UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

CARACTÉRISATION DE LA VOIE D'INFLAMMATION CGAS-STING DANS LES MÉGACARYOCYTES ET LES PLAQUETTES

CHARACTERISATION OF THE CGAS-STING INFLAMMATION PATHWAY IN MEGAKARYOCYTES AND PLATELETS

MÉMOIRE PRÉSENTÉ
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MAÎTRISE EN BIOLOGIE CELLULAIRE ET MOLÉCULAIRE

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Résumé

Impliquées dans la voie de détection de l'ADN, les protéines immunitaires innées cGAS et STING jouent un rôle important dans l'auto-immunité, le cancer, la sénescence et plusieurs autres syndromes auto-inflammatoires. Certaines études ont montré que les mégacaryocytes présentent une instabilité génomique due à un processus naturel appelé polyploïdisation. Il a été démontré que la voie cGAS-STING induisait une immunité antitumorale, une sénescence cellulaire et des maladies auto-inflammatoires dans un contexte de dommages à l'ADN. Nous voulions déterminer l'effet de l'endomitose mégacaryocytaire sur la régulation de la voie cGAS-STING en utilisant des mégacaryocytes de souris extraits de moelle osseuse. Dans ce projet, nous avons montré que les mégacaryocytes peuvent activer la voie de l'inflammation cGAS-STING lorsqu'ils sont stimulés respectivement par l'ADN et le DMXAA, les agonistes de cGAS et STING. Nous avons également démontré que ces cellules sont soumises à des dommages génomiques considérables et activent STING et donc la voie d'inflammation. Aussi, ces cellules qui génèrent les plaquettes, semblent transférer les protéines cGAS-STING aux plaquettes lors de la thrombopoïèse. Nous avons également trouvé que les plaquettes humaines en contact avec le collagène stimulaient une agrégation synergique avec l'ADN et le cGAMP. Collectivement, nos résultats ont révélé que les mégacaryocytes et les plaquettes exprimaient les protéines de la voie cGAS-STING et que la voie y était fonctionnellement active. Ces résultats ont un impact important sur la compréhension des mécanismes sous-jacents conduisant à la thrombopoïèse, un élément important dans la lutte contre la thrombocytopénie et les maladies auto-inflammatoires.

Mots clés : La voie d'inflammation cGAS-STING, mégacaryocyte, plaquette, instabilité génomique, système immunitaire inné.

Abstract

Involved in the DNA sensing pathway, the innate immune proteins, cGAS and STING, play an important role in autoimmunity, cancer, senescence and several other autoinflammatory syndromes. Few studies showed that megakaryocytes display genomic instability due to a natural process called polyploidization. cGAS-STING pathway was shown to contribute to anti-tumor immunity, cellular senescence, and auto-inflammatory diseases in a context of DNA damage. We wanted to determine the effect of megakaryocyte endomitosis on the activation of the cGAS-STING pathway using bone marrow extracted mouse megakaryocytes. In this project, we showed that megakaryocytes can activate the cGAS-STING inflammation pathway when stimulated with DNA and DMXAA, cGAS and STING agonists respectively. We also demonstrated that these cells are subjected to increase level of DNA damage during maturation, which correlated to the activation of STING signaling. In addition, these cells which generate platelets, seemed to transfer cGAS-STING proteins to platelets during thrombopoiesis. We have also found that human platelets in contact with collagen stimulated a synergic aggregation with DNA and cGAMP. Collectively, our results revealed that megakaryocytes and platelets expressed the cGAS-STING pathway proteins, and that the pathway is functionally active in them. These results have important impacts and bring new insights into the biology of megakaryocytes and platelets.

Keywords: cGAS-STING pathway, megakaryocyte, platelet, genomic instability, Innate immune system.

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PRR	Pattern Recognition Receptors
PAMPS	Pathogen-Associated Molecular Patterns
DNA	Deoxyribonucleic acid
DAMP	Damage associated molecular pattern
TLR	Toll-like receptors
NLR	Nod-like receptors
NOD	Nucleotide-binding oligomerrization domain
cGAS	Cyclic GMP-AMP Synthase
TLR9	Toll-like receptor 9
AIM2	Absent In Melanoma 2
ALR	AIM2-like receptor
cGAMP	2′3′ cyclic GMP–AMP
STING	Stimulator of Interferon Genes
TBK1	TANK-Binding Kinase 1
IKK	Nuclear Factor Kappa Kinase
IRF3	Interferon Regulatory Factor 3
NF-κB	Nuclear Factor Kappa B
IFN-I	Type I interferons
ER	Endoplasmic reticulum
MK	Megakaryocyte
MKs	Megakaryocytes

TPO	Thrombopoietin
HSC	Hematopoietic stem cells
LPS	Lipopolysaccharide
ROS	Reactive oxygen species
PLC	Phospholipase C
PKC	Protein kinase C
PI3K	Phosphatidylinositide-3-kinase
PSGL2	P-selectin glycoprotein ligand-1
NETs	Neutrophil extracellular traps

Chapter I

1. Introduction

1.1 Sources and recognition of endogenous DNA

The immune system helps protect your body from harmful substances, pathogen and cell alteration that could make you ill. It is composed of various organs, cells, and proteins. Immune cells include lymphocytes, macrophages, neutrophils, platelets, and many others [1]. These many cells and components of the immune system adapted to perceive the self from the non-self and steered into a self tolerance against self-proteins, nucleic acids, and other molecules [2]. Pattern Recognition Receptors (PRR) can recognize microbial molecules like LPS among many others that are found in pathogens and non-pathogens alike [3]. These molecules are called Microbial-Associated Molecular Patterns (MAMPS). Similarly, cells may also detect Damage-Associated Molecular Patterns (DAMPs), these are endogenous danger molecules that are released from the damaged or dying cells and activate the innate immune system by interacting with the Pattern Recognition Receptors (PRRs). Usually, in the absence of infection, healthy cells do not induce an inflammatory response [4]. On the other hand, when a healthy cell ages and becomes senescent or when it is subjected to cellular stresses, radiation, cancer among others, the nucleus or mitochondria that contains deoxyribonucleic acid (DNA) may lose their integrity and releases this DNA into the cytoplasm of the cell which is then detected as a DAMP [5]. There are many PRRs that can induce a DNA driven immune response like Toll-like Receptors (TLRs), Nod-like Receptors (NLRs), Nucleotide-binding Oligomerization Domain (NOD) and many more [2]. So far, Toll-like Receptor 9 (TLR9) is only known to recognizes unmethylated CpG sequences of bacterial DNA [6]. On the other hand, Cyclic GMP-AMP Synthase (cGAS), Absent In Melanoma 2 (AIM2), AIM2-like Receptor (ALRs) are the main players in recognizing DNA as a PAMP from pathogens and as a DAMP due to cellular damage, including mitochondrial and nuclear damage self-DNA [7]. While TLR9, AIM2 and ALRs are important receptors who play a crucial role in the fight against pathogens, the focus of this research will be on the cGAS cytosolic DNA receptor, a key player in the maintenance of cellular homeostasis [8].

