Study of calcium transport processes in blood platelets using knock out mouse models. Regulation and role of “store-operated calcium entry” (SOCE)

Ph.D. Thesis

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ABBREVIATIONS

AC   adenylyl cyclase
ACD  acid-citrate-dextrose
ADP  adenosine diphosphate
AM   acetoxy methylester
aPTT activated partial thromboplastin time
ATP  adenosine triphosphate
BM   bone marrow
BSA  bovine serum albumin
[Ca^{2+}] calcium concentration
[Ca^{2+}]_i intracellular calcium concentration
cAMP cyclic adenosin monophosphate
c  coiled-coil (domain)
cGMP cyclic guanosin monophosphate
CIF  calcium influx factor
CLEC-2 C-type lectin-like receptor 2
CRACM calcium release activated calcium modulator
CRP  collagen related peptide
CVX  convulxin
cyt  cytoplasmic
DAG  diacyl glycerol
DNA  deoxy-ribonucleic acid
dys  dystrophin
ECL  enhanced chemoluminescence
ECM  extracellular matrix
EDTA ethylenediamine tetraacetic acid
EGTA ethylene glycol tetraacetic acid
ER  endoplasmic reticulum
ES  embryonic stem (cell)
FITC fluorescein-isothiocyanate
FSC/SSC forward scatter/side scatter
GEF  guanine nucleotide exchange factor
GP   glycoprotein
GPCR G protein coupled receptor
HCT  hematocrit
HE   hematoxylin-eosin
HRP  horseradish peroxidase
IFI  integrated fluorescence intensity
Ig   immunoglobulin
int  integrin
IP   immunoprecipitation
IP_3  inositol-1,4,5-trisphosphate
IP_3-R inositol-1,4,5-trisphosphate receptor
ITAM immunoreceptor tyrosine-based activation motif
LAT  linker for activation of T cells
mAb  monoclonal antibody
MCA  middle cerebral artery
MPV  mean platelet volume
MRI  magnetic resonance imaging
mRNA  messenger ribonucleic acid
NCX  Na+/Ca²⁺ exchanger
PBS  phosphate-buffered saline
Plt  platelet
PCR  polymerase chain reaction
PE  phycoerythrin
PGI₂  prostacyclin
PI-3-K  phosphoinositide-3 kinase
PIP₂  phosphatidilinositol-4,5-bisphosphate
PIP₃  phosphatidilinositol-3,4,5-trisphosphate
PKC  protein kinase C
PLC  phospholipase C
PMCA  plasma membrane Ca²⁺ ATPase
ppp  platelet poor plasma
prp  platelet rich plasma
PT  prothrombin time
RC  rhodocytin
RNA  ribonucleic acid
RNAi  RNA interference
ROC  receptor operated calcium (channel)
ROCE  receptor operated calcium entry
RT  room temperature
RT-PCR  reverse transcriptase polymerase chain reaction
SAM  sterile α-motif
Sax  Saxcoburggotski
SCID  severe combined immunodeficiency
SD  standard deviation
SERCA  sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase
SLP76  Src homology 2 domain-containing leukocyte protein of 76 kDa
SOC  store-operated calcium (channel)
SOCE  store-operated calcium entry
STIM  stromal interaction molecule
TBHQ  2,5-di-[(t-butyl)-1,4-hydroquinone
TBS  tris-buffered saline
TCT  thrombin clotting time
TG  thapsigargin
Thr  thrombin
tMCAO  transient middle cerebral artery occlusion
TF  tissue factor
TRPC  canonical transient receptor potential (channel)
TTC  2,3,5-triphenyltetrazolium chloride
TxA₂  thromboxane A₂
vWF  von Willebrand factor
A. INTRODUCTION

Blood platelets are 2-3 µm big anuclear cells deriving from bone marrow megakaryocytes. The exact mechanism of platelet production is not fully understood; according to the currently accepted hypothesis, the microtubule system of megakaryocytes forms cytoplasmic protrusions – so called proplatelets – the marginal of which will be shed by shear forces in the microcirculation of the bone marrow(1;2). Once produced, discoid platelets circulate in the blood stream for up to ten days in a resting state. Most of them never get activated and undergo firm adhesion during this time and will be removed from the circulation by macrophages in the spleen, liver and lung. Only when the endothelial cell layer of blood vessels is damaged by injuries or pathological alterations, such as found in atherosclerosis, the adhesive potential of platelets becomes evident. Under these conditions, components of the subendothelial extracellular matrix (ECM) are exposed and trigger sudden platelet activation and adhesion. In addition, various soluble stimuli are produced and released from platelets which strengthen platelet adhesion and, together with locally produced thrombin, recruit more platelets into the growing thrombus by exposing or activating receptors on the platelet surface which allow platelets to adhere to each other, a process termed aggregation (Fig.1). These events are crucial to prevent posttraumatic blood loss by providing a platelet plug that leads to the closure of smaller defects of the vessel wall and are required for primary hemostasis. On the other hand, uncontrolled thrombus formation in diseased vessels can lead to vascular occlusion resulting in myocardial infarction or stroke, two of the leading causes of mortality in industrialized countries(3). This duality of platelet function requires tight regulation of the platelet activation process. It must be on one hand sufficient to provide a platelet plug big enough to seal the wounded artery; however, on the other hand unnecessary platelet activation has to be terminated and thrombus growth must not continue uncontrolled to avoid undesired vessel occlusion.
Figure 1. Platelet adhesion and aggregation on the extracellular matrix (ECM). At sites of vascular injury the ECM becomes exposed to the flowing blood, allowing platelet adhesion and aggregation. Under high shear flow conditions, the initial contact (tethering) to the ECM is mediated predominantly by GPIbα binding to von Willebrand factor (vWF) immobilized on collagen. In a second step, GPVI–collagen interactions initiate cellular activation followed by the shift of integrins to a high-affinity state and the release of second-wave agonists, most importantly adenosine diphosphate (ADP) and thromboxane A2 (TxA2). GPIb-mediated signaling may amplify GPVI-induced activation pathways. In parallel, exposed tissue factor (TF) locally triggers the formation of thrombin, which in addition to GPVI/GPIb mediates cellular activation. Cellular activation and upregulation of integrin affinity is proposed to be a pre-requisite for firm adhesion which is mediated by activated β1 integrins and αIIbβ3. Released ADP and TxA2 in turn mediate thrombus growth by activating additional platelets. (Varga-Szabo D, Pleines I, Nieswandt B Arterioscler Thromb Vasc Biol 2008)

A.1 Thrombus formation

The first step in the hemostatic cascade is platelet interaction with the exposed ECM, which contains a large number of adhesive macromolecules, such as laminin, fibronectin, collagens and von Willebrand factor (vWF). The mechanisms of platelet adhesion at sites of injury are to a large extent determined by the prevailing rheological conditions. Blood flows with a greater velocity in the centre of the vessel than near the wall, thereby generating shear forces between adjacent layers of the fluid that become maximal at the wall. The drag, which opposes platelet adhesion and aggregation, increases with the prevailing shear rates. Under conditions of high shear, such as is found in small arteries and arterioles, the initial tethering of platelets to the ECM is mediated by the interaction between the platelet receptor glycoprotein (GP)Ib and vWF bound to collagen. While mandatory at high shear, this interaction may not be relevant under conditions of low shear as found in veins and large arteries(4). The binding of GPIb to vWF has a fast off-rate and is therefore insufficient to mediate stable adhesion
but rather maintains the platelet in close contact with the surface, although it continuously translocates in the direction of blood flow. During this “rolling”, platelets establish contacts with the thrombogenic ECM protein collagen through its main receptor GPVI, which belongs to the immunoglobulin superfamily(5). While GPVI binds collagen with low affinity and thus is unable to mediate adhesion by itself, it triggers intracellular signals that shift platelet integrins to a high-affinity state and induce the release of the second wave mediators, adenosine diphosphate (ADP) and thromboxane A₂ (TXA₂). These agonists together with locally produced thrombin contribute to cellular activation by stimulating receptors that couple to heterotrimeric G proteins (G₄, G₁₂/G₁₃, Gᵢ), which induce different signaling events and act synergistically to induce full platelet activation (Fig.2).

Under conditions, when high concentrations of these soluble agonists are present, this may be sufficient to mediate platelet activation independently of GPVI, as shown experimentally in a model of laser-induced arterial injury in mice, where thrombus formation occurs independently of collagen receptors(6). Firm adhesion to the ECM is mediated by high-affinity β₁-integrins which bind to collagen (α₂β₁), fibronectin (α5β₁) and laminin (α6β₁), as well as the major platelet integrin, αIIbβ₃, interacting with fibronectin and collagen-bound vWF.

Second wave mediators released by activated platelets not only act in an autocrine manner, they also activate further platelets, thereby recruiting them to a growing thrombus. Platelet-platelet interactions are then mediated by the active form of integrin αIIbβ₃ through the plasma protein fibrinogen (Fig.1).

**A.2 Platelet calcium homeostasis**

A central event in the above detailed platelet activation process is an increase in the intracellular calcium concentration ([Ca²⁺]ᵢ). Calcium is an essential second messenger in virtually all cells, regulating a wide range of fundamental cellular processes like cell differentiation, cell division, gene transcription and many others(7). An elevation in [Ca²⁺]ᵢ is also a prerequisite of platelet activation(8). This supports – among others – through calmodulin the reorganization of the actin cytoskeleton necessary for shape
change(9) or, for instance, the inside-out activation of integrin αIIbβ3, indispensable for platelet aggregation(10). In the latter process the CalDAG-GEFI/Rap1 signaling pathway – also regulated by an elevation in [Ca\(^{2+}\)]\(_i\) and diacyl glycerol – has just recently been reported to play an essential role(11;12).

The required elevation in cytosolic Ca\(^{2+}\) concentration can derive from two major sources: the release of compartmentalized free Ca\(^{2+}\) and the entry of extracellular free Ca\(^{2+}\) through the plasma membrane.

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**Figure 2.** Signaling mechanisms linking various platelet receptors to the inside-out activation of integrins. GPVI is the main receptor mediating platelet activation by collagen. Binding of collagen to GPVI results in tyrosine phosphorylation of the FcRγ-chain by the kinases Lyn and Fyn, leading to recruitment of the tyrosine kinase Syk. Syk phosphorylates linker for activation of T cells (LAT) which in turn recruits phospholipase Cγ2 (PLCγ2) and Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76) to the cell membrane. LAT also associates with phosphoinositol-3 kinase (PI-3-K) resulting in the formation of phosphatidylinositol-3,4,5-P3 (PIP3). There is evidence that the vWF receptor GPIb-V-IX, after ligand binding, also induces intracellular signaling processes. Stimulation of heptahelical G protein-coupled receptors by thromboxane A\(_2\) (TxA\(_2\)), thrombin and ADP leads to the stimulation of various signaling pathways involving the G proteins Gq,Gz and G12/13. AC, adenylyl cyclase; DAG, diacyl glycerol; IP\(_3\), inositol-1,4,5-trisphosphate; PI-3-K, phosphoinositide-3-kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PKC\(_\gamma\), protein kinase C; PLC\(\gamma\), phospholipase C\(\gamma\); RhoGEF, Rho-specific guanine nucleotide exchange factor. (Varga-Szabo D, Pleines I, Nieswandt B Arterioscler Thromb Vasc Biol 2008)
A.2.1 Calcium release from intracellular stores

The way of calcium release from intracellular stores is a rather well established process. Agonist binding of different platelet surface receptors leads to the activation of phospholipase (PL) C isoforms, which hydrolyze phosphoinositide-4,5-bisphosphate (PIP₂) to inositol-1,4,5-trisphosphate (IP₃) and diacyl-glycerol (DAG). IP₃ in turn releases the calcium content of the intracellular stores while DAG is involved in calcium entry from the extracellular compartment(7;13) (Fig.3).

Figure 3. The calcium toolkit of platelets. Upon receptor agonist binding, different phospholipase (PL) C isoforms hydrolyze phosphoinositide-4,5-bisphosphate (PIP₂) into inositol-1,4,5- trisphosphate (IP₃) and diacyl glycerol (DAG). IP₃ releases the intracellular calcium store content through its channel receptor (IP₃-R), whereas DAG mediates store-independent calcium entry. Store-release in turn leads to store-operated calcium (SOC) entry through SOC channels. In addition, receptor-operated calcium entry (ROCE) also exists in platelets through the purinergic receptor P2X₁. The intracellular calcium level will be returned by sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPases (SERCAs), plasma membrane Ca²⁺ ATPases (PMCA) and by a Na⁺/Ca²⁺ exchanger (NCX).
In platelets, two subfamilies of the PLC enzymes are expressed; namely the PLCβ and the PLCγ subfamily. Both subfamilies consist of more isoforms (β1-4 and γ1-2) of which the β1-3 and the γ2 are expressed in human platelets; whereas in mouse platelets the expression of all isoforms has been reported(14;15). The predominant isoforms are the β2/3 (in mouse platelets β1 and β3) and the γ2. The PLCβ isoforms are solely regulated by G-protein coupled receptors (GPCRs) through the α-subunit of Gq, whereas PLCγ2 is located downstream of the major platelet collagen receptor GPVI(5), the newly identified platelet activating receptor, C-type lectin-like receptor-2 (CLEC-2)(16) and the adhesion receptor integrins α2β1 and αIIbβ3(17).

IP3 produced by these PLCs releases Ca^{2+} from the intracellular stores by directly activating the Ca^{2+} channel IP3 receptors (IP3-R). Three different isoforms of the IP3-Rs have been cloned in mammalian cells, the IP3-R1, IP3-R2 and IP3-R3(18). Their relative binding affinity to IP3 is as follows: IP3-R2 > IP3-R1 > IP3-R3(19). In platelets two of the three isoforms have been found, namely IP3-R1 and IP3-R2.

Although, there are several intracellular organelles – like the endoplasmic reticulum (ER), the Golgi apparatus, mitochondria, different secretory granules and lysosomes – which contain higher [Ca^{2+}] than the surrounding cytoplasm, representing possible releasable Ca^{2+} stores, there is consensus that the major intracellular Ca^{2+} pool is the ER. Based on the expression of two different sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) isoforms(20) and on their sensitivity towards the SERCA inhibitor thapsigargin (TG), there is experimental data supporting the presence of two distinct Ca^{2+} stores in platelets(21;22). One expressing a 100 kDa SERCA isoform – being identified as SERCA2b(23) – shows high sensitivity to TG and is corresponding to the dense tubular system (DTS), the equivalent of the ER in platelets, and another expressing a 97 kDa SERCA isoform (SERCA3)(24;25), which shows low sensitivity to TG but is sensitive to 2,5-di-(t-butyl)-1,4-hydroquinone (TBHQ)(22). This latter has been identified as an acidic organelle, although its true identity remains to be clarified(26).
A.2.2 Store-operated calcium entry

In non-excitable cells, such as platelets, the major way of Ca\textsuperscript{2+} entry from the extracellular compartment is triggered by the IP\textsubscript{3} mediated Ca\textsuperscript{2+} release from the intracellular stores. This has first been described in neutrophils(27) in 1986 and is referred to as store-operated calcium entry (SOCE) (Fig.4). So the process has been known for over two decades but the underlying mechanisms have remained elusive. Several hypotheses evolved during the years regarding the possible way connecting Ca\textsuperscript{2+} store release to SOCE. These include intracellular Ca\textsuperscript{2+}(27), cGMP(28), cytochrome P450 metabolites(29) or inositol-1,3,4,5-tetakisphosphate(30) acting as second messengers, the production of an undefined soluble “calcium influx factor” (CIF)(31), tyrosine phosphorylation(32), insertion of Ca\textsuperscript{2+} channels into the plasma membrane by vesicle trafficking(33) and – in platelets – conformational coupling between the IP\textsubscript{3}-R2 and a store-operated calcium (SOC) channel candidate – canonical transient receptor potential (TRPC) channel 1(34;35).

Figure 4. Schematic drawing of store-operated calcium entry (SOCE). Calcium release from the intracellular stores through the IP\textsubscript{3}-receptor (IP\textsubscript{3}-R) leads to the opening of store-operated calcium (SOC) channels in the plasma membrane, allowing calcium to enter the cell from the extracellular compartment.
Despite the efforts, neither the identification of the exact mechanism and the key proteins succeeded, nor could be the relevance of SOCE in cell physiology elucidated. Not only the mechanism of store-operated calcium entry but the identity of the platelet SOC channel as well remained a matter of considerable debate. As mentioned briefly above, the conformational coupling model proposed TRPC1 to fulfill this function based mainly on in vitro studies, where a blocking antibody against TRPC1 (obtained from Alomone Labs, Israel) reduced SOCE in response to thrombin and thapsigargin (TG)(36). The model suggested that upon store release a de novo conformational coupling between the IP$_3$-R2 and TRPC1 should happen, which in turn would open TRPC1 as SOC channel in the plasma membrane to allow Ca$^{2+}$ entry(34;37).

