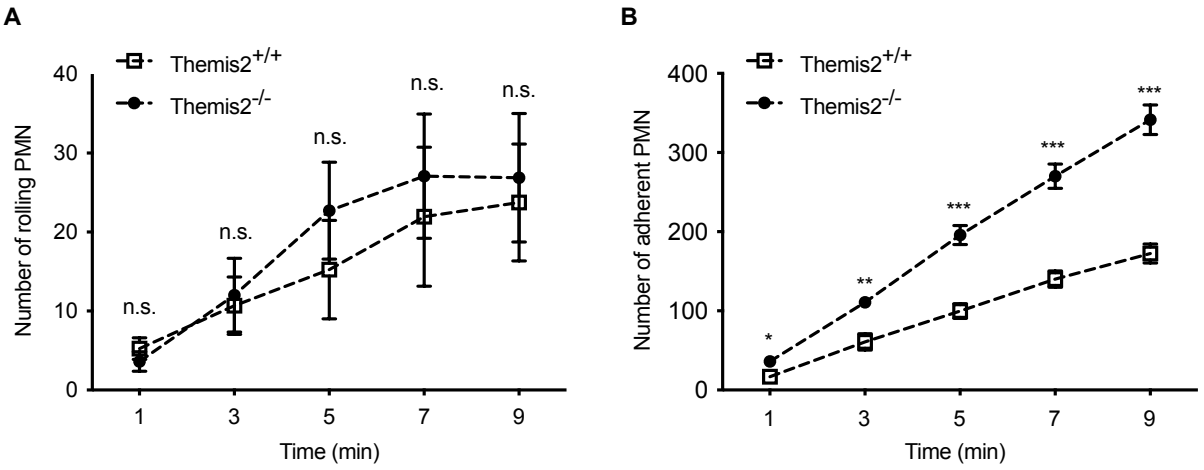


Figure 11. Rolling and adhesion of Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs under flow conditions. Number of (A) rolling and (B) adherent Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs under flow conditions (1 dyne/cm^2) on rmP-selectin (10 µg/mL), rmICAM-1 (12.5 µg/mL) and CXCL1 (5 µg/mL) at the indicated time points. n = 4. Mean ± SEM. * $p < 0.05$, ** $p \leq 0.01$, *** $p < 0.001$, n.s. not significant.

Strikingly, the number of adherent PMNs was significantly increased in the absence of Themis2. This effect was already measurable after 1 min and dramatically increased with time. After 9 minutes the number of adherent Themis2$^{-/-}$ PMNs was increased to 341 ± 19, compared to 172 ± 12 of Themis2$^{+/+}$ PMNs (Figure 11B). This effect was already observed at earlier time points of 1, 3, 5 and 7 minutes with 36 ± 5, 111 ± 4, 196 ± 12 and 270 ± 15 adherent cells in the Themis2$^{-/-}$ PMNs compared to 16 ± 6, 61 ± 10, 100 ± 9 and 140 ± 10 adherent cells in Themis2$^{+/+}$ PMNs. Taken

together, these results suggest that although Themis2 has no significant effect on the number of rolling PMNs, it has a negative impact on PMN adhesion under flow.

5.2.2 Adhesion strengthening of mPMNs under flow conditions

In order to investigate whether the absence of Themis2 affects also the next steps of the leukocyte recruitment cascade, adhesion strengthening was analysed. An effective activation of β_2 integrins leading to a conformational change of the integrin´s extracellular domains into the extended, high affinity conformation is a prerequisite for PMNs to resist shear stress[120,121]. However, in particular the formation of an adhesion complex and a connection with the cytoskeleton is essential for shear stress resistance of PMNs. To analyse the impact of Themis2 on β_2 integrin activation and furthermore on the linkage of the ligand-bound β_2 integrins to the cytoskeleton, isolated mPMNs of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice were allowed to adhere on the previously coated chambers (rmICAM-1 and rmCXCL1), followed by a subsequent gradual increase in shear stress from 0.5 to 8.0 dyne/cm^2 within the flow chambers. The percentage of adherent cells in relation to the number of cells at the start point, which was set after allowing the cells for 10 min to adhere, was calculated for both Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs (Figure 12).

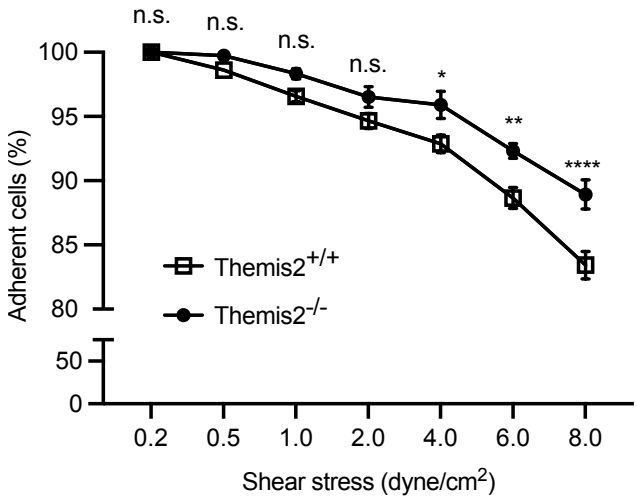


Figure 12. Adhesion strengthening of Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs.
Percentage of adherent mPMNs, relative to the amount of adherent mPMNs before the onset of flow, at indicated shear stress rates. Flow chambers were coated with rmICAM-1 øFc (3 µg/mL), rmCXCL1 (5 µg/mL) and P-selectin (10 µg/mL). n = 4. Mean ± SEM. * $p < 0.05$, ** $p \leq 0.01$, **** $p < 0.0001$, n.s. not significant.

Up to 2 dyne/cm^2, both mutant and control mPMNs showed equal resistance to shear stress, but with increasing shear stress from 4 dyne/cm^2 onwards, mPMNs lacking Themis2 exhibited significantly more adhesion to the surface. At 8 dyne/cm^2 89.0 ± 1.1% of Themis2$^{-/-}$ mPMNs were resistant to the shear stress, compared to 83.4 ± 1.1% of Themis2$^{+/+}$ mPMNs. These results confirmed the enhanced adhesion in Themis2$^{-/-}$ mPMNs and suggests that a more stable adhesion complex may be formed in the absence of Themis2.

5.2.3 Crawling of mPMNs under flow conditions

Next, the crawling behaviour of mPMNs of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice was analysed in order to test whether loss of Themis2 affects PMN migration. Cells were allowed to adhere for 10 min on coated flow chambers (rmP-selectin, rmICAM-1, rmCXCL1) before a constant flow of 1 dyne/cm^2 was applied. Migration of control and Themis2 knockout PMNs were then recorded by time-lapse videos microscopy (Figure 13). The quantification of Euclidian distance, crawling velocity as well as percentage of crawling cells showed no significant difference between wildtype and mutant cells. In summary these results indicate, that Themis2 plays no essential role in PMN crawling and is dispensable for this step of PMN recruitment.

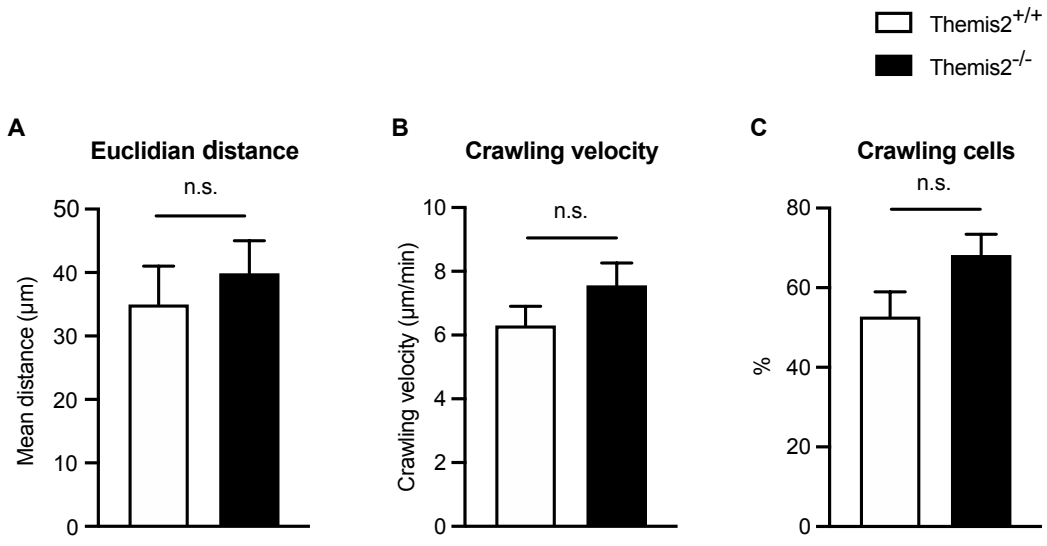


Figure 13. Migration of Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs under flow conditions.
Mechanotactic migration of Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs under flow conditions (1 dyne/cm^2) using flow chambers coated with ICAM-1 øFc (3 µg/mL) and rmCXCL1 (5 µg/mL). Quantitative analysis of (A) mean Euclidian distance, (B) mean crawling velocity and (C) percentage of crawling cells. n = 4. Mean ± SEM. n.s. not significant.

5.2.4 LFA-1-specific binding of ICAM-1 to mPMNs

Since PMN adhesion requires a change in the integrin´s ectodomain towards a high affinity state, I wondered whether the increased adhesion of Themis2 deficient PMNs is caused by altered LFA-1 (CD11a/CD18) affinity regulation. In order to investigate this, a LFA-1-specific ICAM-1 binding assay in the presence of a function-blocking Mac-1 (CD11b/CD18) antibody was performed. The binding of soluble ICAM-1 by Themis2$^{+/+}$ and Themis2$^{-/-}$ PMNs was analysed in non-stimulated cells and upon stimulation with CXCL1, fMLP, and Mn^{2+} by flow cytometry (Figure 14). The threshold for LFA-1-specific rmICAM-1/Fc binding was set by the anti-CD11a antibody-treated control defining 95% of mPMNs as negative for LFA-1 binding to rmICAM-1/Fc.

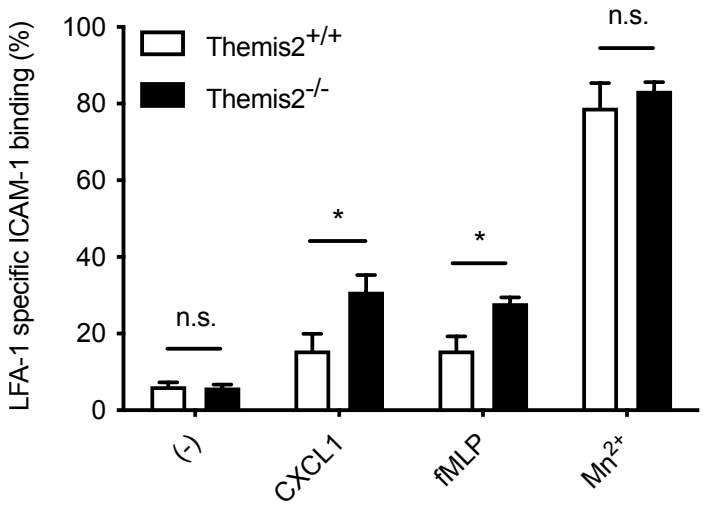


Figure 14. LFA-1-specific binding of ICAM-1 in Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs.
Flow cytometric analysis of LFA-1-specific ICAM-1 binding in mPMNs of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice upon stimulation for 3min with CXCL1 (100 ng/mL), fMLP (10 µM) or Mn^{2+} (3 mM) or without stimulation (-). Data show the percentage of cells with LFA-1-specific rmICAM-1/Fc binding. Threshold was set by the anti-CD11a antibody-treated control defining 95% of PMNs as negative for LFA-1 binding to rmICAM-1/Fc. n = 4. Mean ± SEM. * $p < 0.05$, n.s. not significant.