1.2 The cGAS-STING Pathway

cGAS is activated by binding to cytosolic dsDNA [9]. Upon activation, it synthesizes the second messenger 2′3′ cyclic GMP–AMP (cGAMP) from ATP and GTP [9]. Stimulator of Interferon Genes (STING), which is located on the membrane of the endoplasmic reticulum binds cGAMP, dimerizes, and translocate to the Golgi to then activate TANK-Binding Kinase 1 (TBK1) and Inhibitor of Nuclear Factor Kappa Kinase (IKK){ [10]. These kinases then phosphorylate respectively the transcription factor Interferon Regulatory Factor 3 (IRF3) and IκBα, the repressor protein for Nuclear Factor Kappa B (NF-κB) [10]. P-IRF3, the active form of IRF3, and NF-κB are translocated to the nucleus where they induce the expression of genes that encode type I interferons (IFN-I) and some pro-inflammatory cytokines such as interleukin 6 (IL6) [10]. IFN-I play an important role in the defence against viruses by initiating the production of antiviral effector molecules [11]. Low levels of IFN-I may also be required in case of bacterial infections to induce a cell-mediated immune response [11].

Type I IFN family in humans consists of 14 IFNα species, and one specie of each IFNβ, IFNκ, IFNω, and IFNε [12]. IFNα and IFNβ are mainly released from cells infected with pathogens and IFNγ which is part of the IFN-II family is released from white blood cells triggered by interferon-alpha and interferon-beta to help fight the pathogens. Other roles of interferons include signaling and helping the immune system to fight cancerous cells, activating immune cells to attack, stopping virus and cancer cells growth and division, and helping healthy cells fight infections. All in all, IFN-I are part of a complex network which protects the host against infectious diseases, immunoregulation, tumor cells recognition, and help in T-cell responses [11].

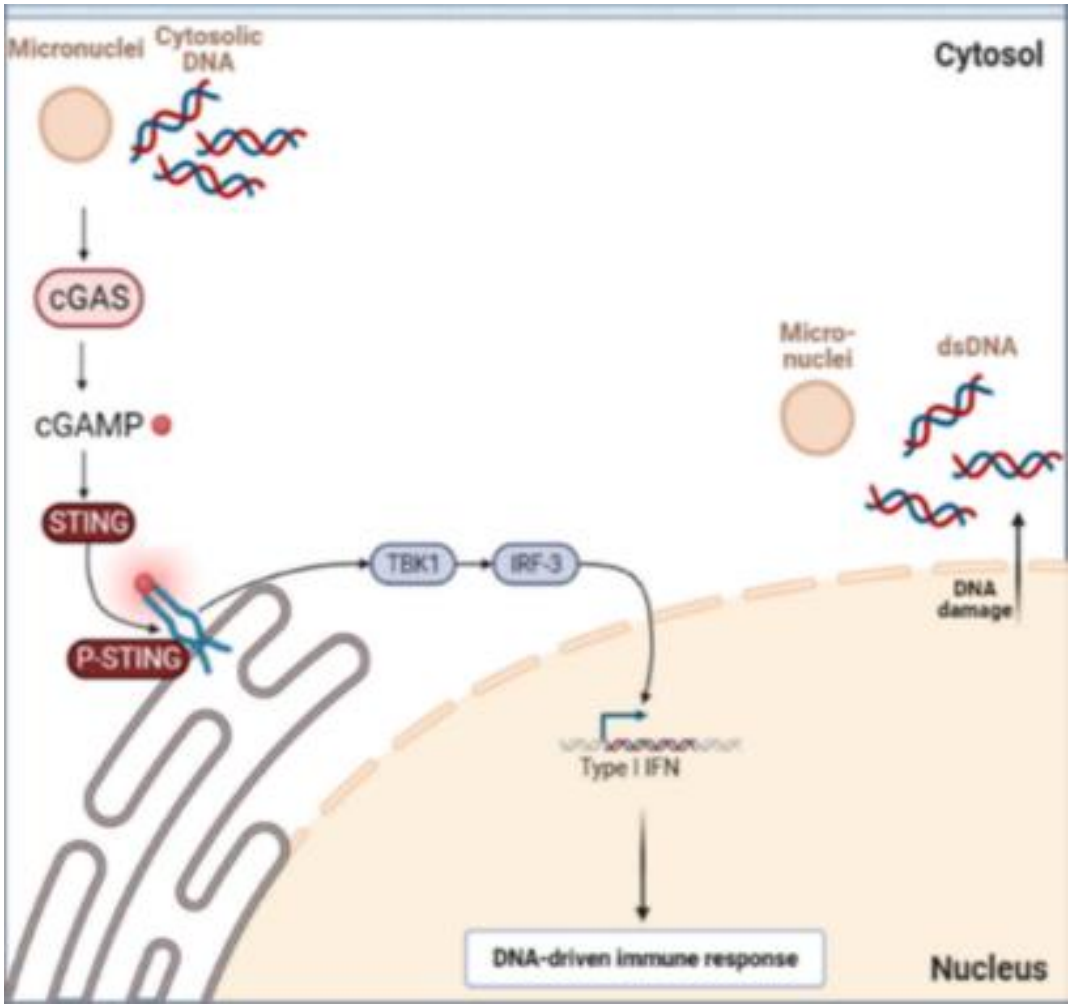

Figure 1.1: The cGAS-STING Pathway

Schematic representation of the cGAS-STING pathway response to DNA damage which leads to a production of type-I IFN and a DNA-driven immune response as described in section 1.2. Upon release of double stranded DNA in the cytosol due to a cellular stress and upon release of micro-nuclei due to mis segregation of the chromosomes, cGAS binds itself to the double stranded DNA or the Micronuclei and synthetize cGAMP. STING binds cGAMP, dimerizes, and translocate to the Golgi to then activate TBK1. TBK1 phosphorylate IRF-3 which translocate to the nucleus where it induces the expression of genes that encode IFN-I and a few other pro-inflammatory cytokines. Figure made in bio render.

1.3 cGAS localisation in the cell

cGAS was first discovered as a cytosolic DNA receptor that can recognize dsDNA at least 45 bp in length, retrovirus-transcribed cDNA, and y-form DNA [13]. The cellular receptor cGAS is now known to localise in the nucleus, in the cytosol and bound to the cell membrane [13]. The N-terminus of cGAS modulate its activity [13]. For instance, in terminally differentiated cells or during interphase, cytosolic cGAS can be activated by dsDNA [13]. During the same phase, nuclear cGAS can bind exogenous or naked DNA that is not chromatin bound [13]. Upon entering mitosis, the barrier between the nucleus and the cytosol breaks down and during this phase, cGAS is tightly tethered to the chromatin at the interface between histones H2A-H2B known as the acidic patch to prevent activation with genomic DNA [14]. The acidic patch is a conserved region which functions as an interaction site for many proteins which binds to chromatin [14]. During interphase, this strategic localisation of cGAS serves as a guard to sense and help protect against upcoming cell stress before pathogens can cause major damage [13]. Also, during this phase, nuclear cGAS is phosphorylated at N-terminus which can be done by kinases like Aurora B Kinase [13]. Neither endogenous, exogenous DNA or naked DNA can activate this form of cGAS [13].