**A.2.2.1 Canonical transient receptor potential channel 1 (TRPC1)**

TRPC1 is a mammalian homologue of the *Drosophila* TRP protein. This latter one has been identified in *Drosophila* photoreceptor cells as a non-voltage gated Ca$^{2+}$ channel with characteristics of SOC channels(38). Mammalian TRPC1 is a ~85 kDa protein with 6 predicted transmembrane domains and intracellular N and C termini(39) (Fig.5). Its putative ion-selective pore is situated between the 5$^{th}$ and 6$^{th}$ transmembrane domain and the protein is suggested to fulfill its channel function as a homo- or heterotetramer. Since its description, much effort has been made to confer the SOC channel function of TRPC1 with mixed success. Although striking store-operated signals have been shown in one study(40), others found TRPC1 to be a non-selective cation channel(41). A possible explanation for these seemingly controversial results could be provided by tissue specific differences in the store-operated calcium entry process – as also suggested by studies on knock out mice. Whereas no alterations in SOCE could be found in vascular smooth muscle cells of *TRPC1*$^{-/-}$ mice(42), an attenuation of store-operated calcium currents in salivary gland cells of the same knock out mice has been reported(43).
There are numerous controversies in the literature regarding the TRPC channel family and its role in platelet function as well. The first controversy concerns already the localization of TRPC1 in platelets. While TRPC1 has been reported to be expressed in lipid rafts of the platelet plasma membrane associated with TRPC4 and TRPC5(44), others found it to be expressed only in internal membranes(45). It is also noteworthy that there are no further reports of TRPC4 or 5 detection at protein level in platelets and TRPC5 has not even been detected at mRNA level in human megakaryocytes and platelets(46) or in mouse megakaryocytes(47). Concern has also been raised regarding the specificity of the Alomone anti-TRPC1 antibody with reported lack of recognition of overexpressed TRPC1 in heterologous systems(48). Finally, there are reports stating no effect of anti-TRPC1 treatment on thrombin or TG induced SOCE(49).

A.2.2.2 Stromal interaction molecule 1

The identification of stromal interaction molecule 1 (STIM1) as the Ca\(^{2+}\) sensor molecule in the ER membrane, has revolutionized the field of store-operated calcium entry. STIM1 has originally been described as a tumor suppressor protein involved in different pediatric malignancies, including Wilms tumor(50). It is a type I single transmembrane protein of ~84 kDa containing two N-terminal EF-hand domains (an unmasked canonical one and a “hidden” EF-hand), followed by a sterile α-motif (SAM) domain, the transmembrane region, two coiled-coil region and at the C-terminal end a serine/proline-rich and a lysine-rich domain(51-53).

In 2005 two independent groups identified STIM1 – based on RNA interference (RNAi)-based screening and knock-down – as a conserved component of SOCE in *Drosophila S2* cells, HeLa cells and Jurkat T cell lines(54-56). They found STIM1 to be
present mainly in the ER membrane with the Ca\textsuperscript{2+}-sensing N-terminal EF-hands situated in the lumen of the ER. Introduction of a point mutation in this calcium-binding domain resulted in permanent activation of the protein leading to a continuously opened state of the SOC channels in the plasma membrane\cite{54,56}. Based on these results, they suggested STIM1 to be the long sought Ca\textsuperscript{2+}-sensor of the ER, which upon store-release moves to plasma membrane near puncta and signals to the plasma membrane SOC channels. It has to be mentioned here, that some studies found a small portion of STIM1 to be present in the plasma membrane and suggested this plasma membrane STIM1 to have a direct regulatory role on the SOC channels\cite{57}; however, others could not confirm this\cite{54}. There is still ongoing discussion whether or not STIM1 is present in the cell membrane and the relevance of this is also unclear.

\textbf{A.2.2.3 Orai1}

Parallel to STIM1, calcium-release activated calcium modulator 1 (CRACM1 or Orai1) – a plasma membrane protein with four predicted transmembrane domains and intracellular C- and N-termini – has been identified as another essential component of SOCE in T cells of patients with severe combined immunodeficiency (SCID) and in \textit{Drosophila} cells\cite{58,59}. At this time it was still unclear whether Orai1 is part of the SOC channel or a central regulator of it, but shortly after the first publications further studies on \textit{Drosophila} S2 cells provided evidence that Orai1 itself is the pore forming unit of the SOC channel\cite{60,61} and that Orai1 multimerizes with itself to create the channel\cite{62}. 
B. AIM OF THE STUDY

The aim of the study was to investigate the store-operated calcium entry process in blood platelets. In light of the above detailed advances in our understanding of SOCE and as there is considerable disagreement regarding the role of TRPC1 in platelet physiology, I wanted to explore the molecular machinery behind platelet SOCE. Furthermore, the relevance of calcium entry in platelet function in vivo remained so far largely unknown. Using knock out mouse models, I aimed to investigate this aspect of platelet physiology in more detail.
C. MATERIALS AND METHODS

C.1 Materials

C.1.1 Chemicals

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Oregon Green 488 Bapta-1 AM    Molecular Probes (Oregon, USA)
PD-10 column    Pharmacia (Uppsala, Sweden)
penicillin/streptomycin    PAN (Aidenbach, Germany)
Pluronic F-127    Invitrogen (Karlsruhe, Germany)
polyethylene glycol 1500 (PEG 1500)    Roche Diagnostics (Mannheim, Germany)
prostacyclin    Calbiochem (Bad Soden, Germany)
protein G sepharose    Pharmacia (Uppsala, Sweden)
R-phycoerythrin (PE)    EUROPA (Cambridge, UK)
Thapsigargin    Molecular Probes (Oregon, USA)
Thrombin    Roche Diagnostics (Mannheim, Germany)
3,3,5,5-tetramethylbenzidine (TMB)    EUROPA (Cambridge, UK)
U46619    Alexis Biochemicals (San Diego, USA)

Collagen related peptide (CRP) was kindly provided by S.P Watson (University of Birmingham, UK). Convulxin was obtained from the venom of the tropical rattlesnake *Crotalus durissus terrificus* and was kindly provided by M. Leduc and C. Bon (Institute Pasteur, Paris, France).

All other chemicals were obtained from Sigma (Deisenhofen, Germany) or Roth (Karlsruhe, Germany).

**C.1.2 Monoclonal antibodies (mAbs)**

Anti-STIM1 mAbs were purchased from BD Transduction (GOK/Stim1, clone number 44) and Abnova (clone 5A2, H00006786-M01), anti-α-tubulin mAb was from Chemicon (clone YL1/2) and the TRPC1 recognizing monoclonal antibody 1F1 was a generous gift from Dr. L Tsiokas (Oklahoma, USA). Every other mAbs used were from Emfret Analytics (Würzburg, Germany),
<table>
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<th>described in</th>
</tr>
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<td>(63)</td>
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<tr>
<td>BRU1</td>
<td>IgG1</td>
<td>P-selectin</td>
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C.1.3 Polyclonal antibodies (pAbs)/secondary reagents

Anti-Orai1 antibodies were from ProSci Incorporated (Poway, USA) and the anti-XTRP-1 antibody was a generous gift from G. Barritt (Adelaide, Australia).
Goat anti-rabbit, rabbit anti-mouse and rabbit anti-rat IgG (-HRP) were purchased from DAKO (Hamburg, Germany).

C.1.4 Animals

Specific-pathogen-free mice (C3H, C57Bl/6) 5 to 10 weeks of age were obtained from Harlan (Sulzfeld, Germany). C3H mice bearing an activating mutation in the EF-hand domain of STIM1 (Sax) were from Ingenium Pharmaceuticals AG (Munich, Germany), mice deficient in TRPC1 were from Alexander Dietrich (Philipps-University Marburg, Department of Pharmacology and Toxicology, Marburg, Germany), whereas mice deficient in STIM1 and Orai1 were generated in our laboratory.
C.1.4.1 Generation of STIM1- and Orai1-deficient mice

C.1.4.1.1 Generation of Stim1−/− mice

A gene trap ES cell line containing an insertional disruption in the Stim1 gene (RRS558; RZPD Clone ID: IRAKp961K1818Q)) was purchased from BayGenomics. Male chimeras from this ES cell line were bred to C57Bl/6 females to generate Stim1+/− mice, which were intercrossed to produce Stim1−/− mice. In the ES cells, the gene-trap vector pCMV-SPORT6, which is composed of a splice-acceptor site followed by a "βGeo" cassette encoding a fusion of β-galactosidase and neomycin phosphotransferase II, was inserted 600 bp downstream of exon 7, into intron 7 of the Stim1 gene. The integration site was confirmed by PCR and sequencing of amplified genomic DNA fragment using exon 7 forward (5′-GCTGCACAAGGCCAGGAGG-3′) and geo1 reverse (5′-ATCGGCCTCAGGAAGATCGC-3′) primers. The transcript would result in the generation of a fusion protein between truncated Stim1 (extracellular region plus transmembrane domain) and β-galactosidase. This fusion protein was, however, not detectable in platelets by western blotting using anti-β galactosidase antibodies or anti-STIM1 antibodies that recognize the N-terminus of the protein(66) (GOK/Stim1, clone 44; BD Transduction), suggesting that it is rapidly degraded.

C.1.4.1.2 Generation of Orai1−/− mice

Orai1−/− mice were generated as described by Vig et al(67). Briefly, ES cell clone (XL922) was purchased from BayGenomics and microinjected into C57Bl/6 blastocysts to generate Orai1 chimeric mice. After germ line transmission heterozygous and knockout animals were genotyped by Southern blot and PCR using mouse tail DNA. Homologous recombinant and wild type alleles were detected by external probe which is located in upstream region of exon1. External probe was amplified by PCR. ExtpFor: 5′-GCTAGGGGAATCTCAGAAAC-3′
ExtpRev: 5′-CATCGGCTACCTCAGAACG-3′
C.2 Methods

C.2.1 Buffers and Media

All buffers were prepared and diluted using aqua bidest.

**Phosphate-buffered saline (PBS), pH 7.14**
- NaCl 137 mM (0.9 %)
- KCl 2.7 mM
- KH$_2$PO$_4$ 1.5 mM
- Na$_2$HPO$_4$·2H$_2$O 8 mM

**PBS/EDTA**
- PBS
- EDTA 5 mM

**Tris-buffered saline (TBS), pH 7.3**
- NaCl 137 mM (0.9%)
- Tris/HCl 20 mM

**Storage buffer, pH 7.0**
- Tris 20 mM
- NaCl 0.9 %
- BSA 0.5 %
- NaN$_3$ 0.09 %

**Tyrode’s buffer, pH 7.3**
- NaCl 137 mM (0.9 %)
- KCl 2.7 mM
- NaHCO$_3$ 12 mM
- NaH$_2$PO$_4$ 0.43 mM
- Glucose 0.1 %
- Hepes 5 mM
BSA 0.35 %
CaCl₂ 1 mM
MgCl₂ 1 mM

**Acid-citrate-dextrose (ACD) buffer, pH 4.5**
Trisodium citrate dehydrate 85 mM
Citric acid anhydrous 65 mM
Glucose anhydrous 110 mM

**IP buffer**
Tris/HCl (pH 8.0) 15 mM
NaCl 155 mM
EDTA 1 mM
NaN₃ 0.005 %

**SDS sample buffer, 2X**
β-mercaptoethanol (for red. conditions) 10 %
Tris buffer (1.25 M), pH 6.8 10 %
Glycerin 20 %
SDS 4 %
Bromophenolblue 0.02 %

**Laemmli buffer**
Tris 40 mM
Glycin 0.95 M
SDS 0.5 %

**Blotting buffer A**
Tris, pH 10.4 0.3 M
Methanol 20 %
Blotting buffer B
Tris, pH 10.4 25 mM
Methanol 20 %

Blotting buffer C
ε-amino-n-caproic acid 4 mM
Methanol 20 %

Washing buffer
PBS
Tween 20 0.1 %

DNA digestion buffer
Tris-HCl (pH 8.0) 50 mM
EDTA 100 mM
NaCl 100mM
SDS 1 %
Proteinase K (10 mg/mL) 35 μL

Coomassie Stain
Acetic acid 10 %
Methanol 40 %

Destaining solution
Acetic acid 10 %
in H₂O

C.2.2 Genomic DNA isolation from mouse ears

Approximately a 5 mm² part of one ear was dissolved in 500 μL DNA digestion buffer by overnight incubation at 56°C under shaking conditions. Samples were then vortexed for 5 min, followed by the addition of 250 μL of saturated NaCl (6 M) solution.
Samples were then centrifuged at 14000 rpm for 10 min. 750μL supernatant was taken and 500μL isopropanol was added and samples were shaken for 2 min. After centrifugation at 14000 rpm for 10 min, the DNA pellet was washed twice with ice cold 70% ethanol. The DNA pellet was left to dry and finally resuspended in 50μL H₂O.

**C.2.3 mRNA isolation from mouse and human platelets**

Platelets were isolated and washed from 3 mL freshly drawn blood as described under C.2.5 and C.2.7 and finally lysed in 250 μL IP buffer containing 1% NP40. Platelet mRNA was isolated using Trizol reagent (Invitrogen GmbH, Karlsruhe, Germany) and extracted with chloroform. The mRNA was precipitated with isopropanol and washed with 70% ethanol, dried at room temperature and solved in DEPC-treated H₂O. cDNA was synthesized using 1 μg platelet total mRNA and Super Script Reverse Transcriptase (Invitrogen GmbH, Karlsruhe, Germany) as described by the manufacturer. Gradient PCR was performed to amplify the cDNA of interest.

Primers were used:

**mouse TRPC1:**
- TRPC1f: 5’-CATGGAGCATCGTATTTCAC-3’
- TRPC1r: 5’-GAGTCGAAGGTAACTCAGAA-3’

**human Orai1:**
- hOrai1f: 5´-AGCAACGTGCACAATCACAA-3´
- hOrai1r: 5´-GTCTTTATGGCTAACCAGTA-3´

**human Orai2:**
- hOrai2f: 5´-CGGCCATAAGGGCATGGATT-3´
- hOrai2r: 5´-TTGTGGATGTTGCTCACG-3´

**human Orai3:**
- hOrai3f: 5´-CTCTTCCTTGCTGAAGTTGT-3´
- hOrai3r: 5´-CGATTCAAGTTCCTCTAGTT-3´
mouse Orai1:
mOrai1f 5’-CTTCGCCATGGTAGCGATGG-3’
mOrai1r 5’-ACCAGGGAGCGGTAGAAGTG-3’

mouse Orai2:
mOrai2f: 5’-CTTCGCCATGGTGGCCATGG-3’
mOrai2r: 5’-ACCAGGGAACGCTTAGAAGTG-3’

mouse Orai3:
mOrai3f: 5’-CTTCGCCATGGTGGCCATGG-3’
mOrai3r: 5’-ACCAAGGATCGGTAGAAGTG-3’

human actin:
hActinf: 5’-GGGTCAGAAAGATTCCTACGTG-3’
hActinr: 5’-TCGCCATATCGTTCTCGAAGTC-3’

mouse actin:
mActinf: 5’-GTGGGCCGCTCTAGGCACCAA-3’
mActinr: 5’-CTCTTTGATGTCACGCACGATTTC-3’

C.2.4 Genotyping protocols

Primers (Stim1-/- mice)
Detection of the wild-type allele:

wtF: 5’-GTCATAGCCTGTAAACTAGA-3’
wtR: 5’-GTAGCTGCAGGTAGCACTAG-3’

Detection of the knock out allele:
GeoF: 5’-TTATCGATGAGCGTGGTTATG-3’
GeoR: 5’-GCGCGTACATCGGCAAAATAATATC-3’
Two PCR reactions are needed; one to detect the wild-type band, and the other to detect the knock-out band.

**PCR buffer (10x)** 5μL  
**MgCl₂ (10x)** 5μL  
**dNTPs (10 mM)** 1μL  
**primer A (100 ng/μL)** 1μL  
**primer B (100 ng/μL)** 1μL  
**DNA** 50ng  
**Taq polymerase** 1 U  
**H₂O** ad 50μL

**PCR program wild-type and knock out allele (**Stim1⁻/⁻ mice**)**

1. 96°C 3 min  
2. 94°C 30 sec  
3. 51°C 30 sec  
4. 72°C 1 min  
5. repeat step 2.-4. 35x  
6. 72°C 10 min  
7. 4°C hold

**Primers (Orai1⁻/⁻ mice)**

Detection of the wild-type allele:  
wtF: 5’-GATCCCTAGGACCCATGTGG-3’  
wtR: 5’-AAGTGTGCATGCACACTTGC-3’

Detection of the knock out allele:  
GeoF: 5’-TTATCGATGAGCGTGGTGGTTATG-3’  
GeoR: 5’-GCGCGTACATCGGCGAAAATAATATC-3’
Two PCR reactions are needed; one to detect the wild-type band, and the other to detect the knock-out band.