As expected, ICAM-1 binding to mPMNs was increased upon stimulation compared to unstimulated controls. However, LFA-1 activation was significantly increased upon CXCL1 and fMLP stimulation in mPMNs lacking Themis2 compared to Themis2$^{+/+}$ mPMNs: The percentage of Themis2$^{-/-}$ mPMNs with LFA-1-specific rmICAM-1/Fc binding was 31.0 ± 4.4% upon CXCL1 and 28.0 ± 1.5% upon fMLP-stimulation compared to 15.6 ± 4.3% and 15.6 ± 3.6% of Themis2$^{+/+}$ mPMNs, respectively. In contrast, after stimulation with $Mn^{2+,}$ which stabilises the high-affinity conformation of integrins independent of intracellular signals, there was no significant difference in ICAM-1 binding between wild-type and Themis knockout mPMNs.

These data suggested, that Themis2 is involved in the intracellular signalling events leading to LFA-1 activation. Thus, Themis2 might act as negative regulator during integrin inside-out signalling. The fact that this effect was not present upon stimulation with Mn^{2+} suggested, that the β_2 integrin itself is not defective in the absence of Themis2 and can still be normally activated.

5.3 Role of Themis2 for β_2 integrin affinity regulation in the Hoxb8 cell system

To decipher the role of Themis2 for β_2 integrin affinity regulation, we generated Hoxb8 cells from the bone marrow of Themis2$^{-/-}$ and Themis2$^{+/+}$ mice. The Hoxb8 cells were generated in our lab by Annette Zehrer. Hoxb8 cells are immortalized hematopoietic progenitor cells that can be differentiated in vitro into various myeloid cell types, such as PMNs, macrophages and DCs. Importantly, these cells functionally resemble their primary counterparts. The Hoxb8 cell system was first described by the group of Hans Häcker[122,123]. This cell system depends on a virally expressed estrogen-dependent variant of the transcription factor Hoxb8 (ER-Hoxb). Upon binding of estrogen, the ER-Hoxb8 fusion protein translocates into the nucleus leading to transcription of proteins, which are crucial to keep the cells in the progenitor state and prevent differentiation. Upon removal of estrogen, the differentiation of the immortalized progenitor cells into PMNs or other myeloid cell types can be directed by adding certain cytokines to the culture medium.

5.3.1 Characterization of Themis2$^{+/+}$ and Themis2$^{-/-}$ Hoxb8 cells

To examine, whether the absence of Themis2 affects proper Hoxb8-SCF cell differentiation or integrin expression, Themis2$^{+/+}$ and Themis2$^{-/-}$ Hoxb8 cells were analysed on d0 and d4 of differentiation by flow cytometry for specific cell surface markers. The expression of different progenitor and mature neutrophil markers as well as integrins were analysed to assess a differentiation profile (Figure 15).

The progenitor cell markers CD34 and c-Kit were found to be expressed in both cell lines at d0 and were strongly downregulated during the differentiation indicating proper progenitor cell differentiation (Figure 15A). Gr-1, Ly6G and CXCR2 were strongly upregulated in both Themis2$^{+/+}$ and Themis$^{-/-}$ dHoxb8 cells, indicating proper differentiation into mature neutrophils (Figure 15B).

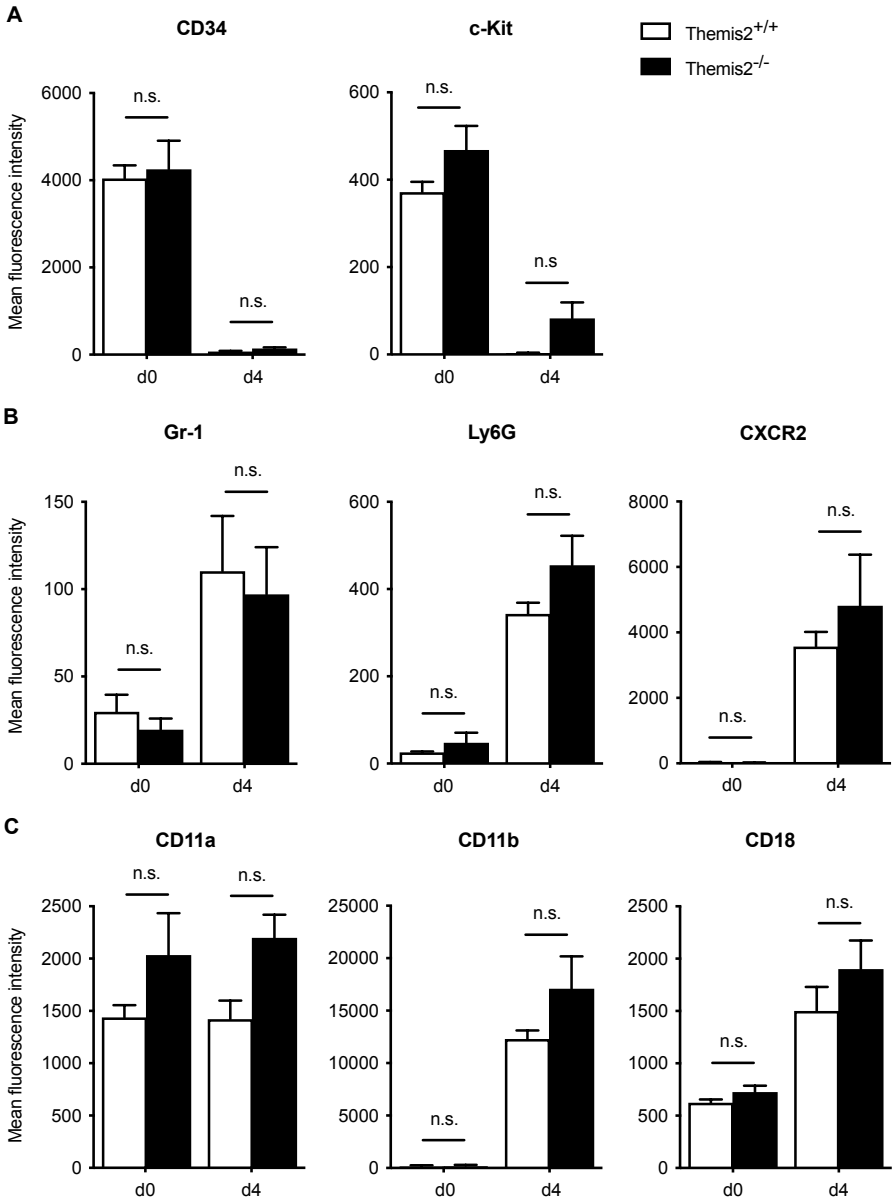


Figure 15. Differentiation profile of bone-marrow-derived Themis2$^{+/+}$ and Themis2$^{-/-}$ Hoxb8-SCF cells.

Hoxb8SCF cells from bone-marrow of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice were analysed on d0 and d4 by flow cytometry after incubation with fluorescence-labelled antibodies, specific for several differentiation markers: (A) progenitor markers CD34 and c-Kit, (B) differentiation markers Gr-1, Ly6G and CXCR2 for mature PMNs and (C) integrin subunits CD11a, CD11b and CD18. Mean fluorescence activity is shown upon correction with isotype control. n ≥ 4. Mean ± SEM. n.s. not significant.

The expression of the integrin subunits CD11a, CD11b and CD18 were also not significantly altered in Themis2$^{+/+}$ Hoxb8 cells compared to Themis2$^{-/-}$ Hoxb8 cells at both d0 and d4 (Figure 15C). Together, these data demonstrated, that Themis2$^{-/-}$ Hoxb8 cells and Themis2$^{-/-}$ dHoxb8 cells show similar phenotypical characteristics regarding adhesion molecule, differentiation marker and

CXCR2 expression as Themis2$^{+/+}$ dHoxb8 cells, thereby pointing towards proper differentiation of Themis2$^{-/-}$ dHoxb8 cells.

5.3.2 Adhesion under flow and phagocytic potential of Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells

As in vitro differentiated neutrophils from Hoxb8 cells functionally resemble their primary counterparts, we wanted to use this cell system to confirm our previous observation that loss of Themis2 promotes neutrophil adhesion. To this end, Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells were perfused through flow chambers coated with CXCL1, rmICAM-1 and rmP-selectin, at a shear stress of 1 dyne/cm^2. As shown in figure 15A, also Themis2-deficient dHoxb8 cells showed increased adhesion similar to isolated mPMNs from Themis$^{-/-}$ mice (Figure 16A).

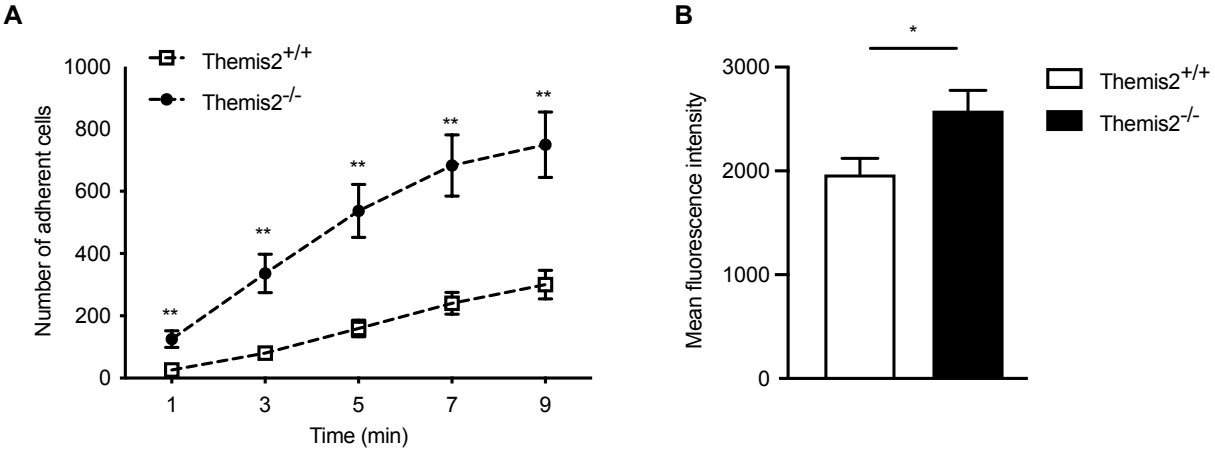


Figure 16. Adhesion under flow conditions and phagocytic potential of Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells.
(A) Number of adherent Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells to flow chambers coated with rmP-selectin (10 µg/mL), rmICAM-1 (12.5 µg/mL) and CXCL1 (5 µg/mL) at a shear stress of 1 dyne/cm^2 at the indicated time points. n = 6. Mean ± SEM. * $p < 0.05$, ** $p \leq 0.01$, n.s. not significant. (B) Phagocytotic activity of Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells. Data represent mean ± SEM. n = 4. Mean ± SEM. * $p < 0.05$.