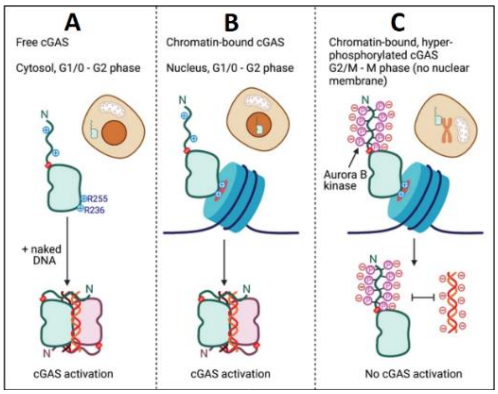

Figure 1.2: The three forms of cGAS depending on the cell cycle

(A) cGAS in the cytosol during G1/G0-G2 phase in a non-hyperphosphorylated form at its N-terminus. (B) Chromatin-bound cGAS in the nucleus during G1/G0-G2 phase also in a non-phosphorylated N-terminus form. (C) Chromatin-bound hyper-phosphorylated cGAS during G2/M-M phase (no nuclear membrane) which render it inactive in this form. (Figure taken from Anna-Maria Herzner, M. S. E. B. (2021). The many faces of cGAS: how cGAS activation is controlled in the cytosol, the nucleus, and during mitosis. *Nature*. https://doi.org/10.1038/s41392-021-00684-3)

1.4 STING localisation in the cell

The anti-viral PRR STING is a protein with four transmembrane domains and resides in the endoplasmic reticulum (ER) [16]. It is expressed in non-immune cells and in both innate and adaptive immune cells [17]. After it's binding to cGAMP and dimerization, STING is activated and translocate from the ER to the perinuclear area with the help iRhom2 [17]. A study has shown a new role for STING in the nucleus [18]. STING can translocate to or from the nucleus during an innate immune response [18]. Since most cGAS is nuclear [13], this could suggests that nuclear STING could be activated before ER STING in case nuclear viral dsDNA is present. This hypothesis is also supported by the finding that STING translocate from the nucleus region to the perinuclear region during an infection with nuclear-localized replication of the virus HSV-1 [18]. Surprisingly, this same translocation of nuclear STING to perinuclear foci was seen in cells transfected with polyinosinic:polycytidylic acid (poly(I:C)) which is structurally similar to dsRNA and is used to simulate a viral infection [18]. This suggests that STING could have a role in a different functional pathway other than the dsDNA immune response.

1.5 cGAMP transfer between cells through connexins

An important biological insight into the cGAS-STING pathway is that it's role in cellular protection goes further than just cytosolic DNA sensing. In fact, this pathway has been proven to help combat pathogens in a way that significantly protects uninfected cells adjacent to the cell that is afflicted by a viral infection [19]. It is achieved via the transfer of cGAMP through connexins between cells which transactivates STING to modulate an antivial response including in cells where cytoplasmic dsDNA is not available [20].

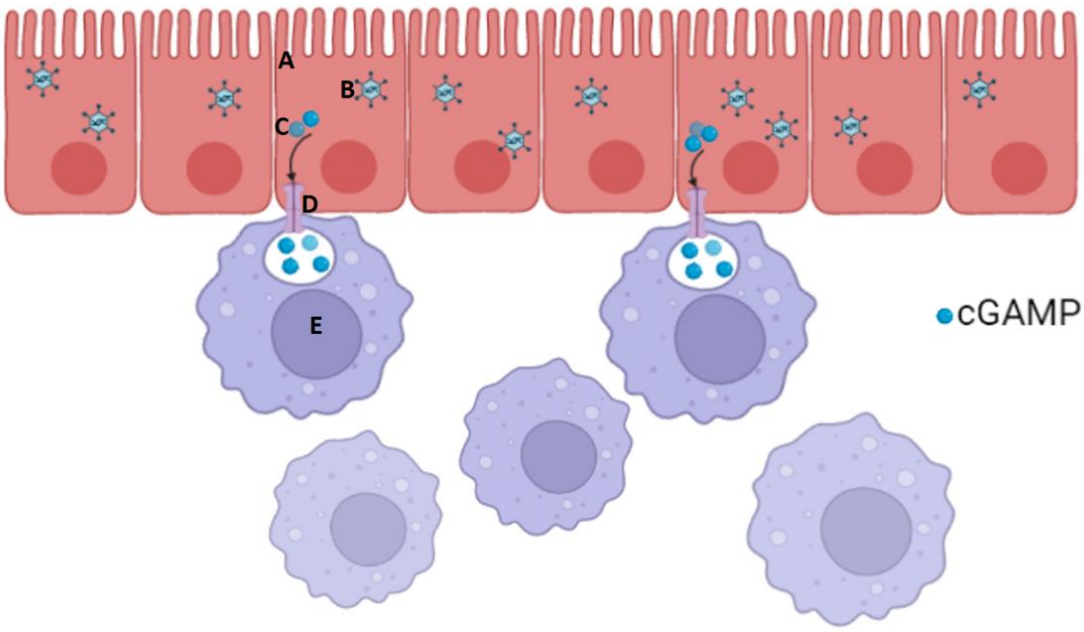

Figure 1.3: Virus infected epithelial cells transfer cGAMP to macrophage through connexins

In epithelial cells(A) infected with viruses(B), cGAS can bind to the virus dsDNA and synthesize cGAMP(C). This cGAMP can then transfer through neighbor cells using connexins(D) and to macrophage(E) and induce an innate immune response.

1.6 The relationship between autophagy and cGAS-STING

To go back to homeostasis, immune responses need to be shut down. To end its signalling, the cGAS-STING pathway can induce autophagy [21-22]. Autophagy induced by cGAS–STING helps eliminate pathogens and DNA from the cytosol while breaking down the molecules of cGAS and STING [21-22]. There are three pathways in which cGAS, and STING can induce autophagy. The first is through the interaction of cGAS and/or STING with LC3 which is a vital protein in the autophagy pathway where it accomplishes the role of autophagosome biogenesis and autophagy substrate selection [21-22]. The second is through cGAS interaction with Beclin-1 another protein that has a central role in autophagy [23]. The third is through STING-triggered ER stress–mTOR signaling [24]. Autophagy induced by the cGAS-STING pathway is important for multiple reasons among preserving intracellular homeostasis. A recent study even pointed to the crucial role of autophagy induced by the cGAS-STING pathway in micronuclei clearance [25], therefor helping in preventing chromosomal instability.

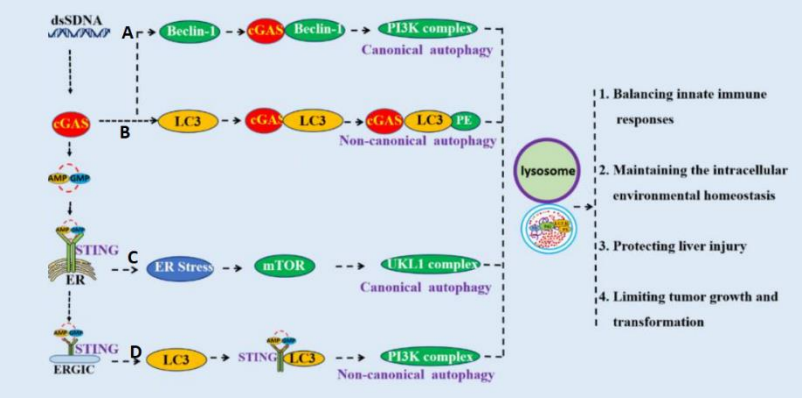

Figure 1.4: The different pathways for cGAS-STING induced autophagy

(A) cGAS interact with Beclin-1 to induce canonical autophagy. (B) cGAS interacts with LC3 to induce non-canonical autophagy. (C) STING-triggered ER stress–mTOR signaling induce a canonical autophagy. (D) STING interact with Beclin-1 to induce non-canonical autophagy. (Adapted from Mengmeng Zhao, F. W., Juehui Wu, Yuanna Cheng and Baoxue Ge (2021). CGAS is a micronucleophagy receptor for the clearance of micronuclei. Autophagy. https://doi.org/10.1080/15548627.2021.1899440)