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<tr>
<td>MgCl₂ (10x)</td>
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<td>dNTPs (10 mM)</td>
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<td>primer A (100 ng/μL)</td>
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<td>primer B (100 ng/μL)</td>
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<tr>
<td>DNA</td>
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<tr>
<td>Taq polymerase</td>
<td>1 U</td>
</tr>
<tr>
<td>H₂O</td>
<td>ad 50μL</td>
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</table>

**PCR program wild-type allele (Orai1⁻/⁻ mice)**

1. 96°C 3 min
2. 94°C 30 sec
3. 65°C 30 sec
4. 72°C 2 min
5. repeat step 2.-4. 35x
6. 72°C 10 min
7. 4°C hold

**PCR program knock out allele (Orai1⁻/⁻ mice)**

1. 96°C 3 min
2. 94°C 30 sec
3. 51°C 30 sec
4. 72°C 1 min
5. repeat step 2.-4. 35x
6. 72°C 10 min
7. 4°C hold
Primers (*TRPC1*<sup>−/−</sup> mice)

Detection of the wild-type allele:

wtF: 5’-GGCAACCTTTGCCCTCAAAGTGGTGGC-3’
wtR: 5’-AGTGAATATATATATATCAGACATAGATTTGGG-3’

Detection of the knock out allele:

koF: 5’-TCTATGGCTTCTGAGGCGGA-3’
koR: 5’-GCATTATTAATATCTGAGTCATTTTTCTTATTGGGAAAAATGAGG-3’

Two PCR reactions are needed; one to detect the wild-type band, and the other to detect the knock-out band.

- PCR buffer (10x) 5µL
- MgCl₂ (10x) 5µL
- dNTPs (10 mM) 1µL
- primer A (100 ng/µL) 1µL
- primer B (100 ng/µL) 1µL
- DNA 50ng
- Taq polymerase 1 U
- H₂O ad 50µL

**PCR program wild-type and knock out allele (*TRPC1*<sup>−/−</sup> mice)**

1. 96°C 3 min
2. 94°C 30 sec
3. 55°C 30 sec
4. 72°C 1 min
5. repeat step 2.-4. 35x
6. 72°C 10 min
7. 4°C hold
C.2.5 Platelet preparation and washing

Mice were bled under ether anesthesia from the retroorbital plexus. Blood was collected into an Eppendorf tube containing 300µl heparin in TBS (20 U/ml), pH 7.3. Blood was centrifuged at 1800 rpm for 5 min. The supernatant was centrifuged again at 800 rpm for 6 min at to obtain platelet rich plasma (prp). To wash platelets, prp was centrifuged at 2500 rpm for 5 min in the presence of prostacyclin (PGI₂) (0.1 µg/mL) and the pellet was resuspended in Ca²⁺-free Tyrode’s buffer containing PGI₂ (0.1 µg/mL) and apyrase (0.02 U/mL). Repeating the washing two times, the platelet pellet was resuspended in Tyrode’s buffer containing apyrase (0.02 U/mL) (500µL) and left to incubate for at least 30 min at 37 °C before analysis.

C.2.6 Platelet counting

For determination of platelet counts and size, blood (50 µL) was obtained from the retroorbital plexus of anesthetized mice using heparinized microcapillaries and collected into an Eppendorf tube containing 300µl heparin in TBS (20 U/ml), pH 7.3. Platelet counts and size were determined using a Sysmex KX-21N automated hematology analyzer (Sysmex Corp, Kobe, Japan).

C.2.7 Preparation and washing of human platelets

Blood from aspirin free healthy volunteers was collected in TBS-EDTA (5 mM) containing syringes. To obtain prp, blood was centrifuged at 300 g for 20 min and supernatant (prp) was collected carefully. To wash platelets, PGI₂ (0.1 µg/mL) was added to prp which was then centrifuged at 500 g for 10 min and platelet pellet resuspended in PGI₂-containing Tyrode’s buffer (0.1 µg/mL). The centrifugation step was repeated once and platelets were finally suspended in Tyrode’s buffer and adjusted to 2 x10⁸ platelets/mL.
C.2.8 Western-blotting

For Western blot analysis, platelets were washed 3x in PBS/EDTA and finally solubilized in 150 μL IP buffer containing 1% NP-40. Samples were separated by 12 % SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. To prevent non-specific antibody binding, membrane was incubated in 10 % fat-free milk (dissolved in washing buffer) for 1 h at RT. After that, membrane was incubated with the required antibody (5 μg/mL) for 1 h at RT. For washing, the membrane was incubated 3x with washing buffer for 10 min at RT. After the washing steps, HRP-labeled secondary reagent was added and left to incubate for 1 h at RT. After several washing steps, proteins were visualized by ECL.

C.2.9 In vitro analysis of platelet function

C.2.9.1 Flow cytometry

Platelets ($1 \times 10^6$) were activated with the indicated agonists or reagents (10 min, RT), stained for 10 min with saturating amounts of fluorophore-conjugated antibodies. The reaction was stopped by addition of 500 μL PBS, and samples were immediately analyzed on a FACScalibur (Becton Dickinsin). For a two-color staining, the following settings were used:

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Compensation:

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<td>0 % of Fl3</td>
</tr>
<tr>
<td>Fl3</td>
<td>0 % of Fl2</td>
</tr>
</tbody>
</table>

C.2.9.2 Aggregometry

To determine platelet aggregation, light transmission was measured using washed platelets adjusted to a platelet concentration of $3 \times 10^8$ platelets/mL with Tyrode's buffer. Alternatively, heparinized prp was used. Agonists or reagents were added as 100-fold concentrates and light transmission was recorded over 10 min on an Apact 4-channel optical aggregation system (APACT, Hamburg, Germany). Before starting the measurements, Tyrode’s buffer (for washed platelets) or plasma (for prp) was set as 100% aggregation and washed platelet suspension (for washed platelets) or prp (for prp) was set as 0% aggregation.

C.2.9.3 Adhesion under flow conditions

Blood (700µL) was collected into 300µL TBS buffer, pH 7.3, containing 20 U/mL heparin, labeled with a Dylight-488 conjugated anti-GPIX Ig derivative (0.2 µg/ml, 5
min) and diluted 2:1 with Tyrode’s buffer. Coverslips (24 x 60 mm) were coated with fibrillar type-I collagen (Horm) (0.25 mg/mL, Nycomed, Munich, Germany) and blocked for 1 h with 1% bovine serum albumin. Perfusion studies were performed as follows. Transparent flow chambers with a slit depth of 50 μm, equipped with the coated coverslips, were connected to a syringe filled with the diluted whole blood. Perfusion was performed using a pulse-free pump under high shear stress equivalent to a wall shear rate of 1000 s⁻¹ (4 min). Thereafter, chambers were rinsed by a 10 min perfusion with Tyrode’s buffer at the same shear stress and phase-contrast and fluorescent pictures were recorded from at least five different microscope fields (40x objectives). Image analysis was performed off-line using MetaVue® software. Thrombus formation was expressed as the mean percentage of total area covered by thrombi, and as the mean integrated fluorescence intensity per mm².

C.2.9.4 Intracellular calcium measurements

C.2.9.4.1 Intracellular calcium measurements by flow cytometry

Washed platelets (as described above) were incubated in Ca²⁺-free Tyrode’s buffer with 5 μM Oregon Green 488 Bapta-1 AM and 5 μM Fura Red AM (both from Molecular Probes) for 20 minutes at 37°C. Labelled platelets were suspended in Tyrode’s buffer containing either 1 mM Ca²⁺ or 0.5 mM EGTA. Changes in intracellular calcium – as ratios of Oregon Green Bapta-1 fluorescence over Fura Red fluorescence – upon agonist induced stimuli were real-time monitored and recorded on a FACSCalibur using CellQuest v3.3 software (Becton Dickinson). Data were offline analyzed by the shareware FCSPress. The system was calibrated and [Ca²⁺]ᵢ was calculated from the fluorescent ratios as previously described(68). Briefly, fluorescence ratios were converted to relative cytosolic calcium concentration ([Ca²⁺]ᵢ) according to the following equation:

\[
[Ca^{2+}]_i = 170 \times \frac{(R-R_{\text{min}})}{(R_{\text{max}}-R)} \times \frac{F_{\text{max}}}{F_{\text{min}}}
\]

where 170 is the \(K_d\) value of Oregon Green BAPTA-1 Ca²⁺ binding; \(R\) represents the actual fluorescence ratio; \(R_{\text{max}}\) is the maximum fluorescence ratio determined from cells
suspended in Tyrode's buffer supplemented with 0.1 % Triton-X100 + 10 mM CaCl$_2$; $R_{\text{min}}$ is the mean fluorescence ratio determined from cells preincubated with 70 µM BAPTA, AM and resuspended in Tyrode's buffer supplemented with 5 mM EGTA; $F_{\text{max}}$ and $F_{\text{min}}$ represent the fluorescence values (arbitrary units) of Oregon Green BAPTA-1 for $R_{\text{max}}$ and $R_{\text{min}}$, respectively.

**C.2.9.4.2 Intracellular calcium measurements by a fluorimeter**

Platelets isolated from blood and washed as described above, were suspended in Tyrode’s buffer without calcium and loaded with fura-2 AM (5 µM) in the presence of Pluronic F-127 (0.2 µg/ml) (both from Molecular Probes) for 30 min at 37°C. After labeling, platelets were washed once and resuspended in Tyrode’s buffer containing 1 mM Ca$^{2+}$ or 0.5 mM EGTA. Stirred platelets were activated with different agonists and fluorescence was measured with a PerkinElmer LS 55 fluorimeter. Excitation was alternated between 340 and 380 nm, and emission was measured at 509 nm. Each measurement was calibrated using 1% Triton X-100 and EGTA.

**C.2.10 In vivo experiments**

**C.2.10.1 Bleeding time experiments**

Mice were anesthetized by intraperitoneal injection of a mixture of fentanyl (Janssen-Cilag GmbH, Neuss, Germany), midazolam (Roche Pharma AG, Grenzach-Wyhlen, Germany) and medetomidine (Pfizer, Karlsruhe, Germany), and a 2mm segment of the tail tip was cut off with a scalpel. Tail bleeding was monitored by gently absorbing blood with filter paper at 20 second intervals, without making contact with the wound site. When no blood was observed on the paper, bleeding was determined to have ceased. Experiments were stopped after 20 minutes and the anaesthesia was antagonized with atipamezol (Pfizer, Karlsruhe, Germany), flumazenil and naloxon (both from Delta Select GmbH, Dreieich, Germany).
C.2.10.2 Intravital microscopy of thrombus formation in FeCl₃ injured mesenteric arterioles

Four weeks old mice or bone marrow chimeras 4-5 weeks after bone marrow transplantation were anesthetized by as described previously and the mesentery was exteriorized through a midline abdominal incision. Arterioles (35-60µm diameter) were visualized with a Zeiss Axiovert 200 inverted microscope (x10) equipped with a 100-W HBO fluorescent lamp source and a CoolSNAP-EZ camera (Visitron, Munich, Germany). Digital images were recorded every second and analyzed off-line using Metavue® software. Injury was induced by topical application of a 3 mm² filter paper saturated with FeCl₃ (20%) for 10 sec. Adhesion and aggregation of fluorescently labelled platelets (Dylight-488 conjugated anti-GPIX Ig derivative) in arterioles was monitored for 30 min or until complete occlusion occurred (blood flow stopped for > 1min).

C.2.10.3 Mechanical injury model of the abdominal aorta

A longitudinal midline incision was performed to open the abdominal cavity of anesthetized mice and expose the abdominal aorta. A Doppler ultrasonic flow probe was placed around the vessel and thrombosis was induced by a single firm compression with a forceps upstream of the flow probe. Blood flow was monitored until complete occlusion occurred or 30 minutes had elapsed.

C.2.10.4 Transient middle cerebral artery occlusion model

Experiments were conducted on 10-12 weeks old bone marrow chimeras according to published recommendations for research in mechanism-driven basic stroke studies(69). Transient middle cerebral artery occlusion (tMCAO) was induced under inhalation anesthesia using the intraluminal filament (6021PK10; Doccol Company) technique(70). After 60 min, the filament was withdrawn to allow reperfusion. For measurements of ischemic brain volume, animals were sacrificed 24 h after induction of
tMCAO and brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, Germany). Brain infarct volumes were calculated and corrected for edema as described(70). Neurological function and motor function were assessed by two independent and blinded investigators 24 h after tMACO as described(70).

C.2.10.4.1 Assessment of infarction and hemorrhage by MRI

MRI was performed 24 h and 7 d after transient ischemia on a 1.5 T unit (Vision; Siemens) under inhalation anesthesia. A custom made dual channel surface coil was used for all measurements (A063HACG; Rapid Biomedical). The MR protocol included a coronal T2-w sequence (slice thickness 2 mm), and a coronal T2-w gradient echo CISS sequence (Constructed Interference in Steady State; slice thickness1 mm). MR images were transferred to an external workstation (Leonardo; Siemens) for data processing. Visual analysis of infarct morphology and search for eventual intracerebral hemorrhage was performed in a blinded manner. Infarct volumes were calculated by planimetry of hyperintense areas on high-resolution CISS images.

C.2.10.5 Splenectomy

Mice were anesthetized as described above. The spleen was identified from a left subcostal laparotomy and removed after appropriate blood vessel ligation. The wound was closed with 5-0 Prolene.

C.2.10.6 Platelet life span experiments

Circulating platelets were labelled in vivo with the intravenous injection of 5 µg Alexa-488-anti-GPIX antibody (2 µg in the mutant) in 200 µL PBS. 30 min after antibody injection (and every 24 hours for 5 days) 50 µL blood was taken from the retroorbital plexus of the treated mice and diluted 1:20 in heparin containing PBS. The diluted whole blood was in turn incubated with 10 µL PE-Cy5-anti-GPIb for 10 minutes at
room temperature. The Alexa-488 positive platelet population was determined as the percentage of the whole PE-Cy5 positive population by flow cytometry.

C.2.10.7 Generation of bone marrow chimeras

5-6 weeks old C57Bl/6 female mice were lethally irradiated with a single dose of 10 Gy. Bone marrow cells from 6 weeks old wild type, $\text{Stim1}^{\text{Sax/}}$, $\text{Stim1}^{\text{Sax/}}$, or $\text{Orai1}^{\text{Sax/}}$ mice were washed out of isolated femora with ice cold PBS and injected intravenously into the irradiated mice at a cell number of $4 \times 10^6$ cells/mouse. All recipient animals received acidified water containing 2g/L Neomycin sulphate for 6 weeks after transplantation. Platelet counts reached physiological levels four weeks after transplantation.

C.2.11 Histology

Formalin-fixed brains and aortas embedded in paraffin (Histolab Products AB) were cut into 4-μm thick sections and mounted. After removal of paraffin, tissues were stained with hematoxylin and eosin (Sigma-Aldrich).

C.2.12 Data analysis

The results shown are mean ± S.D. Statistical analysis were performed using Student’s $t$ test with $p < 0.05$ taken as the level of significance.
D. RESULTS

D.1 Analysis of a mouse line bearing an activating EF-hand mutation in STIM1 indicate an important role for the protein in platelet function

D.1.1 A single amino acid change in the EF-hand domain of STIM1 causes a dominant negative phenotype in mutant mice

We have received mice from Ingenium Pharmaceuticals AG (Munich, Germany) with a single point mutation in the EF-hand domain of STIM1. They induced genome wide random mutations using the chemical mutagen N-ethyl-N-nitrosourea. Offsprings bred for homozygosity of the induced mutations were tested for abnormalities by visual inspection, a battery of neurological and behavioural tests, and in parameters of hematology and clinical chemistry. A mouse line was established with dominant inheritance of elevated mean platelet volume (MPV) and reduced platelet counts (Plt) in comparison to wild-type controls (Fig.6A). All other hematological parameters, such as red and white blood cell counts and erythrocyte indices, were unaltered in 6-week and 6-months old mutant animals, just like the expression of platelet surface glycoproteins (Table 1).

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<td>CD9</td>
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Table 1. Platelet membrane glycoprotein expression in Stim1Sax/+ platelets. Diluted whole blood was stained with fluorophore-labeled antibodies at saturating concentrations for 15 min at RT and analyzed directly on a FACScalibur (Becton Dickinson, Heidelberg). Platelets were gated by FSC/SSC characteristics. Results are given as the mean fluorescence intensity ± SD of 6-12 mice per group. (Grosse J, Braun A, Varga-Szabo D et al. J Clin Invest 2007)
The mouse line was named Saxcoburggotski (Sax). Both parameters, MPV and Plt, were later shown to reliably predict the genotype (100% penetrance). Using animals out- and back-crossed to C57BL/6, the phenotypic trait was mapped to chromosome 7 between the SNP marker Omp and microsatellite marker D7Mit220 (data not shown). Sequencing of genes located in the mapping region identified an A to G transition at nucleotide 444 in exon 2 of the gene of stromal interaction molecule 1 (Stim1, NM_009287) resulting in an amino acid exchange in the EF-hand motif at position 84 (Asp84Gly, or D84G). The identified mutation, Stim1\textsuperscript{Sax}, was absent in the parental strains and co-segregated with the Sax phenotype in all animals tested.

To explore the phenotype of homozygous animals, heterozygous breeder pairs were set up. Only one homozygous animal was detected among 72 offspring deviating from a mendelian birth rate (Chi square exact test, $p < 0.001$), whereas the expected frequency was observed for heterozygotes (66%) and wild-type (33%) after exclusion of homozygotes. The hematological parameters of the homozygous animal were within the 95% percentile of the heterozygotes suggesting that STIM1 might play a critical role in embryonic development. Many residual bodies were detected at embryonic day E7 - E9 and only few Stim1\textsuperscript{Sax/Sax} embryos survived until E13 - E14 which, however, displayed severe hemorrhages in different regions of the body (Fig.6B). At later stages, only material obtained from resorption sites but not morphologically normal embryos were genotyped as Stim1\textsuperscript{Sax/Sax}. This indicates that Stim1\textsuperscript{Sax/Sax} mice suffer from additional, most likely vascular, defects that contribute to the observed embryonic lethality.