After 1 min already 126 ± 27 Themis2$^{-/-}$ dHoxb8 cells were adherent compared to 26 ± 8 Themis$^{+/+}$ dHoxb8 cells. The difference in cell adhesion between both genotypes became even more pronounced with time. After 9 min, 750 ± 105 Themis$^{-/-}$ dHoxb8 cells were adherent compared with 300 ± 46 Themis$^{+/+}$ dHoxb8 cells. In order to analyse whether other β_2 integrin dependent

functions of PMNs are affected by the absence of Themis2, a phagocytosis assay was conducted. To this end, mouse serum opsonized Texas Red™ labelled *E.coli* BioParticles® were incubated with Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells for 15 min at 37°C. Subsequently, the phagocytotic uptake of the fluorescent particles was measured via flow cytometry. Quantification of the MFI values revealed significantly higher phagocytic activity of Themis2$^{-/-}$ dHoxb8 cells compared to Themis2$^{+/+}$ dHoxb8 cells (Figure 16B). Themis2$^{-/-}$ dHoxb8 cells revealed a MFI of 2582 ± 194 whereas the MFI of Themis2$^{+/+}$ dHoxb8 cells was 1967 ± 155. These results indicate that Themis2 is not only involved in the regulation of β_2 integrin adhesion but also contributes to the regulation of phagocytosis of PMNs.

5.3.3 LFA-1-specific binding of ICAM-1 in Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells

In order to confirm, whether the effect of Themis2 on LFA-1 activation in primary PMNs can also be observed in dHoxb8 cells, an LFA-1-specific ICAM-1 binding assay in the presence of a function-blocking Mac-1 antibody was conducted in Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells. Similar to the experiment on primary PMNs, the binding of soluble ICAM-1 by the cells was analysed upon stimulation with CXCL1, fMLP, Mn^{2+} or without stimulation via flow cytometry (Figure 17). LFA-1-specific ICAM-1-binding was calculated relative to the anti-CD11a antibody-treated control, which defined 95% of PMNs as negative for ICAM-1-binding. Themis2$^{-/-}$ dHoxb8 cells bound significantly more ICAM-1 upon CXCL1 stimulation with 28.4 ± 3.5% ICAM-1 binding compared to 8.7 ± 1.0% ICAM-1-binding in Themis2$^{+/+}$ dHoxb8 cells. Moreover, ICAM-1 binding in response to fMLP stimulation was markedly higher with 49.0 ± 4.5% in Themis2 deficient cells compared to 27.2 ± 4.0% in Themis2$^{+/+}$ dHoxb8 cells. Consistent with the results in mPMNs, stimulation with Mn^{2+}, which bypasses inside-out signalling and stabilizes β_2 integrins in the high-affinity conformation, resulted in no significant difference in ICAM-1 binding between wild-type and knockout cells.

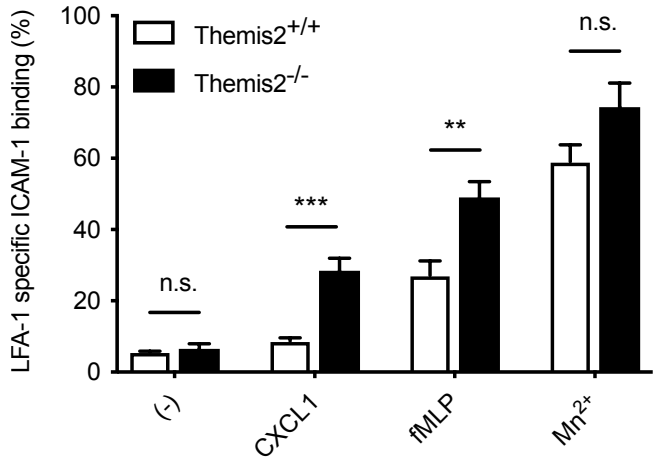


Figure 17. LFA-1-specific binding of ICAM-1 by Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells
Flow cytometric analysis of LFA-1-specific ICAM-1 binding in Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells upon stimulation for 3 min with CXCL1 (100 ng/mL), fMLP (10 µM) or Mn^{2+} (5 mM) or without stimulation (-). Data show the percentage of dHoxb8 cells with LFA-1-specific rmICAM-1/Fc binding. Threshold was set by the anti-CD11a antibody-treated control defining 95% of PMNs as negative for LFA-1 binding to rmICAM-1/Fc. n = 6. Mean ± SEM. ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. not significant.

In conclusion, these results underlined again, that Themis2 plays a role in signalling events that regulate LFA-1 activation and acts as negative regulator in β_2 integrin affinity regulation of PMNs. Again, ICAM-1 binding was comparable between Themis2$^{+/+}$ and Themis2$^{-/-}$ cells upon Mn^{2+} treatment, indicating that an integrin conformational change can normally occur in the absence of Themis2.

Because the results were consistent with our previous findings in mPMNs, they not only underscored the presumed role of Themis2 as a negative regulator of β_2 integrins, but also demonstrated that dHoxb8 cells behave in a functionally similar manner to mPMNs, thereby providing a valuable model system to study the role of Themis2 in PMNs.

5.4 The role of Themis2 in intracellular signaling and PMN function

In order to delineate, whether loss of Themis2 affects the change in integrin conformation towards a high affinity state resulting in increased adhesion, reporter antibodies were used. For human CD18 the reporter antibodies KIM127 and mAb24 are available. KIM127 (which was a generous gift of Markus Sperandio, BMC) binds to the extended conformation (E^+) of integrins. mAb24 binds to an epitope of the open headpiece (H^+) of integrins, indicating the high affinity LFA-1 conformation. Using these tools, it is possible to investigate differences in the integrin

conformation during activation. In order to use those antibodies a cooperation with Markus Moser and Thomas Bromberger was initiated. They knocked-out CD18 in the Themis$^{+/+}$ and Themis2$^{-/-}$ Hoxb8 cells using the CRISPR/Cas technology and re-expressed human CD18 with a retroviral vector[124].

5.4.1 Characterization of humanised CD18 Themis2$^{+/+}$ and Themis2$^{-/-}$ Hoxb8 cells

First, we tested whether simultaneous deletion of murine β_2 integrin and re-expression of human β_2 integrin affected differentiation of the Hoxb8 cells. To check this, hCD18 Themis2$^{+/+}$ and hCD18 Themis2$^{-/-}$ Hoxb8 cells on days d0 and d4 of differentiation were analysed by flow cytometry for specific cell surface markers.

Markers for progenitor and mature PMNs as well as integrin expression were analysed like in 5.3.1 to assess the differentiation profile. CD34 and c-Kit, expressed on myeloid progenitors, were found to be expressed in both cell lines at d0 and were strongly downregulated at d4 of differentiation (Figure 18A). Gr-1, Ly6G and CXCR2 were strongly upregulated in hCD18 Themis2$^{+/+}$ and hCD18 Themis$^{-/-}$ dHoxb8 cells, indicating proper differentiation to mature neutrophils (Figure 18B). The integrin subunits CD11a, CD11b and hCD18 were also equally expressed in hCD18 Themis2$^{+/+}$ and hCD18 Themis2$^{-/-}$ Hoxb8 cells at both d0 and d4 (Figure 18C). Taken together, these data showed that the deletion of the endogenous murine CD18 gene and expression of hCD18 in Themis2$^{+/+}$ and Themis2$^{-/-}$ Hoxb8 cells did not alter their differentiation into PMN-like cells as indicated by normal surface expression of adhesion molecules, differentiation markers and CXCR2 at d0 and d4.

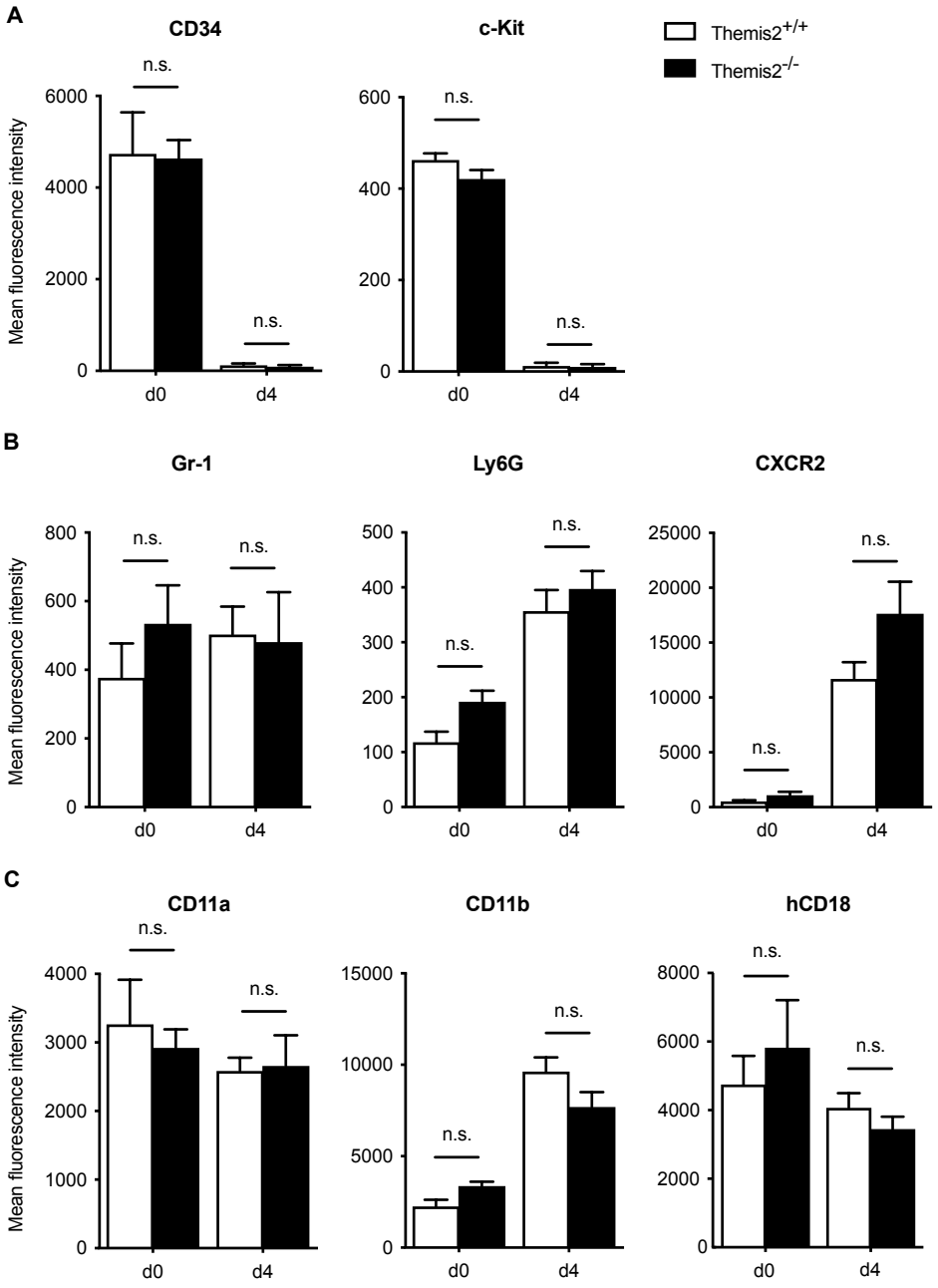


Figure 18. Differentiation profile of hCD18 Themis2$^{+/+}$ and hCD18 Themis2$^{-/-}$ Hoxb8 cells.
Hoxb8 SCF cells from bone-marrow of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice expressing human CD18 were analyzed on d0 and d4 by flow cytometry, after incubation with fluorescence-labelled antibodies, specific for several differentiation markers. (A) CD34 and c-Kit, (B) Gr-1, Ly6G and CXCR2 and (C) CD11a, CD11b and hCD18. Mean fluorescence activity was corrected by isotype controls. n = 3. Mean ± SEM. n.s. not significant.