1.7 Chromosomal instability

Chromosomal instability results from errors in chromosomal segregation during mitosis leading to chromosomal aberrance [26]. Chromosomal instability is a defining trait in human cancer and is associated with metastasis and therapeutic resistance [26]. Chromosomal instability can also occur as a consequence of polyploidization [27]. In some cases, polyploids cells, which have an increased number of chromosomes, are formed in case of a rare mitotic or meiotic catastrophe that causes the formation of cells that have a double set of chromosomes [26]. On other cases, polyploidization has also been observed to occur normally in physiological contexts such as during megakaryocyte's endomitosis. Endomitosis is a process in which there is the replication of DNA followed by an abortive mitosis which results in polyploidy. There are two cell types that are better known that have been observed to be polyploid [28]. The first are trophoblasts, cells that are formed during the first stage of pregnancy and constitute the outer layer of the blastocyst and then form the placenta [29]. These cells are capable of endoreplication and/or cell fusion. The second are the megakaryocytes (MKs) [28]. MKs have been characterized to do endomitosis and form a high ammount of micronuclei which are associated to chromosomal instability [28]. It has been shown that sustained DNA replication is often accompanied by genomic instability [30].

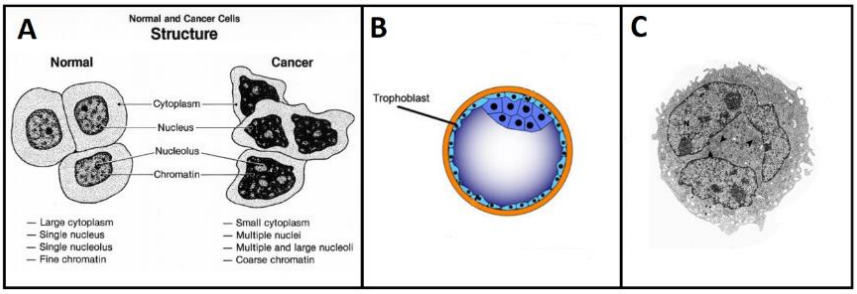

Figure 1.5: Normal cells compared to cancer cells, trophoblast and megakaryocytes

(A) Normal cell structure compared to cancer cells. (B) The cells that constitute the outer layer of the blastocyst: the trophoblasts. (C) An electron microscopy image of a megakaryocyte showing polyploidisation. (Taken from Elisabetta Falcieri, A. B., Sabina Pierpaoli,Francesca Luchetti,Loris Zamai,Marco Vitale,Lia Guidotti,Giorgio Zauli. (2000). Ultrastructural characterization of maturation, platelet release, and senescence of human cultured megakaryocytes. The Anatomical Record. https://doi.org/10.1002/(SICI)1097-0185(20000101)258:1<90::AID-AR10>3.0.CO;2-G)

1.8 Megakaryocyte differentiation

The formation of blood cellular components is called hematopoiesis. Hematopoiesis takes place during embryonic development and throughout adulthood to build up and refill our blood system [31]. Derived from hematopoiesis, MKs are immune cells that generate up to 2000 to 10 000 platelets in response to thrombopoietin (TPO) and other cytokines by a process called thrombopoiesis [32]. MKs differentiate from multipotential hematopoietic stem cells (HSC) which can differentiate into either granulocytic-macrophage, erythroid or megakaryocytic lineages [32]. During their differentiation, MKS are characterized by the upregulation of different MK-specific cell surface markers (CD41, CD42a, CD42b, c-mpl) and the expression of transcription factors associated with MK development like FLI1, RUNX1, GATA1, NFE2, etc. [32]. While maturing, MKs undergo endomitosis which is a process where cells repeatedly duplicate their DNA through abortive mitosis up to 64N in mice and 128N in humans [33]. During endomitosis, MKs grow and amass organelles characteristic of peripheral platelets. Consequently, mature bone marrow MKs are polyploid, granular cells that can reach up to 100 µm of diameter which gives them the title of the largest cells in the bone marrow [28]. After MKs growth, there is a formation of an invaginated membrane system (IMS), then the formation of alpha granules, dense granules which are platelet-specific granules and finally there is the synthesis and endocytosis of RNA and platelet-specific proteins to be dispersed into platelets.

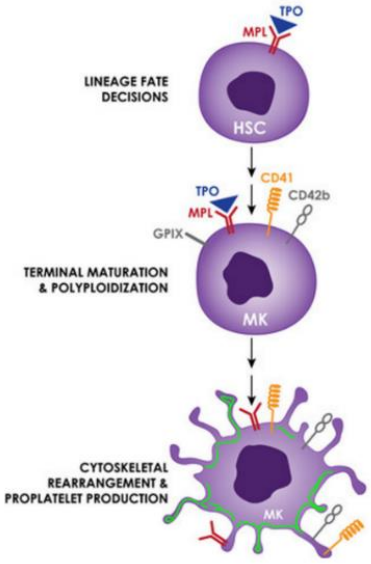

Figure 1.6: Megakaryocytes differentiation from hematopoietic stem cells

Upon stimulation to the hormone thrombopoietin, hematopoietic stem cells undergo differentiation to become immature MKs and after polyploidization mature MKs which are capable of proplatelet production. MK: Megakaryocyte, HSC: hematopoietic stem cell, TPO: thrombopoietin, MPL: myeloproliferative leukemia protein (thrombopoietin Receptor). (Adapted from Leila J Noetzli, S. L. F., and Kellie R Machlus. (2019). New Insights into the Differentiation of Megakaryocytes from Hematopoietic Progenitors. Arterioscler Thromb Vasc Biol. https://doi.org/10.1161/ATVBAHA.119.312129)

1.9 Megakaryocyte localisation

In vivo, MKs are mostly found in the bone marrow, but they have also been observed in the lungs [34]. There are also CD34 stem cells in the blood circulation that when induced with TPO can differentiate into MKs [35]. Since the lungs MKs are in constant interaction with airborne pathogens and high O^2 exposure [34] compared to BM MKs where they are in a sterile and hypoxic environment [34], lung MKs differ in their sensitivity to pathogens and are phenotypically distinct compared to bone marrow MKs. According to a study, lung MKs exhibits greater levels of immune molecules than their bone marrow counterpart and express a superior variety and quantity of cytokines when faced with LPS simulating a bacterial infection [34]. Another observed phenotype is that bone marrow MKs have an average of 16N, but most of the lung MKs have a ploidy of 2N [34].