![Figure 6. The Sax mutation causes macrothrombocytopenia and bleeding in mice. (A) Reduced platelet counts (Plt) and increased mean platelet volume (MPV) in Stim1\textsuperscript{Sax/+} mice. (B) Severe hemorrhage in different regions of the body of Stim1\textsuperscript{Sax/Sax} embryos. (Grosse J, Braun A, Varga-Szabo D et al. J Clin Invest 2007)](image_url)
D.1.2 STIM1 is highly expressed in platelets

STIM1 has been shown to regulate SOC channels in Jurkat T cells but little was known about its expression in other cells of the hematopoietic system. Western-blot analysis revealed high levels of STIM1 protein in platelets whereas lower levels were detected in T cells (2.3-fold) and B cells (10.2-fold), suggesting that STIM1 might play an important role in platelets (Fig.7).

D.1.3 The Sax mutation causes preactivation and reduced life span in platelets due to permanently opened SOC channels in the plasma membrane

D.1.3.1 Permanently opened SOC channels lead to elevated basal calcium levels in Stim1Sax/+ platelets

As STIM1 has been proposed to be the calcium sensor in the ER regulating SOCE in T cells, intracellular Ca\(^{2+}\)-measurements on Stim1Sax/+ platelets were performed. These measurements yielded an ~3 fold increase in the basal [Ca\(^{2+}\)], in mutant platelets as compared to wild-type (54.3 ± 15.2 nM vs. 18.3 ± 7.2 nM, \(n=6\) for wild-type and \(n=14\) for mutant, \(p=0.0000329\)) (Fig.8A,B) raising the possibility of permanently opened calcium channels in the cell membrane. This was further confirmed when the cells were exposed to alternating extracellular [Ca\(^{2+}\)]. In the absence of extracellular Ca\(^{2+}\), the mutant cells showed similar [Ca\(^{2+}\)], to that of the wild-type controls (27.7 ± 8.2 versus 17.3 ± 7.6 nM, respectively; \(n = 3\) per group). Addition of 2 mM Ca\(^{2+}\) had virtually no effect on wild-type platelets but led to an increase of [Ca\(^{2+}\)], up to 78.9 ± 14.3 nM in the
mutant cells. This was reversed upon removal of extracellular Ca\textsuperscript{2+} and re-established when Ca\textsuperscript{2+} was added again (Fig.8C). These results strongly support the idea of continuously opened calcium channels in the plasma membrane of these cells.

**Figure 8.** Permanently opened SOC channels in *Stim1\textsuperscript{Sax/+}* platelets. (A) Basal calcium levels are ~3 fold elevated in mutant platelets in the presence of 2 mM extracellular [Ca\textsuperscript{2+}]. Representative curves of at least 3 measurements per group. (B) Basal [Ca\textsuperscript{2+}] in wild-type and *Stim1\textsuperscript{Sax/+}* platelets were measured in the presence or absence of 2 mM extracellular Ca\textsuperscript{2+}. (C) Oregon Green BAPTA-1/FuraRed-loaded wild-type and *Stim1\textsuperscript{Sax/+}* platelets were exposed to alternating extracellular Ca\textsuperscript{2+} concentrations and intracellular concentrations were monitored by flow cytometry. Representative curves of 4 measurements per group. (Grosse J, Braun A, Varga-Szabo D et al. *J Clin Invest* 2007)

**D.1.3.2 Preactivation and reduced life span of *Stim1\textsuperscript{Sax/+}* platelets**

Platelets are very sensitive cells and respond with activation already to minor stimuli. Regarding the central role of calcium in the activation process, the basal [Ca\textsuperscript{2+}] must be tightly regulated to avoid undesired platelet activation in the circulation. As already an increase of ~50nM in the [Ca\textsuperscript{2+}], results in platelet shape change(71) and the mutant platelets have ~3 fold higher basal calcium levels, the resting state of these platelets was studied. Indeed, using the JON/A-PE antibody(72), I found a 50% increase in the expression of activated integrin αIIbβ3 on the surface of unstimulated mutant platelets (Fig.9A). Furthermore, they responded to epinephrine (10 µM). Whereas, this agonist alone causes no activation of wild-type platelets, it enhances the effect of agonists that mobilize intracellular calcium(73). While wild-type platelets failed to respond to this stimulus, a robust increase in integrin αIIbβ3 activation was detected in the mutant cells (Fig.9A).

Under physiological conditions, activated platelets do not circulate in the flowing blood, because they get rapidly cleared by macrophages in the spleen, liver and lung. If
platelets were produced already preactivated, this could lead to thrombocytopenia through the permanent removal of newly produced platelets from the circulation; a mechanism which could explain the observed macrothrombocytopenic phenotype of the $Stim1^{Sax/+]}$ mice. To test this hypothesis, I administered a non-saturating dose of an Alexa Fluor 488–labeled non-cytotoxic antibody against the platelet-specific GPIb-V-IX complex, and the percentage of fluorescence-tagged platelets in the circulation was monitored for 5 days by flow cytometry. In conjunction with published platelet life times, I detected a decline of the fluorescent population in wild-type animals over 5 days(74) (Fig.9B). In sharp contrast, in $Stim1^{Sax/+]}$ mice the labeled platelet population was reduced to approximately 27% after 24 hours and even further, to approximately 4% and 0% on days 2 and 3, respectively, demonstrating a markedly reduced platelet life span. A similar result was also obtained when the experiment was carried out in lethally irradiated wild-type mice that had been transplanted with wild-type or $Stim1^{Sax/+]}$ bone marrow 4 weeks before the experiment (Fig.9B), excluding an involvement of cells of non-hematopoietic origin in the increased clearing of platelets.

![Figure 9. Platelet preactivation causes reduced life span of the mutant cells.](image)

(A) Increased basal levels of activated integrin $\alpha$IIb$\beta$3 (JON/A-PE) are detectable in unstimulated and epinephrine-stimulated $Stim1^{Sax/+]}$ platelets. A PE-conjugated isotype control antibody yielded identical negative signals in wild-type and mutant platelets (not shown). The results are expressed as mean fluorescence intensity ± SD of 9 experiments. (B) The circulating platelet population was fluorescently labeled in the indicated mice and the portion of labeled cells in the total platelet population was monitored by flow cytometry (n=6 per group). The total platelet count did not change significantly during the experiment. (Grosse J, Braun A, Varga-Szabo D et al. J Clin Invest 2007)
D.1.4 Defective SOCE in Stim1Sax/+/ platelets

The above results suggested the presence of constitutively active SOC channels in Stim1Sax/+/ platelets. To test the consequences of this defect on SOCE, the cells were stimulated with TG. TG-evoked store release occurred significantly faster in the mutant cells but the peak value was reduced by approximately 60% as compared with the wild-type control. Similarly, the subsequent TG-dependent Ca²⁺-influx was reduced by approximately 70% in Stim1Sax/+ cells as compared with wild-type, demonstrating largely defective SOC activation (Fig.10A-B).

Figure 10. Defective SOCE in Stim1Sax/+ platelets. Oregon Green BAPTA-1/FuraRed-loaded platelets were stimulated with 5 µM TG for 10 min followed by addition of extracellular Ca²⁺ and [Ca²⁺]i was monitored by flow cytometry. Representative measurements (A) and maximal [Ca²⁺]i ± SD (n = 6 per group) before and after addition of 2 mM Ca²⁺ (B) are shown. (Grosse J, Braun A, Varga-Szabo D et al. J Clin Invest 2007)

D.1.5 Defective collagen responses and thrombus formation in Stim1Sax/+ mice

D.1.5.1 Defective Ca²⁺ response and platelet activation through PLCγ2 in Stim1Sax/+ mice

Next I tested the consequences of increased basal [Ca²⁺]i and defective SOCE for platelet activation. For this different agonists were used evoking Ca²⁺ entry either via G protein-coupled receptors (GPCRs) – through by activating PLCβ – (thrombin, ADP, TxA2) or via ITAM-coupled receptors – through activating PLCγ2 – (collagen related peptide [CRP], rhodocytin [RC]). Stimulation of wild-type platelets with thrombin (0.1 U/ml) or a combination of ADP (10 µM) and U46619 (1 µM) yielded similar increases
in [Ca\textsuperscript{2+}], in wild-type and mutant platelets (Fig.11A-B). In marked contrast, stimulation with the GPVI-specific agonist CRP or the CLEC-2 agonist RC yielded profound rises in [Ca\textsuperscript{2+}], in wild-type platelets but only minor responses in Stim1\textsuperscript{Sax/+} platelets (Fig.11A-B). These results suggested that Stim1\textsuperscript{Sax/+} specifically interferes with PLC\textgamma{2}-but not PLC\textbeta{-dependent platelet activation. This was confirmed by flow cytometric analysis of integrin \alphaIIb\beta3 activation and P-selectin exposure in response to these agonists. The G protein–coupled agonists thrombin and ADP (Fig.11C) as well as the combination of ADP and the stable TxA2 analogue U46619 (data not shown) induced comparable responses in wild-type and Stim1\textsuperscript{Sax/+} platelets. In contrast, the ITAM-coupled agonists CRP and RC induced strong and irreversible activation of wild-type platelets but virtually no response in the mutant cells (Fig.11C).

Figure 11. Selective ITAM signaling defect in Stim1\textsuperscript{Sax/+} platelets. (A,B) Oregon Green BAPTA-1/FuraRed-loaded platelets were stimulated with the indicated agonists in the presence of 2 mM Ca\textsuperscript{2+} and [Ca\textsuperscript{2+}], was monitored by flow cytometry. Representative measurements (A) and maximal [Ca\textsuperscript{2+}] \pm SD (n = 6 per group) (B) are shown. (C) Wild-type and Stim1\textsuperscript{Sax/+} platelets were stimulated with the indicated agonists, incubated with fluorophore-labeled antibodies to detect integrin \alphaIIb\beta3 activation (JON/A-PE) and degranulation (anti-P-selectin-FITC) and analyzed directly. Platelets were gated by FSC/SSC characteristics and FL4 positivity (anti-GPIb-PE-Cy5). The results shown are representative for 12 experiments. (Grosse J, Braun A, Varga-Szabo D et al. J Clin Invest 2007)
This defect was not a consequence of the elevated basal [Ca\(^{2+}\)]\(i\) in \(\text{Stim1Sax}^{+/+}\) cells, as an increase in [Ca\(^{2+}\)]\(i\), induced for up to 30 minutes in wild-type platelets by the Gq-PLC\(\beta\)-stimulating agonist serotonin (10 \(\mu\)M), did not reduce subsequent CRP- or RC-induced activation of the cells (data not shown).

**D.1.5.2 Defective thrombus formation in \(\text{Stim1Sax}^{+/+}\) mice**

To see what impact the above detailed activation defect has on thrombus formation under flow, the interaction of \(\text{Stim1Sax}^{+/+}\) platelets with fibrous collagen was tested in a whole blood perfusion assay. As mutant mice have only ~15 % of the normal platelet counts, I reduced the amount of platelets in the wild-type blood using differential centrifugation. Control platelets, at appropriately reduced counts readily established firm adhesions on the collagen fibers and rapidly built stable three-dimensional aggregates. In contrast, the vast majority of \(\text{Stim1Sax}^{+/+}\) platelets that initially came in contact with the collagen either detached within a few seconds or translocated along the fibers before stable adhesion was established. Three-dimensional thrombi were not formed in these samples. As a result, the surface area covered by platelets at the end of the experiment was markedly reduced in the mutant mice (Fig.12A). Thus, \(\text{Stim1Sax}^{+/+}\) mice show reduced platelet counts and a selective impairment of the cells to interact with collagen, which is considered the most thrombogenic constituent of the vessel wall and a major trigger of thrombus formation at sites of vascular injury(5). To test the impact of this combined defect on hemostasis, tail bleeding times were determined and found to be significantly prolonged in \(\text{Stim1Sax}^{+/+}\) mice (Fig.12B). In parallel analyses, blood loss was found to be significantly larger in the \(\text{Stim1Sax}^{+/+}\) group (59 \(\mu\)L; 95% CI: 43 – 75 \(\mu\)L) than in wild type littermates (11 \(\mu\)L; 2 – 19 \(\mu\)L). This severe hemostatic defect also translated into defective pathological thrombus formation as assessed in a model of arterial thrombosis where the abdominal aorta is mechanically injured and blood flow is monitored by an ultrasonic flow probe. In this model, thrombus formation is triggered predominantly by collagen and thus occurs in a GPVI/PLC\(\gamma\)2-dependent manner(72). While all wild-type animals formed irreversible occlusions within 15 minutes, no occlusive thrombus formation occurred in the \(\text{Stim1Sax}^{+/+}\) group for up to 30 min (Fig.12C). Next, the mice were tested in a model of ferric chloride-induced injury of mesenteric arterioles where thrombus formation is largely driven by thrombin and
less dependent on GPVI/PLCγ2 signaling(75). Despite their reduced platelet count, 67% (12/18) of the Stim1Sax/+ mice formed occlusive thrombi in this model within the same time frame as wild-type controls (11/12 vessels occluded) (Fig.12D-E). These results demonstrate that the Sax mutation yields selective protection from collagen-dependent thrombosis but also increased bleeding times in mice.

Figure 12. Defective thrombus formation and bleeding in Stim1Sax/+ mice. (A) Wild-type blood with appropriately reduced platelet counts and Stim1Sax/+ blood was perfused over a collagen-coated surface at a wall shear rate of 1000/s for 4 min. Left: surface area covered by platelets/thrombi is given as mean of 6 experiments ± SD. Right: representative phase contrast images taken at the end of the experiment. (B) Tail bleeding times were monitored for 20 min. (C) The abdominal aorta was mechanically injured and blood flow was monitored for 30 min or until complete occlusion occurred. (B, C) Each symbol represents one individual. (D, E) Thrombosis was induced in mesenteric arterioles by topical application of FeCl3 and the time to complete occlusion was determined by intravital microscopy. (D) Mean occlusion times ± SD. The numbers above the bars represent the number of occluded arteries/total arteries operated. (E) Representative pictures of one experiment where occlusion occurred in both the wild-type and Stim1Sax/+ mouse. Indicated time points represent minutes after FeCl3-induced injury. (Grosse J, Braun A, Varga-Szabo D et al. J Clin Invest 2007)

D.1.6. Summary I.

Targeted mutations of the acidic residues coordinating Ca2+ binding to the EF-hand motif of STIM1 have been used to demonstrate its capacity to activate SOC channels in Jurkat T cells(54;56). In line with this model, we found increased basal Ca2+ levels in
the presence but not absence of extracellular Ca\textsuperscript{2+} in $Stim1^{Sax/+}$ platelets. This resulted in a preactivated state of these cells and in turn led to the removal of platelets from the circulation, manifesting in macrothrombocytopenia. We observed reduced SOCE and a selective activation defect through PLC\textgreek{y}2 in $Stim1^{Sax/+}$ platelets. Consequently, mutant platelets showed defective thrombus formation and mutant mice had a bleeding phenotype.
D.2 Analysis of platelet function in Stim1−/− mice

D.2.1 Stim1−/− mice born severely ill and show growth retardation

To address the function of STIM1 in vivo, the Stim1 gene was disrupted in mice by insertion of an intronic gene-trap cassette. Mice heterozygous for the STIM1-null mutation developed normally, while a majority (~70%) of mice lacking STIM1 (Stim1−/−) died within a few hours after birth. Marked cyanosis was noted before death, suggesting a cardio-pulmonary defect. Surviving Stim1−/− mice exhibited marked growth retardation, achieving ~50% of the weight of wild-type littermates at 3 and 7 weeks of age (Fig.13A-B) and dying latest 8 weeks after birth. Western-blot analyses confirmed the absence of STIM1 in platelets (Fig.13C, upper panel) and other tissues (not shown). Blood platelet counts, mean platelet volume (MPV), and expression levels of major platelet surface receptors, including GPIb-V-IX, GPVI, CD9 and α1- and β3 integrins (not shown) were normal, indicating that STIM1 is not essential for megakaryopoiesis or platelet production. Similarly, no differences were found in red blood cell counts, hematocrit, or the activated partial thromboplastin time (aPTT), a method for assessment of plasma coagulation (table 2).

Figure 13. Stim1−/− mice are ill and develop growth retardation. (A) Wild-type and Stim1−/− littermates, 5 weeks old. (B) Body weights of wild-type (+/+) and Stim1−/− (−/−) mice. (C) Western-blot analyses of platelet lysates from mice with the indicated genotypes (upper panel), or mice transplanted with the indicated bone marrow (BM) (lower panel). STIM1 was assessed using an antibody that can recognize the N-terminal region of the protein (66) (GOK/Stim1; BD Transduction). An antibody to β3-integrin served as control. Results from two individuals per group are shown. (Varga-Szabo D, Braun A, Kleinschnitz C et al. J Exp Med 2008)
Table 2. Hematology and hemostasis in Stim1−/− chimeras. Erythrocyte counts per nL and coagulation parameters for control and Stim1−/− chimeras. The abbreviations are hematocrit (HCT), activated partial thromboplastin time (aPTT), prothrombin time (PT), and thrombin clotting time (TCT). Values given are mean values ± SD of 5 mice for each genotype. (Varga-Szabo D, Braun A, Kleinschnitz C et al. J Exp Med 2008)

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<td>Fibrinogen</td>
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Due to the early mortality and pronounced growth retardation in Stim1−/− mice, all subsequent studies were performed with lethally irradiated wild-type mice transplanted with Stim1−/− or wild-type bone marrow. Four weeks after transplantation, platelet counts were normal and STIM1-deficiency in platelets was confirmed by Western-blot (Fig. 13C, lower panel).