5.4.2 Regulation of β_2 integrin affinity

To investigate whether loss of Themis2 expression affects the change in β_2 integrin conformation, which might be the cause for the increased adhesion, reporter assays with KIM127 and mAb24 antibodies on hCD18 Themis2$^{+/+}$ and hCD18 Themis2$^{-/-}$ dHoxb8 cells were conducted (Figure 19). To ensure that potential effects were not due to differences in CD18 expression between the cells, CD18 expression was also analysed in each assay (Figure 19a). Here, both cell lines showed equal expression of the β_2 integrin subunit. After cells were incubated with CD16/CD32 antibody solution

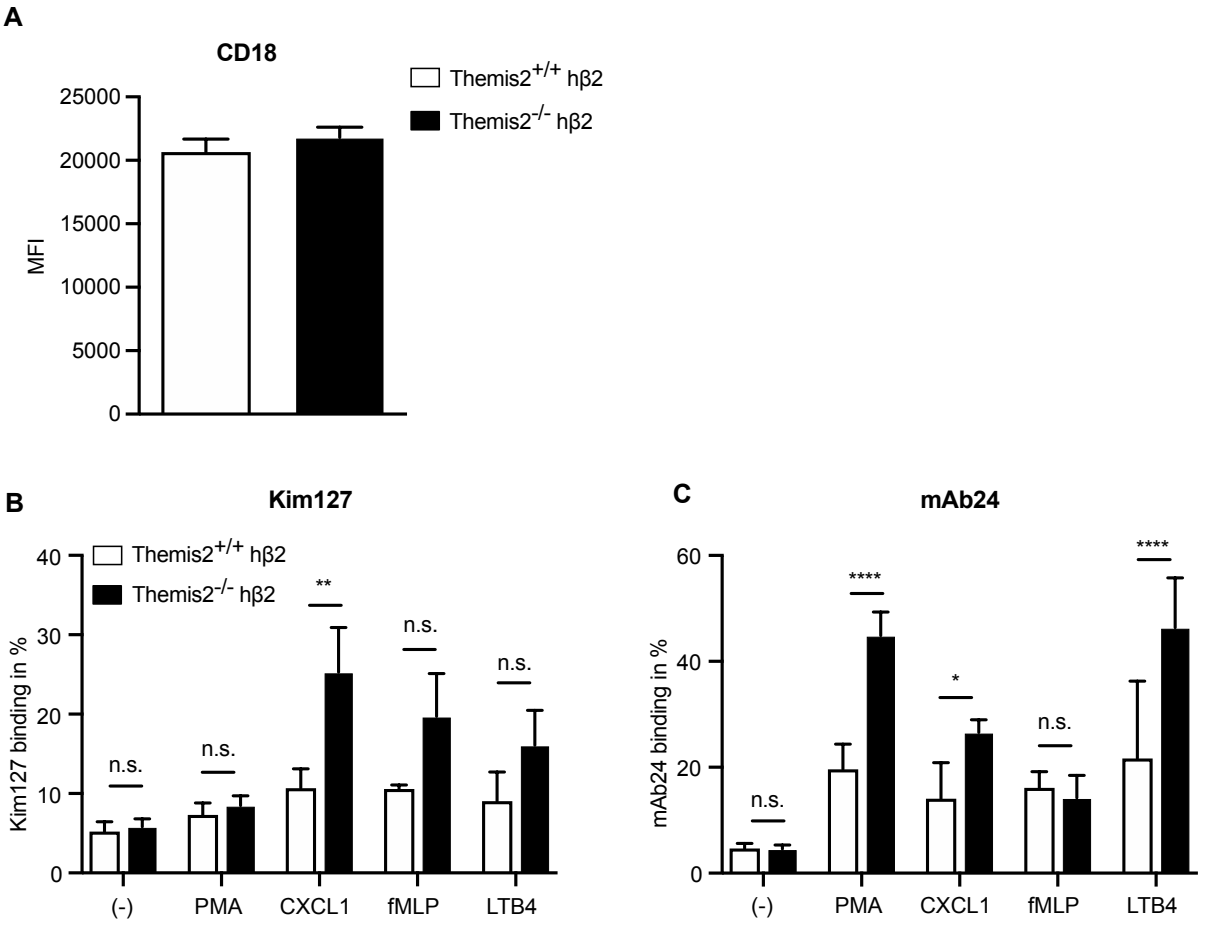


Figure 19. Regulation of β_2 integrin affinity in humanised CD18 Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells.

Flow cytometric analysis of humanised CD18 Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells of (A) CD18 expression and (B) KIM127 and (C) mAb24 staining upon 3 min stimulation with fMLP (10 µM), CXCL1 (100 ng/mL), PMA (1 µg/mL), LTB_4 (100 nM) or without stimulation. n ≥ 3. Mean ± SEM. * $p < 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$, n.s. not significant (Two-way ANOVA followed by Bonferroni's multiple comparison test).

in order to block non-specific Fc-mediated reactions, the conformation-specific antibodies were added and cells were stimulated for 3 min with CXCL1, fMLP, PMA, LTB_4, EDTA (for negative control), or left untreated. The binding of KIM127 was significantly increased in hCD18 Themis2$^{-/-}$ dHoxb8 cells upon stimulation with CXCL1 to 24.2 ± 4.2% of the cells being positive for KIM127 binding in comparison to 12.8 ± 2.8% in hCD18 Themis2$^{+/+}$ dHoxb8 cells (Figure 19b). Upon stimulation with fMLP and LTB_4 KIM127 binding also tend to be increased in the genetic absence of Themis2, however not to a significant extent. mAb24 binding, indicating that LFA-1 is in the high affinity conformation, was massively increased in hCD18 Themis2$^{-/-}$ dHoxb8 cells after stimulation with PMA (Figure 19c). Here, 55.0 ± 6.0% of the cells bound to mAb24, whereas in hCD18 Themis2$^{+/+}$ dHoxb8 cells only 19.2 ± 2.1% were positive for mAb24 binding. After stimulation with LTB_4, 47.9 ± 6.7% of Themis2 knockout cells were positive for mAb24 binding, whereas this was only the case for 19.4 ± 4.1% of the wild-type cells. The same effect was seen upon stimulation with CXCL1. In conclusion, these data indicated that upon activation more PMNs shift their β_2 integrins towards an active conformation in the absence of Themis2.

5.4.3 Rolling velocity of Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs

To investigate whether other steps of the leukocyte recruitment cascade are affected by loss of Themis2 expression in addition to cell adhesion, PMN rolling was examined. In addition to selectin-mediated PMN rolling, intermediate affinity β_2 integrins also contribute to slow rolling of PMN. To this end, flow chambers were coated with E-selectin and rolling velocity of the perfused mPMNs was determined (Figure 20a). Here, no significant alterations in the rolling velocity between Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs were measured.

However, when flow chambers were coated with E-selectin and the β_2 integrin ligand ICAM-1, which allows slow rolling of leukocytes, rolling velocity of Themis2$^{-/-}$ mPMNs was significantly reduced (1.09 ± 0.16 µm/s) compared with Themis2$^{+/+}$ mPMNs (2.02 ± 0.16 µm/s) (Figure 20b).

While selectin-mediated rolling via E-selectin and PSGL-1 is not affected here, β_2 integrin-mediated slow rolling via interaction with ICAM-1, is altered. In conclusion, these data again suggests that Themis2 plays a role in integrin activation.

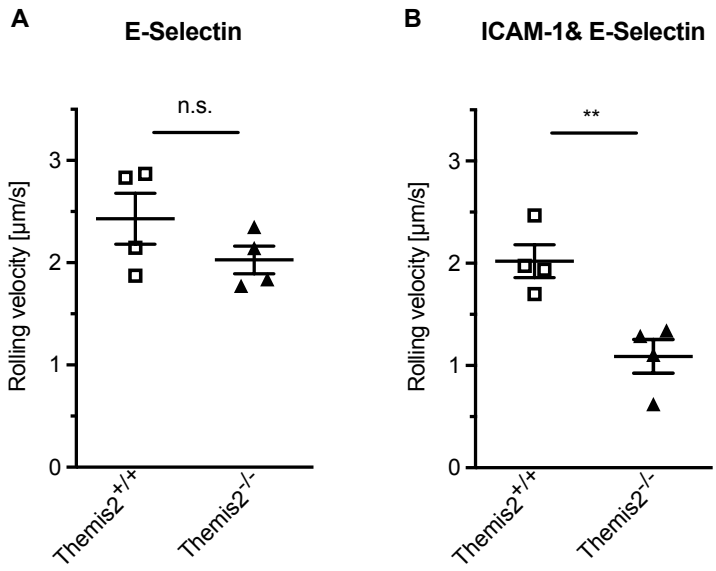
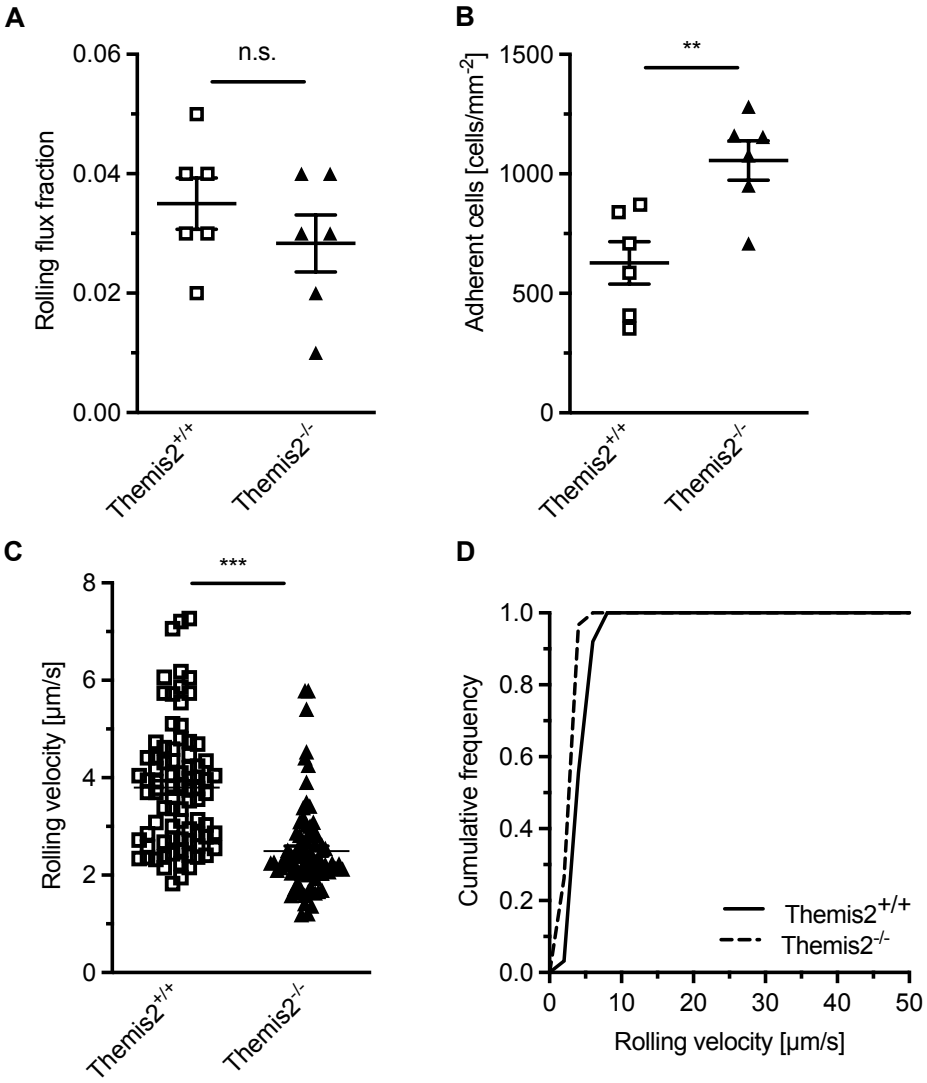