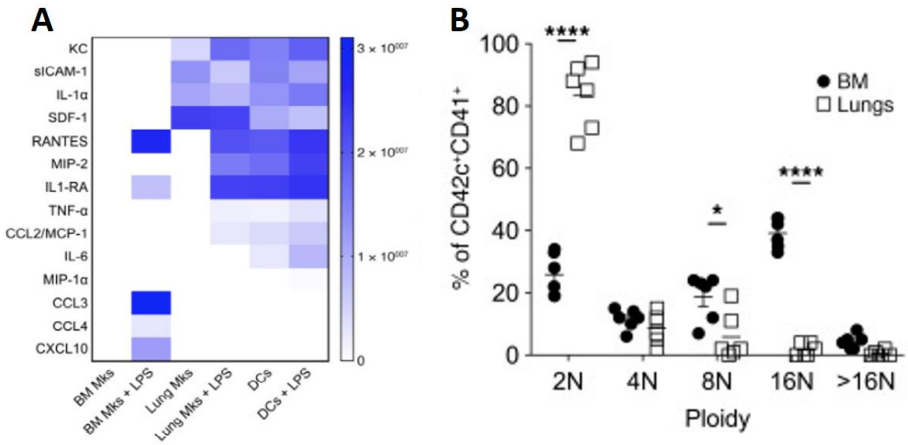

Figure 1.7: Phenotypical distinctions between bone marrow MKs and lung MKS

(A) After an LPS stimulation, lung MKs induce different sets of immune molecules than bone marrow MKS. (B) Most of lung MKs have a ploidy of 2N while bone marrow MKs have an average ploidy of 16N. (Taken from Daphne N. Pariser, Z. T. H. a. C. N. M. (2020). Lung megakaryocytes are immune modulatory cells. The Journal of Clinical Investigation. https://doi.org/10.1172/JCI137377)

1.10 Thrombopoiesis

There are two main mechanisms of platelet formation which are sensitive to the environment in which the MKs are differentiated into [36-37]. The most common model in most MKs *in vitro* is the formation of proplatelets which are extension of the MK's cytoplasm [36-37]. The second mechanism, observed in 53% ± 8% of *in vivo* MKs compared to proplatelet displaying MKS 1% ± 2% [36], is called membrane budding where MKs release a vast number of platelets in the form of buds. A study suggests that MKs can undergo rapid release of platelets via membrane budding when the host is in acute need of platelets after suffering from blood loss, although this finding seems to still be under debate [38].

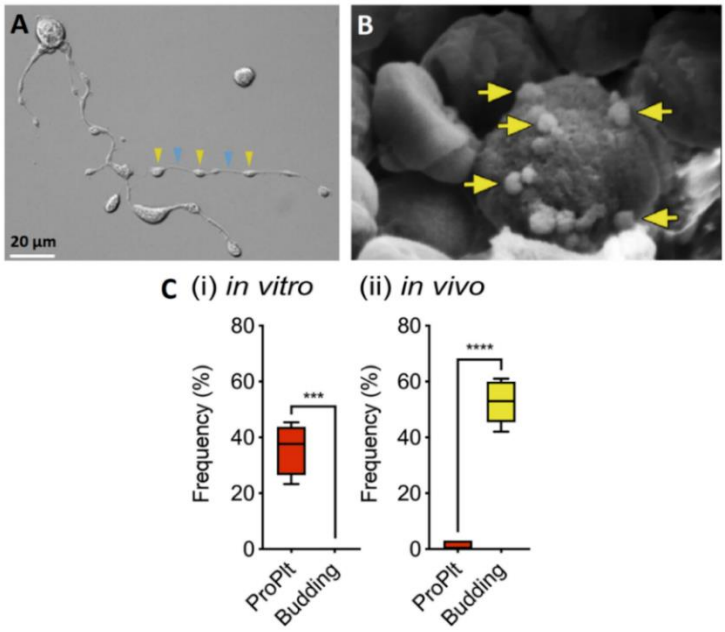

Figure 1.8: The two different mechanisms of thrombopoiesis

(A) Representative example of an in vitro MK forming proplatelets. (B) MK forming buds (arrows) through membrane budding observed using scanning electron microscope; n=3. (C) Quantitation of in vitro (i) and in vivo (ii) platelet forming mechanisms; n = 4–5 independent experiments. (Taken from Kathryn S. Potts , A. F., Caleb A. Dawson, Joel Rimes, Christine Biben, Carolyn de Graaf, Margaret A. Potts, Olivia J. Stonehouse, Amandine Carmagnac, Pradnya Gangatirkar, Emma C. Josefsson, Casey Anttila, Daniela Amann-Zalcenstein, Shalin Naik, Warren S. Alexander, Douglas J. Hilton, Edwin D. Hawkins, Samir Taoudi. (2020). Membrane budding is a major mechanism of in vivo platelet biogenesis. Journal of experimental medecine. https://doi.org/10.1084/jem.20191206)

1.11 MK ROS during differentiation

In a recent study, MKs have been shown to have different mitochondrial reactive oxygen species (ROS) levels depending on the stage of their differentiation [39]. They divided the MKs into 3 differentiation stages: Round MKs, intermediate MKs and terminal proplatelet MKs (Fig. 1.9A). Using MitoSOX fluorescence to quantify ROS intensity, they noticed an increase in ROS levels between round MKs and intermediate MKs [39]. There is then a sharp decrease in ROS levels between intermediate MKs and proplatelet forming MKs [39]. Since ROS are known to regulate actomyosin levels and formation of microtubules in multiple cellular conditions [40] and that proplatelet formation is linked with morphological changes to their cytoskeleton configuration [41], the author have suggested that the burst in ROS contributes to morphological changes. Recent studies also showed that ROS can induce dsDNA breaks and mitochondrial DNA lesions [41].

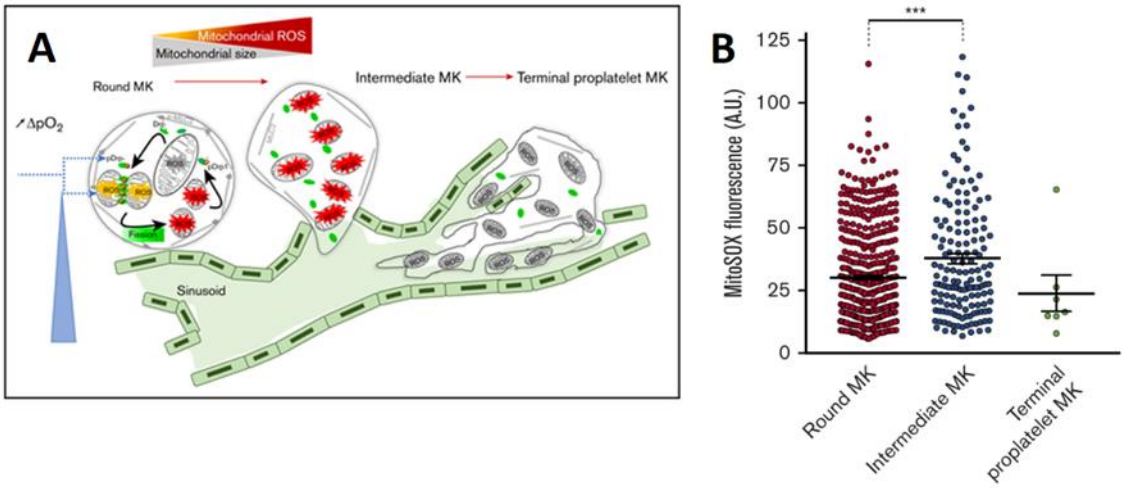

Figure 1.9: Mitochondrial ROS during different stages of the MK

(A) Representation of the level of ROS depending on the stage of the MK. (B) MitoSOX fluorescence was measured in individual cells from the 3 populations of MKs and expressed as AUs normalized to the cell surface; n=3. (Taken from Sonia Poirault-Chassac, V. N.-A., Amandine Houvert, Alexandre Kauskot, Evelyne Lauret, René Lai-Kuen, Isabelle Dusanter-Fourt, Dominique Baruch. (2021). Mitochondrial dynamics and reactive oxygen species initiate thrombopoiesis from mature megakaryocytes. Blood Adv. https://doi.org/10.1182/bloodadvances.2020002847)