D.2.2 STIM1 is a crucial component of SOCE also in platelets

To determine if STIM1 has a role in platelet SOCE, SOC influx was induced in wild-type and Stim1−/− platelets with the SERCA pump inhibitor thapsigargin (TG). Interestingly, already the TG-induced Ca2+ store release was reduced ~60% in Stim1−/− platelets compared to wild-type controls (Fig.14A). Furthermore, subsequent TG-dependent SOC influx was almost completely absent in Stim1−/− cells (Fig.14A). This demonstrated for the first time that STIM1 is essential for SOCE in platelets, and suggested that STIM1-dependent processes contribute to regulation of Ca2+ store content in these cells.

To determine the significance of defective SOCE for agonist-induced platelet activation, changes in [Ca2+]i were assessed in response to ADP, thrombin, a collagen related peptide (CRP) that stimulates the collagen receptor glycoprotein (GP)VI (Fig.14B-C), and the TxA2 analogue U46619 (not shown). Ca2+ release from intracellular stores was
reduced in Stim1−/− platelets compared to control for all agonists, indicating reduced Ca2+ levels in stores in Stim1−/− cells. In the presence of extracellular calcium, Ca2+ influx was dramatically reduced in Stim1−/− platelets. Thus, STIM1-dependent SOCE is a crucial component of the Ca2+ signaling mechanism in platelets for all major agonists, and non-SOCE makes only a minor contribution, at least under the conditions tested.

**Figure 14.** Defective calcium store release and SOCE in Stim1−/− platelets. (A) Fura-2-loaded platelets were stimulated with 5 µM TG for 10 min followed by addition of extracellular Ca2+ and [Ca2+]i was monitored. Representative measurements (left) and maximal increase in intracellular Ca2+ concentrations compared to baseline levels (∆[Ca2+]i) ± SD (n = 4 per group) before and after addition of 1 mM Ca2+ (right) are shown. (B-C) Fura-2-loaded wild-type (black line) or Stim1−/− (grey line) platelets were stimulated with thrombin (0.1 U/ml), ADP (10 µM) or CRP (10 µg/ml) in the presence of extracellular EGTA (0.5 mM) or Ca2+ (1 mM), and [Ca2+]i was monitored. Representative measurements (B) and maximal increase in intracellular Ca2+ concentrations compared to baseline levels before stimulus (∆[Ca2+]i) ± SD (n = 4 per group) (C) are shown. (Varga-Szabo D, Braun A, Kleinschnitz C et al. J Exp Med 2008)
D.2.3 The role of STIM1 in platelet activation and thrombus formation

D.2.3.1 Loss of STIM1 results in a selective GPVI signaling defect

To test the functional consequences of the severe defect in calcium signaling, I performed *in vitro* aggregation studies. Surprisingly, despite the dramatically impaired calcium responses, \( \text{Stim1}^{-/-} \) platelets aggregated normally to the G-protein coupled agonists ADP, thrombin (Fig.15A) and U46619 (not shown) – which activate PLC\( \beta \) –, even at very low concentrations of these agonists (not shown). In contrast, responses to collagen and CRP (Fig.15A) and the strong GPVI agonist convulxin (CVX, not shown) were significantly diminished. The activation defect was confirmed by flow cytometric analysis of integrin \( \alpha I I b \beta 3 \) activation, using the JON/A-PE antibody, and of degranulation-dependent P-selectin surface exposure (Fig.15B). Therefore, loss of STIM1-dependent SOCE impairs GPVI-induced integrin activation and degranulation, whereas G-protein coupled agonists are still able to induce normal activation in \( \text{Stim1}^{-/-} \) platelets in these assays, despite the defect in Ca\(^{2+}\)-signaling.

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**Figure 15. Platelet activation defect in \( \text{Stim1}^{-/-} \) mice through GPVI.** (A) Impaired aggregation of \( \text{Stim1}^{-/-} \) platelets (grey lines) in response to CRP and collagen, but not ADP and thrombin (recording time 10 min). (B) Flow cytometric analysis of \( \alpha I I b \beta 3 \) integrin activation (binding of JON/A-PE, left panel) and degranulation-dependent P-selectin exposure (right panel) in response to thrombin (0.1 U/ml), ADP (10 \( \mu \)M), CRP (10 \( \mu \)g/ml) and CVX (1 \( \mu \)g/ml). Results are means ± SD of 6 mice per group. (Varga-Szabo D, Braun A, Kleinschnitz C et al. *J Exp Med* 2008)
5.2.3.2 Impaired thrombus stability in the absence of STIM1

In vivo, platelet activation on the extracellular matrix (ECM) and thrombus growth occurs in flowing blood, where locally produced soluble mediators are rapidly cleared. Under these conditions, reduced potency of platelet activators may become limiting, particularly at the high flow rates found in arteries and arterioles. Therefore, I analyzed the ability of Stim1−/− platelets to form thrombi on collagen-coated surfaces in a whole blood perfusion system. Under high shear conditions (1,700 s⁻¹), wild-type platelets adhered to collagen fibers and formed aggregates within 2 minutes that consistently grew into large thrombi by the end of the 4 minutes perfusion period (Fig.16A-B). In sharp contrast, Stim1−/− platelets exhibited reduced adhesion, and three-dimensional growth of thrombi was markedly impaired. As a consequence, the surface area covered by platelets and the total thrombus volume were reduced ~42 % and ~81 %, respectively. Similar results were obtained at intermediate shear rates (1,000 s⁻¹, data not shown). These findings indicate that STIM1-mediated SOCE is required for efficient platelet activation on collagen, and on the surface of growing thrombi under conditions of high shear.

Figure 16. Reduced thrombus formation in flow. Stim1−/− platelets in whole blood fail to form stable thrombi when perfused over a collagen-coated (0.2 mg/ml) surface at a shear rate of 1,700 s⁻¹. (A) Representative phase contrast images. Bar, 100 µm. (B) Mean surface coverage (left) and relative platelet deposition as measured by the integrated fluorescent intensity (IFI) per mm² (right) ± SD (n=4). (Varga-Szabo D, Braun A, Kleinschnitz C et al. J Exp Med 2008)

As platelet aggregation may contribute to pathological, occlusive thrombus formation, I studied the effects of STIM1-deficiency on ischemia and infarction by in vivo fluorescence microscopy following ferric chloride-induced mesenteric arteriole injury. In all wild-type chimeras, the formation of small aggregates was observed ~5 minutes after injury, with progression to complete vessel occlusion in 8 of 10 mice within 30 min (mean occlusion time: 16.5 ± 2.8 min) (Fig.17A-C). In contrast, aggregate
formation was significantly delayed in ~50% of the Stim1−/− chimeras (10.6 ± 5.8 min in wild-type and 17.1 ± 7.3 min in Stim1−/− chimeras until the first thrombus > 20 µm in diameter appeared, p<0.05), and formation of stable thrombi was almost completely abrogated. This defect was due to the release of individual platelets from the surface of the thrombi and not to embolization of large thrombus fragments. Blood flow was maintained throughout the observation period in 9 of 10 vessels, demonstrating a crucial role for STIM1 during occlusive thrombus formation. This was confirmed in a second arterial thrombosis model, where the abdominal aorta was mechanically injured and blood flow was monitored with an ultrasonic flow probe. While 10 of 11 control chimeras formed irreversible occlusions within 16 minutes (mean occlusion time: 4.4 ± 4.1 min), occlusive thrombus formation did not occur in 6 of 8 Stim1−/− chimeras during the 30 min observation period (p<0.002) (Fig.17D-E). These results demonstrate that STIM1 is required for the propagation and stabilization of platelet-rich thrombi in small and large arteries, irrespective of the type of injury.

Figure 17. In vivo analysis of thrombosis. (A-C) Mesenteric arterioles were treated with FeCl3, and adhesion and thrombus formation of fluorescently-labeled platelets was monitored by in vivo video microscopy. Representative images (A), time to appearance of first thrombus > 20 µm (B), and time to vessel occlusion (C) are shown. Bar, 50 µm. Each symbol represents one individual. (D-E) The abdominal aorta was mechanically injured and blood flow monitored for 30 min or until complete occlusion occurred (blood flow stopped > 5 min). (D) Representative cross-sections of the abdominal aorta of mice with wild-type or Stim1−/− platelets 30 min after injury. Bar, 250 µm. (E) Time to vessel occlusion. Each symbol represents one individual. (Varga-Szabo D, Braun A, Kleinschnitz C et al. J Exp Med 2008)
D.2.4 STIM1 is an essential mediator of ischemic brain infarction

Ischemic stroke is the third leading cause of death and disability in industrialized countries(76). Although it is well established that microvascular integrity is disturbed during cerebral ischemia(77), the signaling cascades involved in intravascular thrombus formation in the brain are poorly understood. To determine the importance of STIM1-dependent SOCE in this process, in collaboration with the Neurology Department of the University of Würzburg we studied the development of neuronal damage in Stim1−/− chimeras following transient cerebral ischemia in a model that depends on thrombus formation in the microvessels downstream from a middle cerebral artery (MCA) occlusion(78). To initiate transient cerebral ischemia, a thread was advanced through the carotid artery into the MCA and allowed to remain for one hour (transient MCA occlusion - tMCAO), reducing regional cerebral flow by >90 % (70). In Stim1−/− chimeras, infarct volumes 24 hours after reperfusion, as assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining, were reduced to < 30% of the infarct volumes in control chimeras (17.0 ± 4.4 mm³ versus 62.9 ± 19.3 mm³, p < 0.0001) (Fig.18A-B). Reductions in infarct size were functionally relevant, as the Bederson score assessing global neurological function (1.86 ± 0.48 versus 3.07 ± 0.35, respectively; p < 0.0001) and the grip test, which specifically measures motor function and coordination (3.71 ± 0.39 versus 2.00 ± 0.65, respectively; p < 0.0001), were significantly better in Stim1−/− chimeras compared to controls (Fig.18C-D). Serial magnetic resonance imaging (MRI) on living mice was used to confirm the protective effect of STIM1-deficiency on infarct development. Hyperintense ischemic infarcts on T2-w MRI in Stim1−/− chimeras were <10% of the size of infarcts in control chimeras 24 hrs after tMCAO (p < 0.0001, Fig.18E). Importantly, infarct volume did not increase between day 1 and day 7, indicating a sustained protective effect for STIM1-deficiency. Moreover, no intracranial hemorrhage was detected on T2-weighted gradient echo images, a highly sensitive MRI sequence for detection of blood (Fig.18E), indicating that STIM1-deficiency in hematopoietic cells is not associated with an increase in bleeding complications in the brain. Consistent with the TTC stains and MRI images, histological analysis revealed massive ischemic infarction of the basal ganglia and
neocortex in control chimeras, but only limited infarction of the basal ganglia in Stim1−/− chimeras (Fig. 18F). The density of CD3-positive T cell and monocyte/macrophage infiltrates in brain infarcts was low, and did not differ between Stim1−/− and control chimeras at 24h (data not shown).

Figure 18. Stim1−/− chimeras are protected from cerebral ischemia. (A) Representative images of three corresponding coronal sections from control and Stim1−/− chimeras mice stained with TTC 24 hrs after tMCAO. Infarcts in Stim1−/− chimeras are restricted to the basal ganglia (right arrow) in contrast to controls (left arrows). (B) Brain infarct volumes in control and Stim1−/− chimeras (n=7 per group, p < 0.0001). (C-D) Neurological Bederson score and grip test assessed at day 1 following tMACO of control and Stim1−/− chimeras animals (n=7, p < 0.0001). (E) The coronal T2-w MR brain image shows a large hyperintense ischemic lesion at day 1 after tMCAO in controls (left upper arrows). Infarcts are smaller in Stim1−/− chimeras (left lower arrow), and T2-hyperintensity decreases by day 7 during infarct maturation (right arrow). Importantly, hypointense areas indicating intracerebral hemorrhage were not seen in Stim1−/− chimeras, demonstrating that STIM1 deficiency does not increase the risk of hemorrhagic transformation, even at advanced stages of infarct development. Bar, 3 mm. (F) Hematoxylin and eosin stained sections of corresponding territories in the ischemic hemispheres of control and Stim1−/− chimeras. Infarcts are restricted to the basal ganglia in Stim1−/− chimeras but consistently include the cortex in controls. Magnification x 100-fold (left panel) and x 400-fold (right panel). Bar, 200 µm (left panel) and 50 µm (right panel). (Varga-Szabo D, Braun A, Kleinschnitz C et al. J Exp Med 2008)
D.2.5 STIM1 mediates hemostasis

To test whether the defect in \( \text{Stim}1^{-/-} \) platelets impaired hemostasis, I measured tail bleeding times. While bleeding stopped in 28 of 30 (93.3\%) control mice within 10 min, bleeding was highly variable in \( \text{Stim}1^{-/-} \) chimeras, with 11 of 31 (35.5 \%) mice bleeding for >10 minutes (Fig.19) \( p < 0.02 \). These results show that STIM1 is required for normal hemostasis.

D.2.6 Summary II.

My studies on STIM1-deficient mice showed severely defective Ca\(^{2+}\) responses to all major agonists in \( \text{Stim}1^{-/-} \) platelets, clearly establishing SOCE as the major route of Ca\(^{2+}\) entry in these cells and STIM1 as an essential mediator of this process. Besides the severely impaired SOCE I also observed reduced Ca\(^{2+}\) release from intracellular stores upon agonist-induced platelet activation, which likely reflects a lower filling state of the ER, as shown by passively emptying the stores with the SERCA inhibitor TG. Although STIM1-deficiency severely reduced Ca\(^{2+}\) entry in platelets in response to all agonists tested, it did not impair G-protein coupled receptor (GPCR) / PLC\(\beta\) triggered integrin \(\alpha_{IIb}\beta3\) activation or release of granule content in the absence of flow, even at very low agonist concentrations. In contrast, GPVI/PLC\(\gamma2\)-induced cellular activation was impaired under these experimental conditions, even at very high agonist concentrations. The rather moderate activation deficits seen in \( \text{Stim}1^{-/-} \) platelets in the absence of flow translated into severely defective formation of stable three-dimensional thrombi under
conditions of medium and high shear and *in vivo*. Finally, we found that *Stim1*−/− chimeras are protected from neuronal damage following transient cerebral ischemia without displaying an increased risk of intracranial hemorrhage despite prolonged tail bleeding times observed in a subgroup of the animals, which confirms that there is no direct correlation between bleeding time and bleeding risk(79).
D.3 Analysis of platelet function in TRPC1\textsuperscript{−/−} mice

After identifying STIM1 as the calcium sensor molecule in the ER of platelets, I next investigated the question of the store-operated Ca\textsuperscript{2+} channel in these cells. As mentioned already in the introduction, one candidate molecule is the canonical transient receptor potential channel 1 (TRPC1)(36;37), however, there is much debate about the role of this channel in the platelet calcium homeostasis(45;48;49).

To investigate the role of TRPC1 in platelet physiology, I have analyzed TRPC1-deficient mice(42) kindly provided by Dr. Alexander Dietrich from Marburg.

D.3.1 TRPC1 is weakly expressed in platelets

As all the data regarding the function of TRPC1 in platelet physiology come so far from studies on human platelets, and only one publication mentions the presence of TRPC1 in murine megakaryocytes at mRNA level(47), I first investigated the expression of TRPC1 in mouse platelets. The very low levels of the protein in mouse platelet lysates made it necessary to enrich the protein content of the samples using immunoprecipitation. TRPC1 was extracted by immunoprecipitation of platelet lysates from wild-type and TRPC1\textsuperscript{−/−} mice with the anti-XTRP1 antibody (generous gift from G. Barritt, Adelaide, Australia) previously demonstrated to recognize TRPC1(48). The immunoprecipitates were Western-blotted using the TRPC1 recognizing monoclonal antibody 1F1 (generous gift from Dr. L Tsiokas, Oklahoma, USA). The protein was detected as an approximately 85 kDa band in wild-type but not mutant platelets (Fig.20).