Figure 20. Rolling velocity of Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs.
Rolling velocity of isolated Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs under constant shear stress (1 dyne/cm^2) in flow chambers coated with (A) rmE-selectin (20 µg/mL) or (B) rmE-selectin (20 µg/mL) and rmICAM-1 øFc (15 µg/mL). n = 4 mice. Each data point represents mean ± SEM of three flow chambers per one mouse. ** $p \leq 0.01$, n.s. not significant (Unpaired Student's t-test).

5.5 The role of Themis2 for PMN trafficking *in vivo*

5.5.1 PMN extravasation in the TNF-α-inflamed mouse cremaster model

In order to analyse the role of Themis2 for PMN adhesion *in vivo* and to verify the *in vitro* data the TNF-α-induced inflammation model of the mouse cremaster muscle was applied. In this model, PMN rolling, adhesion and extravasation was analysed in venules of the exteriorized mouse cremaster muscle of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice via intravital microscopy. This was conducted in cooperation with the group of Markus Sperandio. An acute inflammation was induced by i.s. injection of TNF-α, which induces P- and E-selectin expression on the endothelium leading to slow leukocyte rolling and adhesion[45]. After 2.5 h Roland Immler prepared the mouse cremaster muscles. After quantification, strongly increased numbers of adherent leukocytes to the vascular wall of Themis2$^{-/-}$ mice were found (Figure 21b). The mean number of adherent cells was 1056.0 ± 82.3 cells compared to 627.9 ± 88.8 cells measured in venules of wild-type mice. In addition, we measured a significantly reduced rolling velocity of Themis2-deficient leukocytes (2.49 µm/s ± 0.1 in Themis2-knockout compared to 3.80 µm/s ± 0.14 in Themis2$^{+/+}$ mice) (Figure 21c–d). However, rolling flux fraction and rheological parameters where not altered in Themis2$^{-/-}$ mice (Figure 21e).



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	Mice	Venules	Diameter (µm)	Length (µm)	Centerline velocity (µm/s)	Shear rate (s^{-1})	WBC (μL^{-1})
Themis2$^{+/+}$	6	24	30±1	257±2	1583±136	1319±120	2627±296
Themis2$^{-/-}$	6	25	31±1	261±3	1564±123	1244±101	2908±300
			n.s.	n.s.	n.s.	n.s.	n.s.

Figure 21. Leukocyte recruitment in the TNF-α-induced mouse cremaster muscle model of acute inflammation.

Intravital microscopy of mouse cremaster muscle venules 2.5 h after i.s. injection of TNF-α (500 ng) (A) Number of adherent cells, (B) rolling flux fraction, (C) rolling velocity and (D) cumulative frequency distribution of rolling velocities. n = 24 venules from 6 Themis2$^{+/+}$ and n = 25 venules from 6 Themis2$^{-/-}$ mice. Mean ± SEM. (E) Rheological parameters of cremaster muscle venules 2.5 h after TNF-α injection. ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. not significant (Unpaired Student's t-test).

To analyse leukocyte recruitment from the circulation into the inflamed tissue, the number of extravasated PMNs in the TNF-α-inflamed cremaster tissue of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice was counted. After the cremaster muscles were exteriorized, they were fixed with 4% PFA and subsequently stained with Giemsa's azur eosin methylene blue. Afterwards the histological preparations were analysed by bright-field microscopy (Figure 22). The microscopic screening of the samples revealed significantly more extravasated PMNs in the perivascular space of the cremaster muscles of Themis2$^{-/-}$ mice compared to cremaster muscles of Themis2$^{+/+}$ mice (Figure 22a).

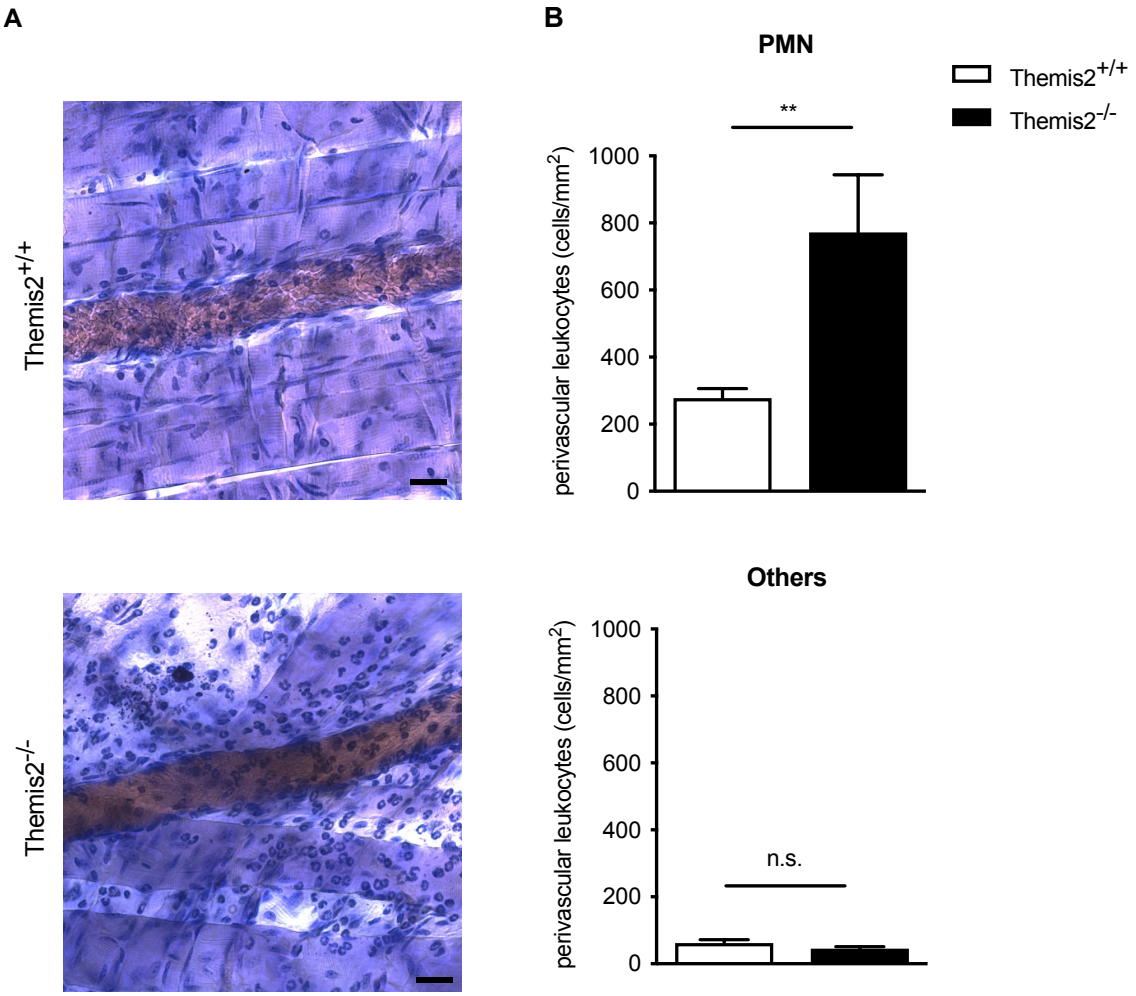

Figure 22. TNF-α-triggered extravasation of Themis2$^{+/+}$ and Themis2$^{-/-}$ PMNs into mouse cremaster tissue.

(A, B) Whole mounts of TNF-α-inflamed mouse cremaster muscles fixed with 4% PFA and stained with Giemsa's azur eosin methylene blue. (A) Representative images of stained postcapillary venules of cremaster muscle whole mounts of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice. Scale bar = 20 µm. (B) Quantification of perivascular PMNs and other leukocyte subtypes (labelled as others) n = 26 venules from 5 Themis2$^{+/+}$ mice, n = 31 from 5 Themis2$^{-/-}$ mice. Mean ± SEM. ** $p \leq 0.01$, n.s. not significant (Unpaired Student's t-test).

A quantification of the extravasated PMNs revealed that their numbers were strongly increased in the cremaster muscles of Themis$^{-/-}$ mice (Figure 22b at top). In the TNF-α-stimulated cremaster muscles of Themis2$^{-/-}$ mice 772.4 ± 171.5 extravasated perivascular PMNs were counted per field of view in comparison to 278.3 ± 28.1 PMNs in those of Themis2$^{+/+}$ mice. However, the number of other extravasated leukocyte subtypes (e.g. eosinophils, monocytes) showed no difference in the cremaster muscles of mice lacking Themis2 (Figure 22b below). These data indicate, that Themis2 not only regulates the rolling velocity and adhesion of PMNs, but it might also act as negative regulator of the extravasation of PMNs in the TNF-α-inflamed mouse cremaster model. Alternatively, the extravasation effect could be due to the increased number of adherent PMN, which subsequently extravasate into the inflamed tissue.