1.12 Platelets

Complex and multifunctional, platelets are anucleate blood cells [42]. Their main functions are hemostasis, thrombosis, and wound healing, but they also have other physiological roles, including immunity and communication with other cells and tissues in blood vessels [42]. Although they have a short lifespan of 7 days, their number remains constant in the blood of the same individual, showing a constant and regulated production in homeostatic conditions [42]. Among the widespread diseases that platelet help fight, there are gram-negative bacterial Infections, Gram-Positive Bacterial Infections, Malaria, Dengue Virus, and many others [43]. Platelets roles in hemostasis and thrombosis relies on an intricate equilibrium of signaling pathways that upregulate or downregulate their activation [44]. During quiescence, platelet activation is suppressed by inhibitory signals such as nitric oxide and prostaglandin I2 which are released from the vascular endothelium and with the help of cAMP-dependent protein kinase (PKA) and the cGMP-dependent protein kinase (PKG) [44]. Following vessel injury, collagen and thrombin initiate activation. This activation leads to platelet secretion of secondary mediators such as ADP and thromboxane A2 (TXA2) [44]. When activated, platelets also release the content of their granules including alpha and dense granules. The next phase is that of the thrombus growth in which phospholipase C (PLC), protein kinase C (PKC), and phosphatidylinositide-3-kinase (PI3K) with the help of integrin αIIbβ3 support platelet activation to induce thrombus formation [44]. To limit an overgrowth of the thrombus and the formation of obstructing thrombi, there is a step of self-regulation in which immunoreceptor tyrosine-based inhibition motif bear negative regulators. Additionally, there is the release of endothelial cell-selective adhesion molecule (ESAM) which limit integrin αIIbβ3 activation v.

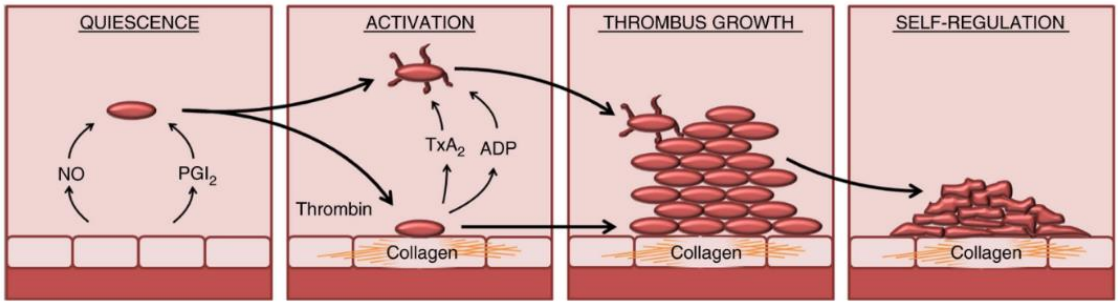

Figure 1.10: Stages of platelet activation and thrombus formation

The four stages of platelet activation and thrombus formation during vascular injury. See text above for detailed description. Adapted from A. P. Bye, A. J. U., J. M. Gibbins. (2016). Platelet signaling: a complex interplay between inhibitory and activatory networks. Journal of thrombosis and haemostasis. https://doi.org/10.1111/jth.13302)

In the case of our study, we focused our research on platelet aggregation in contact with collagen. Platelets express several collagen receptors. Two of these receptors that play a role in collagen-induced platelet activation are Glycoprotein VI (GPVI) and Glycoprotein Ib–IX-V complex (GPIb-IX)[45]. GPVI binding to collagen induce its cross-linking with Fc receptor γ chain (FcRγ). FcRγ is tyrosine phosphorylated on its cytoplasmic domain by GPVI own cytoplasmic domain. This phosphorylation leads FcRγ binding and activation of the Spleen tyrosine kinase (Syk) [45]. Syk initiate a phosphorylation of multiple downstream targets which results in the formation of a signaling complex which includes phospholipase Cγ (PLCγ) among other proteins. This leads to the activation of PKC and the mitogen-activated protein kinase (MAPKs) pathway which in turn activate the synthesis of TXA2 and initiate granule secretion [45]. The other platelet receptor GPIb-IX binds with collagen via von Willebrand factor (VWF) [45]. Upon binding, GPIb-IX cross-linked to the ligand FcRγ and induce the same activation as described earlier. GPIb-IX can also interact with PI3K to induce an early activation signal going through MAPKs leading to TXA2 synthesis and granule secretion [45].

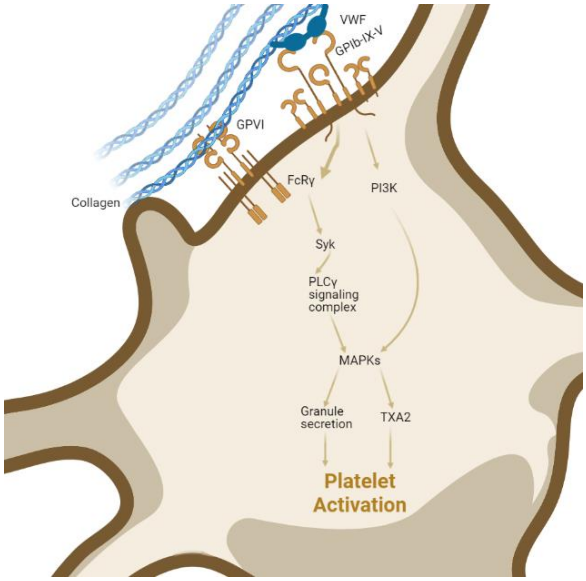

Figure 1.11 Collagen-induced platelet activation

Platelet activation pathway induced by collagen that can happen during vascular injury or stimuli of collagen on platelet. In contact with collagen, platelets activate and with the right amount of collagen, it can even lead to platelet aggregation. See text above for detailed description.

1.13 Platelet and NETs

Platelets express an abundance of surface receptors and molecules and hold granules which are filled with hundreds of biologically active effectors [43]. Platelets and neutrophils form physical interactions with the help of the molecules on their surface and the ones they secrete [46]. During platelet activation, neutrophils have been observed to interact with platelets by binding CD62P expressed in activated platelet to P-selectin glycoprotein ligand-1 (PSGL1) on the surface of neutrophils [46]. This initiates a platelet dependant neutrophil activation and in some conditions like LPS stimulation, this can trigger the production of neutrophil extracellular traps (NETs) through a form of regulated cell death called NETosis [46]. Some of the components of NETs are lattices of chromatin, histones and granule enzymes that help protect against infections, but are also implicated in many immune-mediated conditions [46]. Among the products released from the neutrophils are pro-coagulant molecules that can form thrombosis [46]. This interaction between platelets and neutrophils has a key role in hemostasis and immune-driven responses. A study demonstrated that macrophages and other myeloid cells can phagocytose NETs after trapping invading pathogens [47]. Once in the phagosomes, NETs relocate to the cytosol where cGAS can bind to the chromatin released by NETs and induce a type-I IFN immune response during infection [47]. Additionally, there has been literature suggesting platelets respond to dsDNA by releasing granules and aggregating [43].

Chapter II

Hypothesis and objectives

Objective 1:

MKs undergo endomitosis, which lead to genomic instability, cGAS is activated by cytosolic DNA accumulation in cells with genome instability. We hypothesise that during MKs maturation, cGAS will bind to this DNA and induce an immune inflammatory response. To verify our hypothesis, we will characterize the inflammation pathway of cGAS-STING in bone marrow MKs. We first need to evaluate the presence of the proteins of this pathway, and then evaluate whether it is functional. We will also observe if there is DNA damage during MKs differentiation which could potentially lead to a basal activation of this pathway during endomitosis.