![Figure 20. TRPC1 is present in murine platelets. Immunoprecipitations of lysates from wild-type (+/+) or TRPC1\textsuperscript{−/−} (-/-) platelets were analysed for TRPC1. TRPC1 migrates at approximately 85 kDa. Protein G migrating at 65 kDa (arrowed) and IgG (50 kDa) are also detected. (Varga-Szabo D, Authi K, Braun A et al. Pflug Arch – Eur J Physiol 2008)](image-url)
D.3.2 Loss of TRPC1 does not influence platelet SOCE

To estimate store content and SOCE in TRPC1<sup>−/−</sup> platelets, I performed intracellular Ca<sup>2+</sup> measurements. Upon Ca<sup>2+</sup> release from the ER, a conformational coupling between TRPC1 and the type II IP<sub>3</sub>-receptor has been proposed to happen, which in turn results in the opening of TRPC1 – as SOC channel – and Ca<sup>2+</sup>-entry(34;37). To test this directly, I treated platelets with 5 µM thapsigargin (TG) in Ca<sup>2+</sup>-free buffer to empty the intracellular Ca<sup>2+</sup> stores, and then added 1 mM Ca<sup>2+</sup> to measure the extent of SOCE. Unexpectedly, neither the store content, nor the amplitude of SOCE was different between wild-type and TRPC1<sup>−/−</sup> platelets (Fig.21A), demonstrating that TRPC1 is not required for TG-induced SOCE in platelets.

To test Ca<sup>2+</sup> responses to physiological agonists, I measured changes in [Ca<sup>2+</sup>]<sub>i</sub> upon platelet activation with thrombin – known to activate PLCβ through G-protein coupled receptors – and with collagen related peptide (CRP) which activates PLCγ2 through GPVI. Unexpectedly, both agonists evoked comparable Ca<sup>2+</sup> responses in wild-type and TRPC1<sup>−/−</sup> platelets (Fig.21B-C).

Figure 21. Unaltered store content and SOCE in TRPC1<sup>−/−</sup> platelets. (A) Fura-2-loaded wild-type (black line) or TRPC1<sup>−/−</sup> (grey line) platelets were incubated with 5 µM TG in Tyrode’s buffer without Ca<sup>2+</sup> for 10 min followed by addition of 1 mM extracellular Ca<sup>2+</sup> or (B) stimulated with thrombin (0.1 U/ml) or (C) CRP (10 µg/ml) in the presence of extracellular Ca<sup>2+</sup> (1 mM), and [Ca<sup>2+</sup>]<sub>i</sub> was monitored. The upper panels show representative measurements and the lower panels maximal changes in intracellular Ca<sup>2+</sup> concentrations (mean ± SD, n=4-6). (Varga-Szabo D, Authi K, Braun A et al. Pflug Arch – Eur J Physiol 2008)
D.3.3 Unaltered platelet function in TRPC1-deficient mice both in vitro and in vivo

I further investigated whether the lack of TRPC1 influences agonist-induced platelet activation in vitro. Flow cytometric analysis of integrin αIIbβ3 activation (JON/A-PE) and degranulation, determined as surface expression of P-selectin, yielded indistinguishable results for TRPC1−/− and control platelets in response to all tested agonists and agonist concentrations (Fig.22A). Similarly, no differences in reactivity were noted between TRPC1−/− and control platelets in standard aggregometry (Fig.22B) and in a flow adhesion assay, where whole blood was perfused over a collagen-coated surface at high shear rate (1000 s⁻¹). Wild-type and TRPC1−/− platelets formed stable thrombi to the same extent and with the same kinetics (Fig.22C).

Next, I analyzed the relevance of TRPC1 deficiency for platelet function in vivo in hemostasis and thrombosis. Bleeding times after amputating the tail tip of wild-type and
\(TRPC1^{-/-}\) mice were comparable (3.1 ± 1.1 min vs. 2.7 ± 1.9 min) (Fig. 23A). Similarly, application of 20% FeCl\(_3\) on the exteriorized mesenteric arteries of \(TRPC1^{-/-}\) mice resulted in fast platelet adhesion and thrombus growth leading to irreversible vessel occlusion after (13.1 ± 3.0) min, which was comparable to the kinetics of thrombus formation and vessel occlusion observed in control animals (15.1 ± 3.0 min) (Fig. 23B-C).

**Figure 23. Unaltered hemostasis and thrombosis in \(TRPC1^{-/-}\) mice.** (A) Tail bleeding times in wild-type and \(TRPC1^{-/-}\) mice. Each symbol represents one individual. (B-C) In vivo microscopy of thrombosis in injured arterioles. Mesenteric arterioles were treated with a 3 mm\(^2\) paper tip saturated by 20% FeCl\(_3\) for 10 sec and adhesion and thrombus formation of fluorescently-labeled platelets were monitored by in vivo video microscopy. Representative images (B) and time to vessel occlusion (C) are shown. (Varga-Szabo D, Authi K, Braun A et al. *Pflug Arch – Eur J Physiol* 2008)

**D.3.4 Loss of TRPC1 does not rescue the \(STIM1^{Sax/+}\) phenotype**

Earlier in my thesis I have shown that the heterozygous expression of an activating EF-hand mutant \(STIM1\) (\(Stim1^{Sax/+}\)) constitutively activates SOC channels in murine platelets resulting in elevated basal [Ca\(^{2+}\)]\(_i\), macrothrombocytopenia and bleeding(80). If TRPC1 was the SOC channel on the platelet surface that is regulated by \(STIM1\), lack of the protein should rescue or at least ameliorate the phenotype of the \(STIM1\) mutant platelets. To investigate this, I crossed the two mouse lines and studied their platelets. As shown in Fig. 24, the increase in basal [Ca\(^{2+}\)]\(_i\) and the resulting macrothrombocytopenia were not altered in \(TRPC1^{-/-}/Stim1^{Sax/+}\) as compared to \(Stim1^{Sax/+}\) mice, clearly excluding TRPC1 as the major \(STIM1\)-regulated SOC channel on the platelet surface.
Figure 24. TRPC1 deficiency does not rescue the STIM1^Sax/+ phenotype in platelets. (A) Platelet count was measured by flow cytometry in TRPC1^-/-/Stim1^Sax/+ (DMut) and control mice and are expressed as mean ± SD (n=6). (B) Platelet size was determined in wild-type, Stim1^Sax/+, TRPC1^-/- and TRPC1^-/-/Stim1^Sax/+ (DMut) mice by an automated hematology analyzer (Sysmex Deutschland GmbH, Norderstedt, Germany). Mean platelet size ± SD (n=4). (C) Flow cytometric analysis of basal [Ca^{2+}]_i in wild-type, Stim1^Sax/+, TRPC1^-/- and TRPC1^-/-/Stim1^Sax/+ (DMut) mice. Mean basal [Ca^{2+}]_i ± SD (n=4-6). (Varga-Szabo D, Authi K, Braun A et al. Pflug Arch – Eur J Physiol 2008)

D.3.5 Summary III.

My analyses on mice lacking TRPC1 has found – just like in human – weak expression of the protein in murine platelets. The calcium store content and store-operated calcium entry of these cells were indistinguishable from those of wild-type cells and I found no alterations in platelet activation \textit{in vitro} or thrombus formation \textit{in vivo}. Furthermore, crossing the activating mutant Stim1^Sax/+ and the TRPC1^-/- mouse lines did not rescue the STIM1^Sax/+ phenotype of macrothrombocytopenia due to elevated basal calcium levels in platelets. These results together clearly exclude TRPC1 to be the major SOC channel on the platelet surface.
D.4 Analysis of Orai1-deficient mice

D.4.1 Orai1 is highly expressed in human platelets

After showing that TRPC1 has no major role in SOCE in platelets, I continued the analysis with the investigation of a possible role for Orai1, a recently identified four transmembrane domain channel protein, in the process. First, I have investigated the expression pattern of the Orai channel family in human and murine platelets. Using reverse transcriptase (RT)-PCR analysis, I found Orai1 to be the predominant member of the Orai family present in human/murine platelets at mRNA level; however, very faint bands of Orai2 and Orai3 were also observed. Unfortunately, there is no commercially available antibody that would detect the murine protein, but Western-blot analysis of human platelet lysates demonstrated robust expression of Orai1, indicating that the channel might have a role in Ca\textsuperscript{2+} homeostasis in those cells (Fig.25).

![Figure 25. RT-PCR and Western-blot analysis of human and murine platelets. Orai1, 2 and 3 were assessed with the primer pairs described under materials and methods, and Western-blot was performed using an antibody from ProSci Inc. (Braun A, Varga-Szabo D, Kleinschnitz C et al. Blood 2008)](image)

D.4.2 Generation of a mouse line lacking Orai1

In order to be able to study the function of Orai1 in detail, we have generated a mouse line constitutively lacking the protein. Orai1\textsuperscript{−/−} mice were generated as described by Vig et al(67). Briefly, ES cell clone (XL922) was purchased from BayGenomics and microinjected into C57Bl/6 blastocysts to generate Orai1 chimeric mice. After germ line
transmission heterozygous and knock out animals were genotyped by Southern-blot and PCR using mouse tail DNA as described under materials and methods. Mice heterozygous for the Orai1-null mutation developed normally, while ~60 % of the Orai1-null mice died shortly after birth for unknown reason. Surviving Orai1-null animals developed significantly slower reaching only ~60 % of the body weight of their littermates at 2 weeks of age (Fig.26A-B) and showing still very high mortality as all animals died latest 4 weeks after birth. RT-PCR analysis revealed the presence of wild-type Orai1 mRNA message in control but not in Orai1-null platelets (Fig.26C).

Figure 26. Orai1-deficiency causes perinatal mortality. (A) Wild-type (+/+) and Orai1-null (-/-) littermates, 3 weeks old. (B) Body weights of wild-type and Orai1-null mice. (C) RT-PCR analyses of platelet mRNA from wild-type and Orai1-null mice. Orai1, 2 and 3 specific forward and reverse primers were used (Takashi Y 2007), actin served as control. (Braun A, Varga-Szabo D, Kleinschnitz C et al. Blood 2008)

Due to the early lethality and growth retardation of Orai1-null mice, all further studies were performed with lethally irradiated wild-type mice transplanted with Orai1-null or control bone marrow cells. Four weeks after transplantation, both groups of mice had normal platelet counts (Table 3) and RT-PCR confirmed the virtually complete absence of Orai1 mRNA in platelets from Orai1-null chimeras (data not shown). Furthermore, mean platelet volumes (MPV) and the expression of prominent surface glycoprotein receptors were similar between wild-type and Orai1-null chimeras (data not shown), as were the main hematological and clotting parameters (Table 3). Together, these results demonstrate that megakaryopoiesis and platelet formation occur independently of Orai1.
<table>
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<td>8888 ± 153</td>
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<td>8718 ± 291</td>
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<td>9.8 ± 0.7</td>
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<tr>
<td>INR</td>
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Table 3. Hematology and hemostasis in Orai1<sup>-/-</sup> chimeras. Platelet and erythrocyte counts per nL and coagulation parameters for control and Orai1<sup>-/-</sup> chimeras. The abbreviations are mean platelet volume (MPV), hematocrit (HCT), activated partial thromboplastin time (aPTT), quick test (QT), and international normalized ratio (INR). Values given are mean values ± SD of 5 mice for each genotype. (Braun A, Varga-Szabo D, Kleinschnitz C et al. Blood 2008)

D.4.3 Orai1 is the major SOC channel on the platelet surface

To test the role of Orai1 in SOCE, next I performed intracellular calcium measurements in Orai1<sup>-/-</sup> and control platelets. For this, Fura-2 loaded cells were treated with the SERCA inhibitor thapsigargin (TG) in calcium free buffer followed by addition of extracellular calcium, and changes in [Ca<sup>2+</sup>]<sub>i</sub> were monitored (Fig.27, left panel). Store release evoked by TG was comparable between wild-type and Orai1<sup>-/-</sup> platelets (78.8 ± 25.7 nM and 62 ± 13.4 nM respectively, p=0.17, n=6), whereas it was reduced in Stim1<sup>-/-</sup> platelets (42.3 ± 7 nM, p=0.005, n=6) as reported previously. However, the subsequent SOCE was almost completely blocked in the absence of Orai1 (1438 ± 466 nM vs. 155 ± 44 nM, p<0.0001, n=6; Fig.27, right panel) and this defect was similar to that seen in Stim1<sup>-/-</sup> platelets (Fig.27, right panel). These results establish Orai1 as the principal SOC channel in platelets and show that its loss cannot be functionally compensated by Orai2 or Orai3. Furthermore, these data indicate that Orai1, in contrast to STIM1, is not required for proper store content regulation in platelets.
Figure 27. Almost complete lack of SOCE in Orai1⁻/⁻ platelets. Fura-2-loaded wild-type (+/+ and Orai1⁻/⁻ (-/-) platelets were stimulated with 5 µM TG for 10 min followed by addition of 1 mM extracellular Ca²⁺, and monitoring of [Ca²⁺]. Representative measurements (left) and maximal Δ[Ca²⁺] ± SD (n = 4 per group) before and after addition of 1 mM Ca²⁺ (right) are shown. The white bars represent Stim1⁻/⁻ platelets. (Braun A, Varga-Szabo D, Kleinschnitz C et al. Blood 2008)

To investigate the impact of the Orai1-null mutation on agonist induced Ca²⁺ responses, I measured the changes in [Ca²⁺]; upon platelet activation with different agonists (Fig.28A). In agreement with the results from the TG experiments, store release in response to ADP, thrombin (Fig. 2C) and the stable TxA2 analog U46619 (not shown) which act on Gq/PLCβ-coupled receptors was unaltered in Orai1⁻/⁻ platelets compared to wild-type. Furthermore, only a very mild reduction was seen in response to collagen-related peptide (CRP), a specific ligand of the activating collagen receptor glycoprotein VI (GPVI) that triggers tyrosine phosphorylation cascades downstream of the receptor-associated immunoreceptor tyrosine-based activation motif (ITAM) culminating in the activation of PLCγ2 (69.6 ± 15.9 nM vs. 50.5 ± 14.4 nM, p<0.05, n=6; Fig.28A-B). These results again differ from those obtained with Stim1⁻/⁻ platelets where store release was strongly reduced in response to all these agonists further indicating a direct role for STIM1 in store content regulation. When the experiment was performed in the presence of extracellular calcium, however, a pronounced Ca²⁺ influx was detectable in wild-type platelets which was dramatically reduced, but not abrogated in Orai1⁻/⁻ platelets (Fig.28A-B) and thereby similarly defective as previously seen in Stim1⁻/⁻ platelets. Together, these results demonstrated that Orai1 is essential for efficient agonist induced Ca²⁺ entry in platelets but that it is not required for store content regulation in those cells. As a consequence, due to normal store release, Orai1⁻/⁻ platelets reach significantly higher cytosolic Ca²⁺ concentrations in response to all major agonists than Stim1⁻/⁻ platelets despite equally defective SOCE.
Figure 28. Defective agonist-induced Ca\(^{2+}\)-response in Orai1\(^{-/-}\) platelets. Fura-2-loaded wild-type (black line) or Orai1\(^{-/-}\) (grey line) platelets were stimulated with thrombin (0.1 U/ml), ADP (10 µM) or CRP (10 µg/ml) in calcium-free medium or in the presence of extracellular Ca\(^{2+}\) (1 mM), and [Ca\(^{2+}\)]\(_i\) was monitored. Representative measurements (A) and maximal Δ[Ca\(^{2+}\)]\(_i\) ± SD (n = 4 per group) (B) are shown. (Braun A, Varga-Szabo D, Kleinschnitz C et al. Blood 2008)

D.4.4 Defective integrin activation and granule release through PLC\(\gamma\)2 activation in Orai1\(^{-/-}\) platelets

To test the functional consequences of the defective SOCE, I first performed in vitro aggregation studies. All agonists induced a comparable activation-dependent change from discoid to spherical shape in control and Orai1\(^{-/-}\) platelets, which can be seen in aggregometry as a short decrease in light transmission following the addition of agonists. However, Orai1\(^{-/-}\) platelets aggregated normally in response to the G-protein coupled agonists ADP, thrombin (Fig.29A) and U46619 (not shown), the responses to collagen, CRP (Fig.29A) and the strong GPVI-specific agonist convulxin (CVX, not shown) were diminished at low agonist concentrations, whereas the defect was overcome at intermediate or high agonist concentrations. This selective impairment in GPVI/PLC\(\gamma\)2-mediated activation was confirmed by flow cytometric analysis of integrin αIIbβ3 activation and degranulation-dependent P-selectin surface exposure. As shown in Fig.29B, Orai1\(^{-/-}\) platelets displayed markedly reduced responses to CRP or CVX (p<0.0001), even at high concentrations, whereas the responses to ADP and thrombin were not affected. As expected, the weak agonist ADP failed to induce P-
selectin surface expression in wild-type and Orai1^{-/-} platelets. These results demonstrate that loss of Orai1-mediated SOCE specifically impairs GPVI-induced integrin activation and degranulation, whereas G-protein coupled agonists, despite defective [Ca^{2+}]_{i} signaling, are still able to induce unaltered cellular activation in these assays. Similar observations have been made with Stim1^{-/-} platelets.