5.5.2 PMN extravasation into the inflamed peritoneum

In order to study the role of Themis2 for PMN extravasation, also another in vivo inflammatory model, an acute peritonitis, was used. After i.p. injection of CXCL1 or TNF-α in Themis2$^{+/+}$ and Themis2$^{-/-}$ mice, the number of extravasated PMNs in the inflamed peritoneum was determined. The peritoneal lavage showed no significant difference after CXCL1 injection between Themis2$^{+/+}$ and Themis2$^{-/-}$ mice neither after 2 h nor after 4 h (Figure 23a). In addition, 2 h after TNF-α-induced inflammation no significant difference in PMN extravasation was detected in the genetic absence of Themis2 compared to the wild-type situation (Figure 23b). Thus, the ability of Themis2$^{-/-}$ PMNs to extravasate into the peritoneal cavity was not altered compared to Themis2$^{+/+}$ after this time points. This finding was unexpected, as previous results (Figure 22) indicated increased extravasation in the Themis2$^{-/-}$ situation.

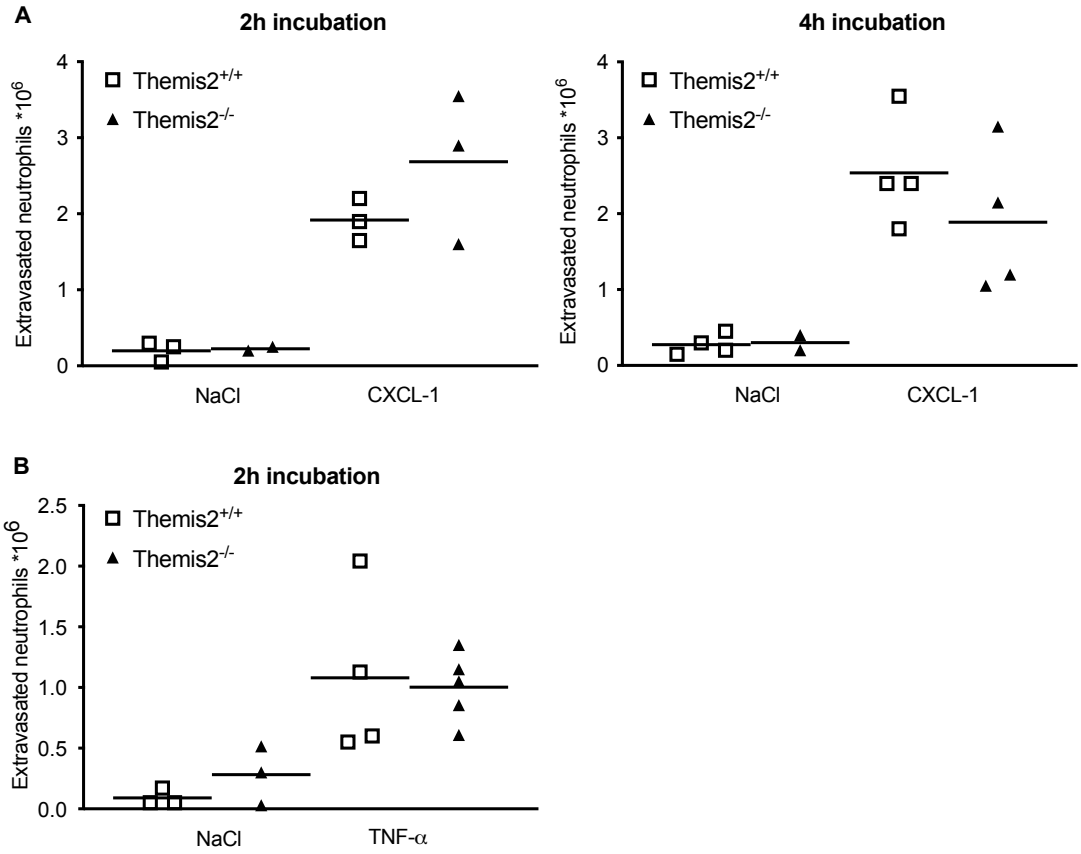

Figure 23. Number of extravasated PMNs in the inflamed peritoneal cavity of Themis2$^{+/+}$ and Themis2$^{-/-}$ mice.

Total number of extravasated Themis2$^{+/+}$ and Themis2$^{-/-}$ PMNs in the peritoneal lavage after i.p. injection of 0.9% NaCl or rmCXCL1 (600 ng) or rmTNF-α (2 µg/mouse). n = 3 (rmCXCL1, 2 h incubation), n = 4 (rmCXCL1, 4 h incubation), n ≥ 3 (TNF-α). Data represents mean with individual data points.

6. DISCUSSION

PMNs play a fundamental role in innate immunity and the acute inflammatory response by being the first leukocytes to arrive at the site of inflammation and defending the host against invading pathogens using various cellular effector functions. The recruitment of PMNs from the bloodstream into inflamed tissue is a tightly controlled multistep process, in which the β_2 integrins LFA-1 and Mac-1 are critically involved[39]. Although several binding partners of the β_2 integrin cytoplasmic domain are known, the mechanism of β_2 integrin regulation in PMNs is poorly understood. In the group of Prof. Walzog the protein Themis2 has been identified as a novel β_2 integrin interactor in hPMN lysates. In this experiment the recombinant cytoplasmic tail of CD18 and whole cell lysates of freshly isolated human primary PMNs were used. Via pull-down assay and subsequent mass spectrometry 59 putative interacting proteins were found. Along with well-known interactors, such as Talin-1 (Figure 4) the protein Themis2 was identified.

Previous studies showed Themis2 to be critically involved in BCR and TCR signaling, which functions similar to integrin signalling[1–3]. Both, the immunoreceptor and the integrin signaling pathways depend on SFKs and Syk tyrosine kinases[125,126]. Moreover, upstream integrin signaling in PMNs functions in an immunoreceptor-like manner via the ITAM-containing transmembrane adaptors Dap12 and FcRγ[127]. In B cells, Themis2 lowers the activation threshold by low-avidity antigens by modulating the intracellular response to BCR stimulation and gets tyrosine phosphorylated by SFKs[2]. Similar to that, Themis1 facilitates positive selection of thymocytes by enhancing TCR signalling[1]. In Themis1$^{-/-}$ mice transgenic expression of Themis2 can compensate for Themis1 and is phosphorylated by SFKs. In addition to that, Themis2 co-precipitates with Grb2 and Vav1 in B cells and in thymocytes of Themis1$^{-/-}$ mice with transgenic Themis2 expression. This suggests a multimolecular complex that comprises Themis2, Grb2, Vav1, and a SFK when immunoreceptors on thymocytes and B cells are activated to mediate further downstream signaling. Due to these facts, the current study investigated the role of Themis2 for PMN biology and trafficking as well as for integrin regulation.

6.1 Applied model systems

First, the results confirmed the expression of Themis2 in human and murine myeloid cells. Moreover, Themis2 protein expression increased during the differentiation of HL-60 cells and Hoxb8 cells to dHL-60 cells and PMN-like dHoxb8 cells (see Figure 7). This result is in line with

previous findings in the literature, which showed that Themis2 expression is up-regulated during development of human acute myeloid leukemia HL-60 cells and macrophages[88,109]. The finding of an interaction of Themis2 with the cytoplasmic tail of CD18 from the screening (see Figure 4) is further supported by the CSRM results. Here, Themis2 co-localized with CD18 during β_2 integrin-mediated adhesion in fMLP-stimulated dHL60 cells and CXCL1-stimulated mPMN (see Figure 8). Co-localization was predominantly detected in the cell front and only very slightly in the uropod of the cells.

Sperm of Themis2$^{-/-}$ mice were provided by V. Tybulewicz and his group that generated and described the Themis2-deficient mouse first[110]. In order to investigate Themis2 function in PMN trafficking, heterozygous Themis2$^{-/+}$ mice were bred to homozygosity. To ensure that the animals used were comparable in age, sex, and genetic background, only littermate controls were used in these studies[128].

In the Themis2$^{-/-}$ mice, exon 4, which is not only the largest exon in the Themis2 gene but more importantly encodes for the C-terminus of the first CABIT domain and the complete second CABIT domain as well as for the NLS and PRR, is deleted (see Figure 9). The deletion of exon 4 causes a frameshift in exon 5 and thus deletion of the C-terminus of the protein including Y660, the proposed SH2 domain-binding site[110]. The lack of the latter prevents tyrosine phosphorylation of Themis2 and thereby presumably an interaction with SFKs, such as Lyn[2,88]. PMNs express the SFKs Lyn, Hck and Fgr and lack or inhibition of these kinases leads to impeded spreading of PMNs and compromised functions such as respiratory burst and degranulation due to adhesion defects[126,129]. Hoxb8 cells generated from the bone marrow of Themis2$^{-/-}$ and Themis2$^{+/+}$ mice were used to study the role of Themis2 for β_2 integrin affinity regulation. Hoxb8 cells are immortalized hematopoietic progenitor cells that can be differentiated into various myeloid cell types in vitro and were initially described by the Hans Häcker group[122,123]. Functionally, these cells resemble their primary counterparts. This cell system is used to investigate myeloid cells *ex vivo* and is based on a virally expressed estrogen-dependent variant of the transcription factor Hoxb8 (ER-Hoxb8). Upon binding of estrogen, the ER-Hoxb8 fusion protein translocates into the nucleus and triggers the expression of proteins that maintain the cells in the progenitor state and prevent differentiation. Upon estrogen removal, differentiation of immortalized progenitor cells into PMNs or other myeloid cell types can be induced by adding specific cytokines to the culture medium.

6.2 The impact of Themis2 on PMN adhesion and β_2 integrin activation

To delineate the role of Themis2 during β_2 integrin-mediated PMN trafficking, one of the first steps of the recruitment cascade, the induction of adhesion under physiological flow conditions was analyzed. In order to comply with the physiological conditions in postcapillary venules, 1 dyne/cm^2 shear stress was applied and chambers were coated with CXCL1, rmP-selectin and rmICAM-1[130,131]. Time-lapse video microscopy revealed that the genetic absence of Themis2 strongly affects mPMN adhesion but does not alter the number of rolling cells (see Figure 11). Themis2$^{-/-}$ mPMNs showed a significantly increased adhesion under flow conditions compared to Themis2$^{+/+}$ mPMNs, which suggests that Themis2 negatively affects this step of mPMN trafficking. The fact that the number of rolling mPMNs is not altered indicates that Themis2 does not affect the initial tethering of mPMNs. This hypothesis is supported by the fact that this step of the cascade is predominantly selectin-mediated[42,132].

Next, adhesion strengthening was examined. Full activation of β_2 integrins with extended conformation and high ligand affinity is prerequisite for shear stress resistance of PMNs but moreover adhesion complex formation, connection to the actin cytoskeleton as well as integrin clustering play a role, since adhesion strengthening is one of the postadhesion functions of PMNs[57,115,120,121,133] Therefore, an increasing shear stress of 0.5–8.0 dyne/cm^2 was applied. Themis2$^{-/-}$ mPMNs were significantly more resistant to the shear stress starting at 4 dyne/cm^2 compared to Themis2$^{+/+}$ mPMNs (see Figure 12). This result indicates a broader role of Themis2 since not only the number of adherent PMNs is increased in Themis2-deficient mPMNs, but they also seem to adhere stronger to coated surfaces as they are more resistant to external shear forces.