Objective 2:

Additionally, our second hypothesis is that cGAS-STING protein will be transferred from the MK to platelets during thrombosis since protein from MK are often transferred to platelets. Hence, we will assess the transfer of the inflammatory proteins in this pathway to platelets and their role in these small anucleate cells. To characterize the inflammation pathway of cGAS-STING in platelets, we will have to evaluate if MKs are transferring these key proteins to platelets during thrombopoiesis and study the role of platelets in dsDNA cGAS-dependant detection.

To proceed with the verification of my hypothesis, I will first have to learn how to properly isolate MKs and platelets and be sure of their purity. To evaluate the presence, functionality, and activity of the proteins in MKs, I will need to do protein analysis and gene expression analysis using WB, qPCR and ELISA. I will also observe using confocal microscopy the localisation of cGAS, STING, P-STING and P-IRF-3 proteins. For platelets, I will evaluate the presence of the proteins using WB and I will do a series of aggregation experiments to see if there is an activation when the inflammation pathway is engaged. To ensure my findings are specific to the cGAS-STING inflammation pathway, I will compare results from MK and platelet isolated from *WT* and *cGAS*$^{-/-}$ mice.

Contribution for authors

Firas El-Mortada did 65% of the experiments; KL 35% of experiments; SBP 5%; FAA and AP helped for some experiments, KM, BK, MPG, JH and ML have supported some experiments and GP has coordinated the project. All authors revised the manuscript.

Chapter III

Non-canonical cGAS-STING signalling drives platelets activation

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Abstract

Platelets display unexpected roles in immune responses and in coagulation. Emerging evidence suggests that the Stimulator of Interferon Genes (STING) is implicated in hypercoagulation during sepsis and severe COVID-19. STING is an adaptor protein downstream of the DNA sensor cyclic GMP-AMP (cGAMP) synthase (cGAS) that is activated by cytosolic microbial and self-DNA during infections and in context of loss of cellular integrity, to instigate the production of type-I interferon (IFN-I) and pro-inflammatory cytokines. To date, whether the cGAS and STING pathway is present in platelets and contribute to platelet functions is not defined. Using a combination of pharmacological and genetic approaches, we demonstrate here that megakaryocytes and platelets possess a functional cGAS-STING pathway. Our results suggest that in megakaryocytes, cGAS is activated and produces a tonic Interferon response. We also found that cGAS localisation changes during the maturation of megakaryocytes and is detected in proplatelets. Finally, we show that both murine and human platelets contain cGAS and STING proteins, and the cGAS-STING pathway contributes to potentiation of platelet activation and aggregation. Taken together, these observations establish for the first time a novel role for the cGAS-STING axis in megakaryocytes and platelets cell populations, beyond IFN-I production, emphasizing the need to study this pathway in specific cell populations to better understand the growing relationship between immune responses and coagulation in contexts of infections and inflammatory disorders.

Introduction

Host defense against infection relies on two important systems, the immune and the coagulation systems. Platelets are anucleate cells produced by megakaryocytes and responsible for blood clotting and hemostasis [1]. They are also very abundant and play unexpected roles in immune responses [2,3]. Hence, platelets are at the crossroads of these critical processes. Platelets possess several immune receptors, including Toll-like receptors (TLRs), that detect microbial molecules and modulate platelet functions, including potentiating their activation and aggregation independently of their canonical transcriptional roles [4]. They also interact directly with other immune cells such as lymphocytes, monocytes, and neutrophils. Hence, through these receptors and cellular interactions, they are implicated in coagulation but also in bacterial clearance, defence against viruses and inflammatory responses [2,3].

Interestingly, early literature suggests that platelets respond to double stranded (ds)DNA, by releasing inflammatory molecules stored in their secretion granules and by aggregating [5]. More recently, it was also observed that platelet activation by DNA leads to increased levels of the cell surface protein P-Selectin and the release of the cytokine Platelet Factor 4 (PF4/CXCL4), two markers of platelet activation and degranulation [6].

The cyclic GMP-AMP Synthase (cGAS) is a cytosolic DNA receptor. cGAS detects microbial DNA during infections and nuclear or mitochondrial self-DNA as a result of loss of cellular integrity [7-11]. Upon DNA sensing, cGAS produces the second messenger, cyclic GMP-AMP (cGAMP) which then binds to Stimulator of Interferon Genes (STING) adaptor protein [12,13]. In cells with a nucleus, activated STING acts as a scaffold protein to recruit Tank Binding Kinase 1 (TBK1) and IKKe to allow for the phosphorylation of the transcription factor Interferon Related Factor 3 (IRF3) and Nuclear Factor kappa B (NF-kB), resulting in the production of type-I Interferon (IFN-I) and pro-inflammatory cytokines [13-16]. Sustained activation of the cGAS-STING pathway underpins the inflammation observed in many pathological conditions including cardiovascular diseases, ageing, obesity, neurological disorder [17]. Using transcriptomic data from macrophages expressing the S365A STING mutant, specifically deficient for IFN-I signalling, a recent study has unveiled Interferon-independent STING activation pathways, emphasising the broad implication of this pathway beyond its canonical role

[18]. Among these IFN-I independent functions of STING, a pivotal role for STING in lethal coagulation during sepsis has recently been reported. Molecularly, this study showed that STING activation in macrophages results in increased cytosolic calcium, which then caused the secretion of Tissue factor and D-dimer triggering a pathological coagulation cascade during sepsis [19]. Despite this first evidence of a role for the cGAS-STING pathway in coagulation, whether the cGAS-STING proteins have a role in other cell populations contributing to coagulation including platelets, remains to be investigated.

Platelets are released into the vasculature from the fragmentation of cytoplasmic protuberances called proplatelets or from the budding of the megakaryocyte plasma membrane [20-22]. Thus, for cGAS-STING proteins to be present in platelets, they first need to be produced within megakaryocytes prior to being translocated and packaged into platelets.

In this work, we initially investigated the presence and functionality of the cGAS-STING axis in megakaryocytes and platelets. We demonstrated that megakaryocytes and platelets possess the cGAS and STING proteins which can be functionally activated, albeit differently. As such, while cGAS/STING drives a tonic IFN-I production in megakaryocytes, it potentiates activation and aggregation of platelets. Taken together, our work demonstrates a previously unrecognised role for the cGAS-STING pathway in megakaryocytes and platelets and raise important questions as to their functions during infections and inflammatory syndromes.