Figure 29. Defective SOCE in response to PLCγ2 activation manifests in an in vitro activation defect. (A) Impaired aggregation of Orai1^{-/-} platelets (grey lines) in response to collagen, but not ADP and thrombin. (B) Flow cytometric analysis of αIIbβ3 integrin activation (upper panel) and degranulation-dependent P-selectin exposure (lower panel) in response to thrombin (0.1 U/ml), ADP (10 µM), CRP (10 µg/ml) and CVX (1 µg/ml). Results are means ± SD of 6 mice per group. (Braun A, Varga-Szabo D, Kleinschnitz C et al. Blood 2008)

D.4.5 Thrombus formation under flow is defective in the absence of Orai1

D.4.5.1 Orai1^{-/-} platelets show reduced thrombus formation ex vivo

Under physiological conditions platelet adhesion and aggregation occur in the flowing blood where high shear forces strongly influence these platelet functions. To test the significance of Orai1-mediated SOCE in thrombus formation under flow, I studied platelet adhesion to collagen in a whole blood perfusion assay at high arterial shear rates (1,700 s^{-1}). Wild-type platelets rapidly adhered to collagen and consistently formed stable three-dimensional thrombi which covered 43.6 ± 6.1 % of the total surface area at
the end of the 4 min runtime (Fig.30). In sharp contrast, platelets from Orai1\(^{-/-}\) mice could barely form three-dimensional thrombi and the overall surface coverage was reduced by \(~60\%\) compared to the control (17.6 ± 5.2, \(p<0.0001\), \(n=5\)) (Fig.30). The defect in three-dimensional thrombus formation became even more evident when the relative thrombus volume was measured and found to be reduced by \(~95\%\) (33 x 10\(^9\) ± 5.8 x 10\(^9\) vs. 2.1 x 10\(^9\) ± 1.8 x 10\(^9\)) integrated fluorescence intensity (IFI) /mm\(^2\), \(p<0.0001\), \(n=5\)) (Fig.30). These results show that Orai1-mediated SOCE is essential for the formation of stable three-dimensional thrombi under high shear flow conditions ex vivo.

**Figure 30. Defective thrombus formation ex vivo.** Orai1\(^{-/-}\) platelets in whole blood fail to form stable thrombi when perfused over a collagen-coated (0.2 mg/ml) surface at a shear rate of 1.700 s\(^{-1}\). Left: representative phase contrast images. Right: Mean surface coverage (left) and relative platelet deposition as measured by the integrated fluorescent intensity (IFI) per mm\(^2\) (right) ± SD (\(n=4\)). Bar represents 30 \(\mu\)m. (Braun A, Varga-Szabo D, Kleinschnitz C et al. Blood 2008)

### D.4.5.2 Defective thrombus formation in Orai1\(^{-/-}\) bone marrow chimeras in vivo

To assess the significance of Orai1-mediated SOCE for platelet function in vivo, wild-type and Orai1\(^{-/-}\) chimeras were intravenously injected with collagen/epinephrine (150 \(\mu\)g / kg ; 60 \(\mu\)g / kg), which causes lethal pulmonary thromboembolism(63). While all but one wild-type chimeras died within 20 min after injection due to asphyxia, 6 out of 7 Orai1\(^{-/-}\) chimeras survived the challenge (Fig.31A). This protection was based on reduced platelet activation as platelet counts 30 min after challenge (or shortly before death in the wild-type animals) were significantly higher in Orai1\(^{-/-}\) compared to wild-type chimeras (5.24 ± 0.8 in Orai1\(^{-/-}\) vs. 2.16 ± 0.9 in wild-type x 10\(^5\)/\(\mu\)l, \(p<0.005\), \(n=4\)) and the number of obstructed pulmonary vessels was \(~50\%\) less in the mutant animals (11 ± 2 vs. 19 ± 3 per histological section, \(p<0.005\), \(n=4\)) (Fig.31B).
Figure 31. Orai1<sup>-/-</sup> chimeras are protected from lethal pulmonary embolization. Lethal pulmonary embolization after injection of collagen and epinephrine in anesthetized wild-type (+/+ and Orai1<sup>-/-</sup> bone marrow chimeras. (A) Time to death through asphyxia. Each symbol represents one individual. (B) Occluded arteries in the harvested lungs per visual field. (Braun A, Varga-Szabo D, Kleinschnitz C et al. Blood 2008)

Next I assessed arterial thrombus formation in vivo in a model of arterial thrombosis where the abdominal aorta is mechanically injured and blood flow is monitored by an ultrasonic perivascular Doppler flow meter. In this model, thrombus formation is triggered predominantly by collagen and thus occurs in a GPVI/PLCγ<sub>2</sub>-dependent manner(72). Whereas all wild-type vessels occluded, blood flow stopped only in 6 of 10 Orai1<sup>-/-</sup> chimeras. However, in 4 of these 6 vessels the thrombi embolized and consequently normal blood flow was found in 8 of 10 Orai1<sup>-/-</sup> chimeras at the end of the 30 min observation period (Fig.32 A-D). In contrast, all vessels in wild-type chimeras occluded (Fig.32A-D) and only 2 of 10 vessels embolized and remained open (Fig.32A-D). Next, the mice were tested in a model of FeCl<sub>3</sub>-induced injury of mesenteric arterioles where thrombus formation is highly thrombin-dependent(75). Interestingly, 14 of 15 Orai1<sup>-/-</sup> chimeras were able to form occlusive thrombi in this model and the process showed similar kinetics as compared to the wild-type controls (11/12 vessels occluded) (Fig.32E-F). Together, these results demonstrate that Orai1-mediated SOCE is required for the stabilization of platelet-rich thrombi at sites of arterial injury under conditions where the process is mainly driven by GPIb-GPVI-PLCγ<sub>2</sub>-dependent mechanisms.
Figure 32. Reduced thrombus stability of Orai1<sup>−/−</sup> platelets in vivo. (A-D) Mechanical injury of the abdominal aorta of wild-type (+/+) and Orai1<sup>−/−</sup> (-/-) chimeric mice was performed and blood flow was monitored with a Doppler flowmeter. Representative flow measurements (A), per cent distribution of irreversible occlusion (dark grey), unstable occlusion (light grey) and no occlusion (black) (B), time to final occlusion (each symbol represents one individual) (C) and representative cross-sections of the aorta 30 min after injury (D) are shown. Bars represent 100 µm. (E-F) FeCl<sub>3</sub> induced chemical injury of small mesenteric arteries from wild-type (+/+) and Orai1<sup>−/−</sup> (-/-) chimeras. (E) Time to occlusion. Each dot represents one individual. (F) Representative fluorescent images before and 24 min after injury. (Braun A, Varga-Szabo D, Kleinschnitz C et al. Blood 2008)

D.4.6 Orai1<sup>−/−</sup> chimeras are largely protected against infarct development after ischemia/reperfusion injury of the brain

I have shown previously in my thesis that STIM1 is an essential mediator in the pathogenesis of ischemic brain infarction indicating that SOCE in platelets is crucial for the stabilization of intravascular thrombi in this setting. To directly test this hypothesis, in collaboration of the Neurology Department of the University of Würzburg we subjected Orai1<sup>−/−</sup> chimeras to occlusion of the middle cerebral artery (MCAO) with a filament as described(70). After one hour the filament was removed to allow reperfusion and the animals were followed for another 24h before the extent of infarctions was assessed quantitatively on 2,3,5-triphenyltetrazolium chloride (TTC)-stained brain slices. In Orai1<sup>−/−</sup> chimeras, infarct volumes 24 hours after reperfusion
were reduced to less than 30% of the infarct volumes in control chimeras (18.15 ± 12.82 mm³ vs. 64.54 ± 26.80 mm³, *p*<0.0001) (Fig.33A). The Bederson score assessing global neurological function (1.69 ± 0.65 vs. 3.43 ± 1.13, *p*<0.01) and the grip test, which specifically measures motor function and coordination (4.5 ± 0.76 vs. 2.14 ± 1.21, *p*<0.01), revealed that *Orai1*<sup>−/−</sup> chimeras developed less neurological deficits compared to controls (Fig.33B-C). Serial magnetic resonance imaging (MRI) on living mice showed that ischemic infarcts on T2-w MRI in *Orai1*<sup>−/−</sup> chimeras were markedly reduced compared to control chimeras 24 hrs after transient MCAO thus confirming our histological findings from TTC stained brain sections. This protective effect was sustained since no delayed infarct growth was observed between day 1 and day 5. Moreover, a highly sensitive MRI sequence for detection of blood was used to assess hemorrhagic transformation. In contrast to increased bleeding complications in this stroke model after GPIIb/IIIa blockade(70), T2-weighted gradient echo images revealed no hypointensities indicative of intracranial hemorrhages after tMCAO in *Orai1*<sup>−/−</sup> chimeras (Fig.33D). This shows that neuroprotection did not occur in expense of bleeding complications despite altered platelet function. Routine histological assessment of infarcts on hematoxylin and eosin-stained paraffin sections confirmed the TTC- and MRI findings. In *Orai1*<sup>−/−</sup> chimeras, infarcts were restricted to the basal ganglia while in control animals the neocortex was regularly involved (Fig.33E). In accordance with the findings of the cerebral ischemia-reperfusion model we found only a minor bleeding tendency of the *Orai1*<sup>−/−</sup> chimeras after amputating the tip of their tail (Fig.33F).
Studies on Orai1-deficient mice showed similarly defective Ca$^{2+}$ responses as seen previously in Stim1$^{-/-}$ platelets, clearly establishing Orai1 as the major SOC channel in platelets and further confirming that SOCE is the major route of Ca$^{2+}$ entry in these cells. In contrast to Stim1$^{-/-}$ platelets, however, Ca$^{2+}$ release from intracellular stores was
unaltered, providing direct evidence, that SOCE is not required for proper filling of the intracellular calcium stores. Similar to Stim1\textsuperscript{-/-} platelets, Orai1-deficiency did not impair G-protein coupled receptor (GPCR)/PLC\(\beta\) triggered integrin \(\alpha{IIb}\beta3\) activation or release of granule content in the absence of flow, despite the severely reduced Ca\(^{2+}\) entry in platelets in response to all agonists tested. In contrast, just like in the case of STIM1-deficiency, GPVI/PLC\(\gamma\)2-induced cellular activation was impaired under these experimental conditions, even at very high agonist concentrations. This moderate activation deficit translated into severely defective formation of stable three-dimensional thrombi under conditions of medium and high shear and \textit{in vivo}. This defect was, however, milder than in Stim1\textsuperscript{-/-} mice, as I found normal thrombus formation in a chemical injury model where thrombus formation is largely dependent on thrombin generation. Furthermore, we found that Orai1\textsuperscript{-/-} chimeras are protected from neuronal damage following transient cerebral ischemia to a similar extent as observed in Stim1\textsuperscript{-/-} chimeras. Finally, tail bleeding times were only mildly prolonged in Orai1\textsuperscript{-/-} chimeras, showing that the protection against thrombosis can be uncoupled from undesired bleeding.
E. DISCUSSION

Platelet activation and aggregation is essential to limit posttraumatic blood loss at sites of vascular injury, but also contributes to arterial thrombosis leading to myocardial infarction and stroke. Agonist-induced elevation of $[\text{Ca}^{2+}]_i$ is a central step in platelet activation, but the underlying mechanisms and its relevance in platelet physiology are barely understood. A major pathway for $\text{Ca}^{2+}$ entry in non-excitable cells involves receptor-mediated release of intracellular $\text{Ca}^{2+}$ stores followed by activation of store-operated calcium (SOC) channels in the plasma membrane. Receptor ligand binding leads to the activation of phospholipase (PL) C isoforms which in turn hydrolyze phosphoinositide-4,5-bisphosphate (PIP$_2$) to diacyl-glycerol (DAG) and inositol-1,4,5-trisphosphate (IP$_3$). DAG opens plasma membrane $\text{Ca}^{2+}$ channels directly, whereas IP$_3$ releases calcium from the intracellular stores which in turn results in calcium entry through the SOC channels. This mechanism, referred to as store-operated calcium entry (SOCE), has been intensively studied in the past two decades without major breakthrough in our understanding of the process and the underlying molecular machinery, until recently. The suggested hypothesis in platelets, the so called conformational coupling model, emphasized the importance of the interaction between the IP$_3$-receptor (IP$_3$-R) type II and canonical transient receptor potential (TRPC) channel 1. It proposed TRPC1 to be the major SOC channel in platelets and its coupling to the IP$_3$-R type II to be the trigger of channel opening. There is, however, considerable debate regarding this hypothesis. For this reason and in light of recent advances in the understanding of SOCE, in my PhD work, I investigated the platelet SOCE process in order to identify the molecular machinery involved and to study its relevance in platelet physiology.

In 2005, stromal interaction molecule (STIM) 1 has been described as the long sought calcium sensor in the ER of Drosophila S2 cells and in Jurkat T cell lines(54-56). Using Western-blot analysis, I found strong expression of the protein also in platelets (Fig.7) raising the possibility, that STIM1 plays a similar role in these cells. In the above mentioned early studies, Zhang et al. and Liou et al. used targeted mutations of the acidic residues coordinating $\text{Ca}^{2+}$ binding to the EF-hand motif of STIM1 in order to demonstrate its capacity to activate CRAC channels in Jurkat T cells(54;56). Mutations
leading to a single amino acid change in the EF-hand domain of STIM1 resulted in permanently opened SOC channels in the plasma membrane due to disrupted Ca$^{2+}$ binding of the N terminus of STIM1.

In line with these results, I found ~3 fold increased basal Ca$^{2+}$ levels in the presence but not absence of extracellular Ca$^{2+}$ in Stim1$^{Sax/+}$ platelets also bearing an activating mutation in the EF-hand domain of STIM1 (Fig.8A). Performing intracellular calcium measurements where the [Ca$^{2+}$] of the surrounding buffer was changed, I could show that these elevated Ca$^{2+}$ levels indeed derive from the constitutive activation of the plasma membrane calcium channels (Fig.8B-C).

Previous studies on human platelets have shown that already an increase in [Ca$^{2+}$]$_{i}$ of ~40-50 nM is enough to induce the change of the discoid shape of platelets into a more spherical one(71). Through this shape change, platelets gain surface to initiate more effectively interactions with extracellular matrix proteins and other platelets; for this reason these platelets are considered preactivated and in order to avoid undesired platelet aggregation and vessel occlusion, macrophages in the spleen, liver and lung remove them rapidly from the circulation. As a consequence of the 3 fold increased basal calcium levels, I found Stim1$^{Sax/+}$ platelets to be ~1.5 times bigger than the wild-type controls and to have a significantly reduced life span in vivo (Fig.6A and Fig.9B).

Furthermore, besides the macrothrombocytopenia, these platelets had partially activated αIIbβ3 integrins expressed on their surface as detected by the JON/A-PE antibody shown to bind only the active conformation of the integrin (Fig.9A).

The functional consequence of these alterations was a selective activation defect through the immunoreceptor tyrosine-based activation motif (ITAM) / PLCγ2 signaling pathway (Fig.11), defective thrombus formation in vivo and bleeding (Fig.12).

These results and the fact that store-operated calcium entry was significantly reduced in Stim1$^{Sax/+}$ platelets (Fig.10) strongly suggest that STIM1 plays a role in platelet SOCE and that SOCE is an important regulator of platelet function.

To directly investigate the function of STIM1 in platelet physiology, we generated STIM1-deficient mice. Most of these mice die perinatally probably due to a cardio-respiratory defect – as indicated by the marked cyanosis of newborn Stim1$^{-/-}$ mice – but those animals which manage to survive are also significantly smaller than wild-type controls and live no longer than 6-8 weeks (Fig.13A-B). They develop an autoimmune-
like disease (Beyersdorf et al. *in revision*), which seems to be responsible for the early lethality.

Intracellular calcium measurements on $\text{Stim1}^{-/-}$ platelets showed \(-90\% \text{ reduced SOCE}\) in these cells when emptying the stores with the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) inhibitor thapsigargin (TG) (Fig.14A). Furthermore, I found severely defective Ca\(^{2+}\) responses to all major agonists in $\text{Stim1}^{-/-}$ platelets (Fig.14B-C), clearly establishing SOCE as the major route of Ca\(^{2+}\) entry in those cells and STIM1 as an essential mediator of this process. The residual Ca\(^{2+}\) influx detected in the mutant cells suggests that other molecules may regulate SOC influx, but only to a minor extent. One candidate molecule is STIM2, which was shown to activate CRAC channels in T cells\((81;82)\), however, its expression and function in platelets have not yet been investigated\((83)\). Alternatively, the residual Ca\(^{2+}\) entry could be mediated by store-independent mechanisms as DAG and some of its metabolites have been shown to induce non-SOCE\((13)\). In line with this, canonical transient receptor potential (TRPC) channel 6 (TRPC6) has been suggested as a candidate mediating non-SOCE in platelets\((45)\).

Besides the severely impaired SOCE I also observed reduced Ca\(^{2+}\) release from intracellular stores upon agonist-induced platelet activation, which likely reflects a lower filling state of the ER, as shown by passively emptying the stores with the SERCA inhibitor TG (Fig.14A). Similar observations have very recently been reported in $\text{Stim1}^{-/-}$ mast cells\((66)\) indicating that STIM1 may be involved in the filling of intracellular Ca\(^{2+}\) stores. However, mast cells lacking the major SOC channel Orai1\((67)\) show normal store content, suggesting a SOC channel-independent role of STIM1 in store refill, possibly through interaction with the IP\(_3\) receptors or SERCA pumps in the ER\((84)\).