However, 2D mechanotactic crawling under flow conditions of mPMNs lacking Themis2 was not altered in comparison with Themis2$^{+/+}$ mPMNs (see Figure 13). Neither Euclidian distance, the crawling velocity, nor percentage of crawling mPMNs changed in the absence of Themis2, suggesting that Themis2 is not critically involved in this step of the recruitment cascade. Since intraluminal crawling on the inflamed endothelium depends predominantly on the interaction between ICAM-1 and Mac-1[134], this result might suggest that Themis2 is not involved or does not affect Mac-1 activity or function.

Using isolated bone marrow-derived Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs, the regulation of β_2 integrin affinity was analyzed *in vitro*. Because LFA-1-specific ICAM-1-binding reports extended conformation of LFA-1 with high ligand-affinity[69,73] this experiment was important to further support the idea of an inhibitory role of Themis2 in the activation of β_2 integrins. Upon stimulation

with CXCL1 and fMLP LFA-1-specific ICAM-1 binding was strongly increased in Themis2$^{-/-}$ mPMNs (see Figure 14). The bacterial peptide fMLP binds to the GPCR FPR and induces mechanisms of innate immunity and is a classical trigger of chemotaxis[135]. Stimulation with Mn^{2+} causes comparable LFA-1-specific ICAM-1 binding in Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs. Mn^{2+} treatment bypasses integrin inside-out signaling and stabilizes β_2 integrins in the extended conformation with high ligand-affinity. These results indicate, that LFA-1 itself as well as its surface expression is not affected by the absence of Themis2[64]. Overall, these results suggest that Themis2 might act as a negative regulator of β_2 integrin affinity regulation. Upon stimulation of inside-out signaling, lack of Themis2 increases LFA-1-specific ICAM-1 binding, supporting the assumption that Themis2 impairs the induction of the high-affinity LFA-1 conformation.

Characterization of PMN-like Themis2$^{-/-}$ Hoxb8 and Themis2$^{-/-}$ dHoxb8 cells showed differentiation towards mature PMN and equal expression of integrins compared to Themis2$^{+/+}$ Hoxb8 and Themis2$^{+/+}$ dHoxb8 cells (see Figure 15). At both day 0 and day 4, Hoxb8 cells lacking Themis2 expressed comparable progenitor or mature PMN surface markers compared with Themis2$^{+/+}$ Hoxb8 cells. In addition, the integrin subunits CD11a, CD11b and CD18 were expressed at normal levels suggesting, that the absence of Themis2 is not affecting normal phenotypical characteristics in terms of adhesion molecule, differentiation marker or CXCR2 expression. The adhesion assay was conducted in the dHoxb8 cells as well, to evaluate the comparability between the PMN-like dHoxb8 cells and mPMNs. Here, the results were consistent with those obtained with bone marrow-derived Themis2$^{-/-}$ mPMNs, further confirming that loss of Themis2 results in increased adhesion. Thus, the results obtained in the PMN-like dHoxb8 cells reinforced the observed effect of increased adhesion in the absence of Themis2 (see Figure 16).

Furthermore, Themis2-deficient dHoxb8 cells also showed significantly increased LFA-1-specific ICAM-1 binding upon stimulation with CXCL1 or fMLP, further supporting the result in mPMNs and thus the hypothesis that Themis2-dependent signals counteract β_2 integrin activation. Upon stimulation with Mn^{2+}, this effect was again absent, confirming that the integrin itself is not defective. Notably, LFA-1-specific ICAM-1 binding was similarly low without stimulation in both Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells and mPMNs, suggesting that there is no difference in steady-state and no basal activation in cells lacking Themis2. However, this finding could also be due to the fact that the assay may not be sensitive enough to detect specific differences at low ICAM-1 binding rates.

Since these findings were in line with the previous findings in mPMNs, it not only strengthened the hypothesis of a potential role of Themis2 in integrin inside-out signaling during PMN

activation, but also confirmed that the produced PMN-like Themis2$^{-/-}$ Hoxb8 cells were a reliable and useful tool to study the role of Themis2 in PMNs since the effects were similar to murine bone marrow-derived Themis2$^{-/-}$ PMNs.

To determine which integrin affinity state is affected by the absence of Themis2, reporter antibodies were used. However, in the murine system this is not possible since no reporter antibodies are available, that can distinguish between the extended intermediate and high ligand affinity conformations of LFA-1[64]. For the human system the CD18 reporter antibodies KIM127 and mAb24 exist. Binding of mAb24 is specific for open β_2 headpiece indicating the high affinity conformation (H^+)[136–138], whereas KIM127 reports β_2 integrin extension indicated as $E^{+139,140}$. To use this method, CD18 was knocked-out in the Themis2$^{+/+}$ and Themis2$^{-/-}$ Hoxb8 cells and human β_2 integrin was retrovirally expressed. Characterization of these humanized PMN-like Hoxb8 cells confirmed that this manipulation did not affect the cells' ability to differentiate or to express CXCR2 or β_2 integrin α-subunits (see Figure 18). Of note, CD18 expression analyzed during each reporter assay was also expressed to the same extent in humanized Themis2$^{+/+}$ and Themis2$^{-/-}$ dHoxb8 cells, ensuring that the effects were not due to differences in β_2 integrin expression (see Figure 19a).

The antibody binding assays were performed after 3-min stimulation with CXCL1, fMLP, PMA, and LTB_4 to initiate several modes of PMN activation. EDTA served as a negative control, as did untreated cells. Both KIM127 binding and mAb24 binding were increased in hCD18 Themis2$^{-/-}$ dHoxb8 cells compared with hCD18 Themis2$^{+/+}$ dHoxb8 cells upon stimulation with CXCL1 (compare Figure 19b and c). Interestingly, stimulation with fMLP and LTB_4 showed only a tendency towards increased KIM127 binding in Themis2-deficient cells. However, mAb24 binding was significantly increased after 3-min stimulation with LTB_4. Fan et al., demonstrating the new conformational model, found PMNs, that were negative for KIM127 binding but positive for mAb24 binding and demonstrated that the opening of the headpiece domain and the extension of the integrin are regulated individually[79]. Integrins with bent conformation but open headpiece (E^-H^+) bind ICAM-1 in *cis*, leading to prolonged rolling time and reduced number of adherent PMN. However, functional data also showed, that freeing β_2 integrins from *cis* interactions increases ligand binding in trans and, as a consequence, PMN adhesion and aggregation. Moreover, they proposed that KIM127$^-$ mAb24$^+$ clusters could be due to steric exclusion: Integrins may be clustered so tightly that KIM127 cannot reach its binding site and an E^+H^+ integrin may be misclassified as bent[79]. Thus, clustered high-affinity integrins that cannot bind KIM127 would

possibly explain increased mAb24 binding without increased KIM127 binding, as was present here upon PMA and also LTB_4 stimulation. In this study, as well as in the findings of Fan et al., Mac-1 cannot influence these results, because Mac-1 is increased on PMNs upon chemokine stimulation by degranulation, which occurs approximately 5 min after chemokine exposure[79,141]. The conformational changes were investigated 3 min after exposure to the different stimuli. Another potential factor that may have influenced the observed outcome is the possibility that KIM127 exhibits generally minor binding efficacy compared to mAb24. All together, these results further support our previous findings that the absence of Themis2 facilitates enhanced integrin activation in PMNs.

6.3 The role of Themis2 for PMN slow rolling

Since intermediate affinity of integrins is critical for PMN slow rolling, this step of the cascade was specifically analysed[142,143]. Fast rolling is mainly dependent on the interaction of endothelial P-selectin with PSGL-1, whereas slow rolling is mostly induced by E-selectin mediated interactions with PSGL-1, CD44 or E-selectin ligand-1, followed by induction of Rap1 and β_2 integrin activation resulting in transient ICAM-1 interactions[144]. Both, rmE-selectin and rmICAM-1 induce rolling of PMNs and thus represent the in vivo situation of PMN rolling on stimulated postcapillary venules[145,146]. To delineate, where exactly in the signaling cascade Themis2 might play a role, flow chambers were coated either with ICAM-1 and E-selectin or with E-selectin only, and the rolling velocity of Themis2$^{+/+}$ and Themis2$^{-/-}$ mPMNs was analyzed. Interestingly, the rolling velocity of mPMNs lacking Themis2 was significantly reduced compared to Themis2$^{+/+}$ mPMNs in the presence of ICAM-1 and E-selectin, but not altered in the chambers only coated with E-selectin (see Figure 20). This finding further undermines the hypothesis, that Themis2 is involved in the signaling pathway leading to LFA-1 activation. Besides that, it is evident that when cells are interacting with E-selectin alone and the rolling process is mediated through PSGL-1, Themis2 is not involved[141,145].

Slow rolling involves the extension of LFA-1, which depends on the Src family kinase Fgr, Syk and the ITAM-containing adapter molecules Dap12 and FcRγ[144,145]. Themis2 contains several putative tyrosine phosphorylation sites, and previous studies have shown that Themis2 gets phosphorylated at Y660 in response to TLR4 stimulation in macrophages. In this case, phosphorylation is critical for its interaction with Lyn[88]. Since Themis2 interacts with SFKs in B cells

and macrophages, it might be possible that Themis2 interacts with Fgr, Hck or Lyn in PMNs as well. Via Lyn, Themis2 could negatively influence or be involved in the regulation of LFA-1 activation.

6.4 Themis2 signaling and potential interacting partners

Src kinases play a controversial role in PMN activation since they can also negatively regulate PMN function[147]. In a model of sterile peritonitis, *in vivo* trafficking of $Hck^{-/-}Fgr^{-/-}$ PMNs was found to be increased. This indicates that Hck and Fgr normally limit the migration of PMNs in response to agonists for CXCR2 and CCR1. The reduction in PMN migration is mediated through the ERK1/2 signaling pathway.

The inhibitory function of Lyn plays primarily a role in integrin, growth factor receptor and FcεRI signaling pathways. Its role as a negative modulator in myeloid cell signaling pathways is mediated by phosphorylating ITIM-containing inhibitory receptors, such as SIRP1α and PIR-B, which in turn recruit inhibitory phosphatases such as SHP-1[147,148]. Hence, in $Lyn^{-/-}$ PMNs recruitment of SHP-1 is reduced due to decreased tyrosine phosphorylation of both SIRP1α and PIR-B upon β_2 integrin engagement[113]. Moreover, Lyn-deficient cells show reduced phosphorylation of SHP-1, while activation of Lyn leads to decreased affinity of LFA-1 for ICAM-1. Accordingly, Lyn-deficient PMNs have a hyper-responsive phenotype: They are hyper-adhesive to integrin-ligand coated surfaces and show a stronger integrin-dependent functional response. SHP-1 has an important function in regulating the balance of protein tyrosine phosphorylation in PMNs[149,150]. In addition, it could potentially interact with Themis2 via its phosphatase domain or SH2 domain and the CABIT domain of Themis2 as it does in thymocytes[91]. Similar to Themis1, Themis2 could thus regulate the catalytic activity of SHP-1 by stabilizing or supporting the oxidation of the catalytic cysteine residue of SHP-1 and thus inhibiting its tyrosine phosphatase activity.

In support of this hypothesis, a hyper-responsive phenotype is observed upon integrin engagement when SHP-1 is down-regulated, which is similar to the findings in Themis2-deficient PMNs. In this context, Lyn phosphorylates SHP-1 at the C-terminal residue Y536, triggering inhibitory signaling. This phosphorylation activates the N-terminal SH2 domain of SHP-1, which removes the basal inhibition and facilitates the binding of Grb2 or Stat5[151,152]. By binding to Grb2 or Stat5, SHP-1 participates in complex formation and modulation of downstream signaling. Themis2, an adaptor protein lacking enzymatic activity, is proposed to bind and stabilize the complex of Lyn, Grb2, and PLCγ2 in B cells[2]. This interaction enhances signaling downstream of

PLCγ2. Additionally, Themis2 interacts with Grb2 and Vav1, further implicating its involvement in signal transduction pathways[88].

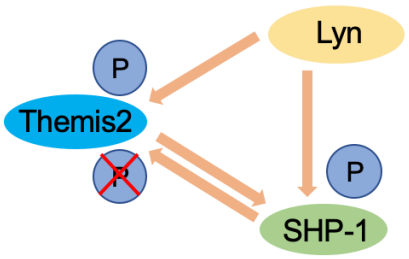

Figure 24. Schematic section of potential Themis2 signaling in PMNs.
The proposed signaling pathway involving Themis2: Firstly, Themis2 undergoes tyrosine phosphorylation at Y660 by Lyn. Additionally, Lyn phosphorylates SHP-1 at Y536, which triggers the inhibitory signaling of SHP-1. Themis2 and SHP-1 are believed to interact through their respective CABIT and phosphatase domains. Through this interaction, Themis2 potentially helps stabilize the oxidation of the catalytic cysteine residue of SHP-1.

In contrast, other negative regulators of integrin activation, such as SHANK1 and SHANK3 scaffold proteins, function by interfering with Rap1 or R-Ras effector binding and competitively limiting the availability of Rap1 on the plasma membrane[153]. Since Rap1 recruits RIAM and Talin-1 to the plasma membrane, this step is crucial for integrin activation[84]. However, the existing data on Themis2 suggests a distinct role in the signaling of integrin activation, implying its involvement in different mechanisms.

6.5 Themis2 in *in vivo* inflammation models

In order to verify the previous results *in vitro* and to analyze the role of Themis2 for PMN adhesion *in vivo*, animal experiments with Themis2$^{+/+}$ and Themis2$^{-/-}$ mice were conducted.

As an *in vivo* model of acute inflammation the TNF-α-induced mouse cremaster model was applied. Due to i.s. injection of the proinflammatory cytokine TNF-α the expression of CXCL1, ICAM-1, P-selectin and E-selectin on the endothelium is increased, which in turn leads to leukocyte slow rolling, adhesion and arrest[45,154]. All of these steps require Talin-1-dependent LFA-1 activation towards the E^{+}H^{-} conformation with intermediate ligand affinity[73,141]. In this model of acute inflammation, genetic absence of Themis2 results in decreased rolling velocity accompanied

by increased numbers of adherent PMNs after i.s. TNF-α injection compared with Themis2$^{+/+}$ mice (see Figure 21). This is consistent with the previous *in vitro* results of strongly increased adhesion in Themis2$^{-/-}$ PMN-like dHoxb8 cells and Themis2$^{-/-}$ bone marrow-derived PMNs and supports the notion that Themis2 acts negatively on LFA-1 extension into the E^+H^- conformation with intermediate ligand affinity. By this, it affects slow leukocyte rolling and arrest[155,156]. The number of white blood cells in the circulation was equal between Themis2$^{+/+}$ and Themis2$^{-/-}$ mice, indicating that the increased number of adherent PMNs in mice lacking Themis2 is not due to enhanced or facilitated recruitment of PMNs from the bone marrow to the bloodstream. Instead, Themis2 plays a role in PMN adhesion and extravasation from the bloodstream to the site of inflammation.

In order to examine the recruitment from the blood vessels into the inflamed tissue the number of extravasated PMNs in the inflamed cremaster tissue of the Themis2$^{+/+}$ and Themis2$^{-/-}$ mice was counted. Bright-field microscopy analysis of whole mount histology revealed that in the cremaster muscles of Themis2$^{-/-}$ mice substantially more PMNs were extravasated (see Figure 22). Quantification confirmed this result and suggested that in the absence of Themis2, not only the rolling velocity and adhesion of PMNs are affected, but extravasation is also enhanced in the TNF-α-inflamed mouse cremaster model. However, the number of other extravasated leukocyte subtypes such as eosinophils or monocytes was not altered in cremaster muscles of Themis2$^{-/-}$ mice compared with Themis2$^{+/+}$ mice, suggesting a specific effect on the extravasation of PMNs following TNF-α-induced inflammation. However, this could be at least partly due to the fact that among the inflammatory cells, mainly PMNs respond to TNF-α stimulation[157].

Aiming to study the impact of Themis2 for the extravasation in another inflamed tissue, the experimental model of acute peritonitis was applied. In general this model works with a wide range of stimuli from pathogens, thioglycolate broth, cytokines or CXCL1[144,158]. In the assays performed here, PMN recruitment into the peritoneal cavity was induced by i.p. injection of CXCL1 and TNF-α. Chemokine signaling via GPCRs triggers binding of both Kindlin-3 and Talin-1 to the cytoplasmic tail of LFA-1 thereby inducing the E^+H^+ conformation of the integrin with high ligand binding affinity. The fully activated LFA-1 interacts and binds strongly to ICAM-1 and ICAM-2 on the endothelium, which leads to firm adhesion of PMNs[69,73]. Interestingly, the number of PMNs was not increased in Themis2$^{-/-}$ mice compared with Themis2$^{+/+}$ mice at either 2 h or 4 h after CXCL1 injection (see Figure 23). Even after TNF-α injection, the number of PMNs in the peritoneal cavity was not significantly altered compared with wild-type. Since PMN recruitment is β_2 integrin-dependent in this experimental model, this is rather unexpected[159–161].

In humans, both significantly reduced PMN recruitment and excessive or inappropriate PMN recruitment lead to severe clinical consequences such as immunodeficiency and chronic inflammation, respectively[38]. Especially, β_2 integrins and selectins have been shown to be critically involved in several inflammatory diseases[162]. Therefore, targeting PMN trafficking via β_2 integrins holds enormous clinical potential as a therapeutic set screw[163]. Moreover, given the differences in PMN recruitment depending on the signaling pathway, organ affected and also the type of disease, this also offers the possibility of targeting only specific PMN phenotypes, since the view of PMNs as a homogenous cell population has been challanged[163–165]. Several PMN phenotypes have been described that have different functions, such as specific involvement in tissue repair, immune regulation and tumor killing[166]. The type of inflammation influences the way of PMN activation and the course of inflammatory states depends on the functionality and activation of PMNs[167–169]. Integrin hyper- or hypoactivation as well as blocking antibodies in different disease models demonstrate the importance of integrin function in acute inflammation in various pathological states such as acute kidney injury, sepsis or pneumonia[170–172]. Integrin targeting shows great promise in animal studies, but major outcome-relevant results in humans are lacking so far or else the therapy is associated with severe adverse effects. Targeting ICAM-1 with antibodies or antisense oligonucleotides, for example, yields negative results, and attempts to directly target β_2 integrins in myocardial infarction have not been successful so far and have not led to the hoped-for results[173–175]. However, blocking LFA-1 and Mac-1 with antibodies has been shown to reduce lung injury in polymicrobial sepsis, and antibodies targeting integrins have been approved for multiple sclerosis and Crohn's disease[170,176]. Although inhibition or even activation of leukocyte trafficking seems promising for the treatment of various diseases, how to circumvent or at least significantly reduce the side effects of such therapies remains to be elucidated. However, the fact that different cell populations are involved and the distinction between the different steps of the cascade involved in integrin recruitment is complex makes this approach a major challenge[38].

However, describing the role of interacting proteins such as Themis2 involved in integrin regulation will primarily lead to a better understanding of PMN trafficking and PMN biology in general, which will provide deeper insights and may also be helpful in the long run to develop or improve such types of therapies.

Given that Themis2 has been shown here to negatively impact the activation of β_2 integrins, enhancing the function of Themis2 could lead to proper integrin regulation or negatively influencing integrin activation. As Themis2 lacks enzymatic activity, a potential way to achieve this is by targeting its phosphorylation sites, such as Y660. Phosphorylation at these sites could

potentially serve as a regulatory mechanism that influences the interaction of Themis2 with key components of the integrin signaling pathway. By modulating the phosphorylation status of Themis2, it may be possible to fine-tune its influence on β_2 integrin activation, leading to a better understanding of the underlying mechanisms and potentially offering new avenues for therapeutic interventions related to integrin-mediated processes. However, as mentioned above, the role of integrins and PMN trafficking in general in disease is complex and context-dependent, and the exact contribution of integrin activation to different pathologies may vary.

In summary, the data presented show that Themis2 plays a role in PMN trafficking and support the concept that Themis2 acts as a negative regulator of β_2 integrin activation. The absence of Themis2 results in enhanced activation of β_2 integrins and in which activation occurs more readily or rapidly and PMNs are hyper-adhesive. The maintenance of homeostasis in health and the restoration of homeostasis in disease are only achieved when the balance between pro-inflammatory and anti-inflammatory functions of the cells involved is normalized, and uncovering all the details that play a role in this process leads to a better understanding of this balance.

7. REFERENCES

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Finally, I want to thank Patrick. Thank you for your patience, encouragement and support in every conceivable way and for always managing to bring a smile to my face.

9. APPENDIX

9.1 Publications and presentations

Lauvai J, **Becker AK**, Lehnert K, Schumacher M, Hieronimus B, Vetter W, Graeve L. (2019): The furan fatty acid 9M5 acts as a partial ligand to peroxisome proliferator-activated receptor gamma and enhances adipogenesis in 3T3-L1 preadipocytes, *Lipids*. 54:277-288

Scientific presentations and Conference attendances

03/2018	2nd International Conference on Leukocyte Trafficking, Munich, Germany
11/2018	Annual Retreat of the IRTG of the SFB 914, Günzburg, Germany (poster presentation)
05/2019	53rd Annual Scientific Meeting of the European Society for Clinical Investigation (ESCI), Coimbra, Portugal
11/2019	Annual Retreat of the IRTG of the SFB 914, Herrsching, Germany (oral presentation)
03/2020	Scientific Retreat of the SFB 914, Obergurgl, Austria (oral presentation)

9.2 Affidavit

Anna-Karina Becker
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I hereby declare, that the submitted thesis entitled

"The role of Themis2 for β_2 integrin function and neutrophil trafficking in innate immunity"

is my own work. I have only used the sources indicated and have not made unauthorized use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Munich, 08.02.2024
Place, Date

Anna-Karina Becker
Signature doctoral candidate

9.3 Confirmation of congruency between printed and electronic version of the doctoral thesis

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I hereby declare, that the submitted thesis entitled

"The role of Themis2 for β_2 integrin function and neutrophil trafficking in innate immunity"

is congruent with the printed version both in content and format.

Munich, 08.02.2024
Place, Date

Anna-Karina Becker
Signature doctoral candidate