Although STIM1-deficiency severely reduced Ca\(^{2+}\) entry in platelets in response to all agonists tested, it did not impair G-protein coupled receptor (GPCR) / PLC\(\gamma\) triggered integrin $\alpha$IIb$\beta$3 activation or release of granule content in the absence of flow (Fig.15), even at very low agonist concentrations (not shown). This shows that SOCE is not essential for these processes when the agonist can act on the cells at constant concentrations for a prolonged period of time. In contrast, glycoprotein (GP) VI/PLC$\gamma$2-induced cellular activation was impaired under these experimental conditions, even at
very high agonist concentrations (Fig.15). The reason for this difference is not clear at present but it could be related to the fact that GPVI and GPCRs activate different PLC isoforms in platelets. GPVI ligation triggers tyrosine phosphorylation cascades downstream of the receptor-associated ITAM culminating in the activation of PLCγ2(85), whereas soluble agonists such as thrombin, ADP and TxA₂ stimulate receptors that couple to heterotrimeric G proteins (Gq) and lead to activation of PLCβ(86). Alternatively, STIM1 could play a direct role in the signaling pathway downstream of GPVI; however, this possibility requires further investigation. Clearly, the low [Ca²⁺]ᵢ alone cannot explain the defective activation downstream of GPVI/PLCγ2 as similar or lower levels are seen in response to thrombin and ADP, respectively, without causing this defect. Possibly, elevated [Ca²⁺]ᵢ levels have to act in concert with other transient signals, that may not or no longer be fully present in GPVI/PLCγ2-stimulated Stim1⁻/⁻ platelets. This could also explain why platelets expressing an activating EF hand mutant of STIM1 (Stim1Sax⁺/⁺), that leads to elevated basal [Ca²⁺]ᵢ levels, display a selective GPVI/PLCγ2 signaling defect, whereas Gq/PLCβ-induced store-release and Ca²⁺ entry are largely preserved (Fig.11).

The rather moderate activation deficits seen in Stim1⁻/⁻ platelets in the absence of flow translated into severely defective formation of stable three-dimensional thrombi under conditions of medium and high shear (Fig.16, 17). Such a phenotype has previously been reported in a number of mice with deficiencies in platelet proteins, including those lacking the heterotrimeric G protein subunit G13 or CD40 ligand (CD40-L) which display marked defects in thrombus stability under flow in vitro and in vivo despite only mild defects in activation/aggregation in the absence of flow (reviewed in(87)). This suggests that STIM1-dependent SOCE is particularly important under conditions where agonist potency becomes limiting due to rapid dilution and various stimuli have to be integrated to produce an appropriate cellular response(86). Reduced exposure times rather than low concentrations of these soluble agonists appear to cause the defect under flow, as no reduction in the aggregation response to G-protein coupled receptor activation in Stim1⁻/⁻ platelets was observed, even at very low agonist concentrations (data not shown). In addition, STIM1 may be involved in the generation of Ca²⁺ signals downstream of GPIb, which is essential for adhesion and thrombus formation under
flow but not required for activation and aggregation of platelets in the absence of shear forces(3;88).

Our data indicate that STIM1-dependent SOCE may be of greater relevance for arterial occlusive thrombus formation than for primary hemostasis, although prolonged tail bleeding was observed in a subgroup of Stim1−/− chimeras (Fig.19). This raises the interesting possibility that hemostasis and thrombosis are mechanistically distinct processes. Therefore, the identification of the mechanisms that trigger pathological thrombus formation, but are less essential to arrest bleeding may be the key to the development of safe antithrombotics. This will be of particular importance for the treatment of acute stroke, which is still the third leading cause of death and disability in industrialized countries with very limited treatment options(76). Numerous attempts to attenuate infarct progression in acute stroke patients by conventional platelet aggregation inhibitors or anti-coagulation failed due to an excess of intracerebral hemorrhages(89). We found that Stim1−/− chimeras are protected from neuronal damage following transient cerebral ischemia without displaying an increased risk of intracranial hemorrhage (Fig.18) despite prolonged tail bleeding times observed in a subgroup of the animals, which confirms that there is no clear correlation between bleeding time and bleeding risk(79). These findings are in line with our recent observation that inhibition of the GPIb-GPVI axis is protective in this model, whereas integrin αIIbβ3 (GPIIb/IIIa) inhibition is not but associated with excess intracranial bleeding(70), which corresponds well with data from clinical studies in stroke patients(89). It is important to note, however, that data obtained in the murine tMCAO model cannot be directly extrapolated to the human situation as differences in the pathomechanisms may exist.

As mentioned already, the de novo conformational coupling between the IP3R type II and TRPC1 has been proposed to mediate SOCE in platelets. My results on STIM1 discussed above strongly question this model but this is not the first time that doubts are raised regarding the correctness of this hypothesis(45;49). TRPC1, which was the first mammalian TRPC channel to be identified, has probably been the most intensively studied and has had many functions ascribed to it including a role in SOCE or being a mechanosensitive channel (for a review see(90)). However with the exception of a role in salivary glands where TRPC1 is well expressed(43), its physiological role in other tissues has remained elusive.
To directly study the role of TRPC1 in platelet function, I analyzed platelets of TRPC1-deficient mice in collaboration with Dr. Alexander Dietrich from the Phillips-University Marburg. I demonstrated unaltered \(Ca^{2+}\) homeostasis and cellular activation in \(TRPC1^{-/-}\) platelets, clearly excluding a major role of this channel in SOCE in mouse platelets.

Changes in \([Ca^{2+}]\) are essential during cellular activation and are therefore tightly regulated. As platelets have to respond to vascular injury very rapidly, their signal transduction machinery is optimized for maximal activation within seconds including the rapid occurrence of maximal cytosolic \(Ca^{2+}\) levels upon agonist-induced stimulation(3;5;86). This is perhaps best exemplified by the steep slope of \(Ca^{2+}\) entry following TG treatment of platelets (Fig.10A, 14A, 21A and 27A) which suggests it to be mediated by the activation of a highly abundant SOC channel. In agreement with this notion, STIM1, the principal regulator of SOCE is highly expressed in platelets, even at higher relative density than in T cells (Fig.7). In contrast, TRPC1 is expressed in both mouse (Fig.20) and human platelets at extremely low levels(45;91), making it necessary to enrich the protein by immunoprecipitation for detection. Furthermore, it has previously been reported that most of the TRPC1 detected is not present in the plasma membrane of platelets but is rather localized in intracellular stores, indicating it to perform functions other than SOCE(45). In support of the latter, other investigators have also reported a widespread intracellular location for TRPC1 overexpressed in HEK-293 cells(92) and, additionally, that TRPC1 alone is unable to increase whole cell currents in resting and carbachol-stimulated cells suggesting that it may not fulfill a channel function(41). Furthermore, analysis of store operated currents in freshly isolated smooth muscle cells from cerebral arteries or from thoracic aortas from wild-type and \(TRPC1^{-/-}\) mice revealed no difference, again suggesting an absence of a major role in SOCE(42). In agreement with this, I found that TRPC1-deficiency does not have any effect on the kinetics and extent of TG-induced store depletion or subsequent SOCE or agonist-induced \(Ca^{2+}\) mobilization and entry in mouse platelets (Fig.21). Its absence in platelets has no measurable effect on platelet aggregation or secretion induced by any of the agonists examined (Fig.22A-B), nor on thrombus formation on a collagen-coated surface (Fig.22C), or in a mesenteric arteriolar injury model utilizing ferric chloride where vessel occlusion is largely driven by thrombin stimulation of platelets and fibrin
clot formation (Fig. 23B-C). This data further suggests that the SOC channel in platelets comprises subunits other than TRPC1.

Finally, studies using the TRPC1−/−/Stim1Sax/+ mutation also imply that STIM1 does not interact with TRPC1 in a functional capacity in mouse platelets. The Sax mutation represents a constitutively active protein that is unable to sense the Ca2+ in intracellular stores and leads to activation of the SOC channel resulting in increased basal cytosolic Ca2+ levels (Fig. 8). If TRPC1 was the important entry channel or an important part of the channel complex linked to STIM1 then crossing the two mouse lines would have resulted in rescue of the Stim1Sax/+ phenotype. That the TRPC1−/−/Stim1Sax/+ showed no difference compared to Stim1Sax/+ mice (Fig. 24) confirms without doubt that TRPC1 plays no major role in SOCE in platelets.

Platelets have been reported to express other TRPC isoforms and members of the TRPM subfamily(47). Thus, a possibility exists that the function of TRPC1 may be compensated for by another TRP channel family member. However, this is very unlikely since previous analysis of the TRPC1−/− mouse vasculature suggested that there is no up- or down-regulation of other TRPC channels (42), unlike the situation in TRPC6−/− mice where TRPC3 levels are elevated (93). I have found that levels of TRPC6 in wild-type versus TRPC1−/− platelets are unchanged (not shown). Other members of the TRPC family (TRPC2, TRPC3, TRPC4, TRPC5 and TRPC7) have previously not been detected in mouse megakaryocytes from which platelets are produced (47), and are therefore unlikely to compensate for the absence of TRPC1 in this model system. Further, with the exception of TRPC4 shown in endothelial cells (94), there is little evidence that any other TRPC channel is able to fulfill a SOC channel role. Thus, there is no indication that another TRP channel compensates for the loss of the putative SOC activity of TRPC1 in the knock out platelets.

Very recently the four transmembrane protein, Orai1 (also called CRACM1) has been identified as an essential component of SOCE in human T cells and mast cells (58; 67). Using reverse transcriptase (RT)-PCR analysis, Orai1 was found to be expressed in both human and mouse platelets and to be the predominant member of the Orai family present in human platelets at mRNA level; however, very faint bands of Orai2 and Orai3 were also observed. Western-blot analysis of human platelet lysates demonstrated robust expression of Orai1, indicating that the channel might have a role in Ca2+...
homeostasis in those cells (Fig.25). These findings correlate well with those from Tollhurst et al. Using quantitative RT-PCR analysis of primary murine megakaryocytes, human platelets and human megakaryocytic cell lines, they observed high expression levels of Orai1 in all three cases, well above that of TRPC1 or other TRPC channels(91).

Lack of Orai1 resulted in strongly reduced SOCE in response to TG and all major physiological agonists but in contrast to STIM1-deficiency, it had no effect on the filling state of the store. Similar observations have been made in Orai1-/− and Stim1-/− mast cells(66;67). This shows that functional SOCE is not a prerequisite of proper store refill and indicates that STIM1 presumably plays a direct, yet unidentified, role in this process. Although the difference in agonist-induced Ca2+ store release between Orai1-/− and Stim1-/− platelets is rather small, it may still be physiologically relevant. This became most evident when FeCl3-induced thrombus formation was assessed in mesenteric vessels (Fig.17A-B and 32E-F, respectively). Orai1-/− chimeras were able to form stable thrombi in this model, whereas no occlusive thrombus formation is seen in Stim1-/− chimeras under the same experimental conditions. This indicates that the relatively small increase in [Ca2+]i caused by store release in platelets may be sufficient to drive thrombus formation independently of SOCE under certain conditions. As platelets have to respond to vascular injury very rapidly, it appears plausible that the first adhesion and activation is regulated mainly by Ca2+ from the stores and very fast Ca2+ channels such as the ATP-gated P2X1 channel, which has been shown to be critical for proper platelet recruitment and activation at very high shear rates(95). However, SOCE appears to be of pivotal importance for thrombus stabilization on collagen/vWF substrates under conditions of high shear which is predominantly mediated by the GPIb-GPVI-PLCγ2 axis(3;5). This was also confirmed by the virtually complete protection of Orai1-/− chimeras from tMCAO-induced neuronal damage which was comparable to the protection seen in Stim1-/− chimeras (Fig.18 and 33, respectively). The development of large brain infarcts in this model is known to be highly dependent on functional GPIb and to a somewhat lesser extent also GPVI(70), indicating that STIM1/Orai1-dependent SOCE may indeed occur predominantly downstream of these receptors during intracerebral thrombus formation following transient ischemia. Importantly, this marked protection was not associated with increased occurrence of
intracranial bleeding which is still the major obstacle in current stroke treatment. In line with this, we observed only a minor increase in tail bleeding times in *Orai1*−/− chimeras suggesting that Orai1, like STIM1, may be of greater relative significance for arterial thrombus formation than for primary hemostasis.

In a recent publication Jardin et al. suggest that the function of Orai1 in platelets is to mediate the interaction between STIM1 and TRPC1 and to regulate the function of TRPC1 as SOC channel in the plasma membrane(96). In their studies, they electrotransjected platelets with an anti-Orai1 antibody, which prevented the interaction between STIM1 and TRPC1. Electroporation of platelets to let molecules smaller than 1,000 Da enter the cells was used in the 1980’s in some studies(97;98), however, this procedure did not allow the diffusion of antibodies of 150,000 Da. If such possibility exists, this would accompany with loss of intracellular proteins/ions and thereby platelet integrity. Platelets are extremely sensitive cells, which react to the smallest stimulus with activation; therefore the use of electroporation makes it hardly possible to investigate physiological processes in those cells. Hence, results of such studies must be treated with caution.

Moreover, numerous studies found the co-expression of STIM1 and Orai1 to be obligatory and sufficient to reconstitute store-operated calcium entry(99-101), also arguing against a major role of TRPC1 in the process.
F. CONCLUSION

The existence of store-operated calcium entry in platelets has been known for over a decade, but the underlying mechanisms and its relevance in platelet physiology remained elusive. My results establish STIM1 and Orai1 as essential components of platelet SOCE, where STIM1 is a calcium sensor in the ER membrane that regulates Orai1 as the major SOC channel in the plasma membrane (Fig. 34). The severely reduced calcium response to all major platelet activating agonists shows that store-operated calcium entry is the major source of calcium increase in platelets. Whereas this process seems to be dispensable for megakaryopoiesis and platelet production, it is necessary for complete platelet activation through PLCγ2. Why functional SOCE is obligatory for the PLCγ2 activation pathway and not for the PLCβ pathway is unknown at present. The slower kinetic of PLCγ2 activation, as compared to PLCβ activation, could be responsible for the observed differences; however, further investigation of this is needed.

In my PhD thesis I show that despite the relatively normal platelet activation observed in vitro, platelet SOCE is of major importance for aggregate formation under flow conditions and thrombus formation in vivo. The defect in the formation of stable thrombi was more pronounced in Stim1−/− mice than in Orai1−/− animals most probably due to the fact that Stim1−/− platelets not only display impaired SOCE but also have reduced calcium content in the intracellular stores. This strongly suggests that besides mediating calcium entry, STIM1 also regulates the filling of cytoplasmic calcium stores through a mechanism independent of SOCE and that physiological platelet functions can to a great extent be fulfilled by only using the intracellular calcium content. SOCE seems to be involved in secondary processes leading to the stabilization of platelet rich thrombi.

Finally, our stroke studies indicate that platelet SOCE is of huge relevance in a clinically important disease model. The fact that lack of STIM1 or Orai1 protects bone marrow chimeras from ischemic cerebrovascular events with no or only very mild bleeding prolongation, makes these molecules – or alternatively the whole platelet SOCE process – promising candidates in the prevention and treatment of ischemic cardio- and cerebrovascular events.
Figure 34. STIM1 and Orai1 are the key components of store-operated Ca\textsuperscript{2+} entry in platelets. Different phospholipase C (PLC) isoforms generate inositol-1,4,5-triphosphate (IP3) which releases Ca\textsuperscript{2+} from the intracellular stores. Decrease in the store Ca\textsuperscript{2+} content activate stromal interaction molecule 1 (STIM1) which in turn trigger Ca\textsuperscript{2+} entry through the major SOC channel Orai1 in the plasma membrane (PM).
G. SUMMARY

Platelet activation and aggregation are essential to limit posttraumatic blood loss at sites of vascular injury but also contributes to arterial thrombosis, leading to myocardial infarction and stroke. Agonist-induced elevation of $[\text{Ca}^{2+}]_i$ is a central step in platelet activation, but the underlying mechanisms are not fully understood. A major pathway for $\text{Ca}^{2+}$-entry in non-excitable cells, such as platelets, involves receptor-mediated release of intracellular $\text{Ca}^{2+}$ stores, followed by activation of store-operated calcium (SOC) channels in the plasma membrane.

In my PhD work I investigated the molecular background and the physiological relevance of store-operated calcium entry (SOCE) in platelets. I have identified stromal interaction molecule 1 (STIM1) and Orai1 to be the key components of SOCE in these cells, where STIM1 is the calcium sensor in the endoplasmic reticulum – the major calcium store – that upon store release signals to Orai1 – the major SOC channel in platelets – and open it to allow $\text{Ca}^{2+}$-entry. Furthermore, I could exclude canonical transient receptor potential channel 1 (TRPC1) to have a major impact on this process. Using *in vivo* thrombosis models I could show that SOCE is of huge importance for stable thrombus formation under high shear flow conditions, such as found in diseased vessels, but lack of the process does not significantly increase bleeding risk. These latter findings establish platelet SOCE and the proteins involved in it as promising targets in the prevention and treatment of ischemic cardio- and cerebrovascular events.
REFERENCE LIST


PUBLICATION LIST

Publications involved in the thesis

Braun A, Varga-Szabo D (joint first), Kleinschnitz C, Pleines I, Bender M, Austinat M, Bosl M, Stoll G, Nieswandt B. Orai1 (CRACM1) is the platelet SOC channel and essential for pathological thrombus formation. Blood Prepublished online Oct 2, 2008; DOI:10.1182/blood-2008-07-171611


Further publications


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