Results

STING stimulation of megakaryocytes induce a potent type-I Interferon response

Platelets bud from cytoplasmic protuberances of megakaryocytes and have little translation capacity, so we hypothesise that to be present in platelets, the cGAS and STING proteins would first be translated in megakaryocytes. To investigate this idea, we relied on in vitro differentiation of cells expressing the megakaryocytes lineage specific integrin CD41 (hereafter named CD41+ cells) isolated from adult mouse bone marrow that were then cultured in the presence of thrombopoietin (TPO) to stimulate their maturation. Mature megakaryocytes were easily identified in our brightfield images by

their multilobed nucleus and the expression of the CD41 integrin at the plasma membrane (Figure 1A and 1B). Consistent with the phenotype associated with mature megakaryocytes, we could observe the formation of proplatelets and budding structures in a high number of the megakaryocytes (Figure 1A and 1B). Protein analysis by western blotting of these megakaryocytes revealed that they expressed the canonical proteins of the pathway including cGAS, STING, TBK1 and IRF3 (Figure 1C). When stimulated with DMXAA, a synthetic murine STING agonist [23], the phosphorylated (P) form of STING (P-STING) and of IRF3 (P-IRF3) were detected (Figure 1C). This phosphorylation was accompanied by a significant induction of the Ifnb1 gene expression (Figure 1D) and the release of IFN-b cytokines (Figure 1E). Megakaryocytes express the IFN- I receptor IFNAR and have the capacity to respond to IFN-I treatment [24,25]. In agreement, DMXAA-treated megakaryocytes exhibited a significant up-regulation of several of IFN-stimulated genes (ISGs) [26]. CCL5 (RANTES) expression levels, a direct IRF3 target and known to stimulate thrombopoiesis [27], was also increased (Figure 1D and 1F).

cGAS and STING localise in the nucleus and in the cytoplasm

The unique maturation process of megakaryocytes prompted us to determine the localisation of both cGAS and STING in megakaryocytes. In contrast to the usual localisation of STING in the endoplasmic reticulum, we found STING located both in the nucleus and in the cytosol (Figure 1G). In addition, cGAS intracellular localisation varied depending on the cell being analysed. We could detect cGAS in the nucleus, in the cytoplasm and in bright punctates that resembled micronuclei (Figure 1G), suggesting that cGAS localisation is dynamic. The nuclear localisation of cGAS was consistent with previous studies showing that during mitosis cGAS was located at the chromatin where its activity was strongly reduced [28,29]. While megakaryocytes do not undergo mitosis per se, they do replicate their DNA and go through several rounds of abortive mitosis, a process called endomitosis [21], suggesting that cGAS might be similarly inhibited.

DNA stimulation produces a cGAS-dependent IFN-I response in megakaryocytes

STING activation occurs downstream of cGAS activation by DNA and the production of cGAMP. However, other cellular receptors including TLR9, expressed by

megakaryocytes, can be activated by DNA [30]. To exclude a role for TLR9, we stimulated megakaryocytes isolated from wild type (*WT*) and *cGAS*$^{-/-}$ mice with a synthetic 70 nucleotides dsDNA that that does not contain CpG hereafter referred to as DNA (Figure 2). Consistent with cGAS being the main receptor involves in the response to non-CpG DNA, we found that only cGAS expressing megakaryocytes responded to DNA by inducing the expression of selected ISGs (Figure 2A). This IFN-I response was accompanied by a low but detectable production of cGAMP in *WT* but not in *cGAS*$^{-/-}$ megakaryocytes (Figure 2C), confirming that this response was cGAS-dependant. Lack of response of *cGAS*$^{-/-}$ megakaryocytes could not be attributed to a defect in IRF3 signalling since DMXAA treated cGAS-deficient megakaryocytes displayed a significant induction of the IRF3 target Ifit1 (Figure 2B). Although significant, the increase in ISGs upon DNA stimulation was much lower than what was detected for DMXAA suggesting that cGAS might not be fully active, at least during endomitosis.

STING drives a tonic Interferon response during megakaryocyte maturation

The fact that megakaryocytes express an active cGAS and that cGAS is present in micronuclei, a hallmark of genome instability [31-34], led us to ask whether cGAS could be basally activated during megakaryocytes maturation. To test that, we posited that if cGAS was basally engaged, we should be able to detect P-STING in *WT* megakaryocytes and IFN-I stimulated gene expression should be basally higher than in cGAS-deficient cells. As expected, we detected a higher signal of P-STING in megakaryocytes *WT* than their *cGAS*$^{-/-}$ counterpart (Figure 2D and 2E). Furthermore, *WT* megakaryocytes displayed a higher number of P-STING punctates, a marker of STING activation, strongly suggesting that the cGAS-STING pathway is basally activated in megakaryocytes. To test this idea, we treated megakaryocytes with the STING inhibitor C176 during their differentiation [35]. STING inhibition resulted in a significant reduction in the expression levels of selected ISGs (Figure 3A). These results support a basal activation of STING in megakaryocytes that results in a tonic Interferon response, which has been previously reported for other cell population including for bone marrow-derived macrophages [36]. Taken together these results suggest that genome instability could drive cGAS activation.

Dynamic localisation of cGAS during megakaryocyte maturation

To investigate whether megakaryocytes display genome instability during maturation, we imaged megakaryocytes and separated them in groups representing their maturation (based on their ploidy and their size) (Figure 3D). Besides cGAS itself, we chose to image P-gH2AX and the Lamin A/C to assess the presence of DNA damage and the integrity of the nuclear membrane respectively. By doing so, we unveiled a correlation between cGAS localisation and the amount of DNA damage. In fact, in early stage of maturation, megakaryocytes displayed low levels of DNA damage (P-yH2AX), no signs of nuclear membrane rupture and exhibited a predominantly nuclear distribution for cGAS. However, as megakaryocytes progressed into maturation, they accumulated DNA damage and a high proportion of cGAS was detected predominately in the cytosol near the plasma membrane and in micronuclei. However, in the last stage of maturation, DNA damage was almost undetected and cGAS was found almost exclusively in the nucleus, strongly suggesting that genome instability contribute to cGAS activation (Figure 3B and 3C).

cGAS is detected in platelets and produces cGAMP

In a subset of megakaryocytes cGAS and STING were also detected in budding structures, reminiscent of proplatelet extensions (Figure 4A). These images led us to investigate whether cGAS and STING could also be present in platelets. To investigate this hypothesis, we isolated platelets from *WT* and *cGAS*$^{-/-}$ mice that we depleted from contaminating leukocytes to analyse the presence of STING and cGAS. STING could be easily detected by confocal immunofluorescence of washed platelets in which we used the integrin CD41 to delineate the plasma membrane (Figure 4B). Unfortunately, cGAS signals were too weak and could not be detected above the background signal. However, platelets isolated from *WT* mice but not from *cGAS*$^{-/-}$ mice produced a significant amount of cGAMP when stimulated with DNA (Figure 4C), strongly supporting the presence and functionality of cGAS in platelets despite that we could not visualise the protein. Given that platelets are anucleate, they are not capable of activating the canonical STING signalling in the presence of cGAMP. Nonetheless, upon activation, platelets express proteins at their surface, release inflammatory mediators and aggregate.

cGAS activation of washed platelets potentiates their activation

To investigate whether cGAS and STING stimulation could activate platelets, we relied on the analysis of the platelet activation and degranulation marker P-selectin (CD62P) [37]. Using washed platelets isolated from *WT* and *cGAS*$^{-/-}$ mice, we measured the level of P-selectin at the surface of platelets stimulated with DNA using flow cytometry. DNA alone could not induce a significant translocation of P-selectin at the cell surface. However, when used in combination with a sub-threshold concentration of collagen, DNA stimulation significantly increased the level of P-Selectin at the platelets surface of *WT* compared to *cGAS*$^{-/-}$ platelets (Figure 4D and E), suggesting a synergistic effect.

cGAS and STING stimulation potentiates human platelets aggregation.

We investigated whether the activation of platelets by cGAS or STING was conserved between mice and humans and could lead to platelets aggregation. To test this effect, we isolated platelets from healthy volunteers and confirmed the presence of cGAS and STING by western blotting using washed platelets (Figure F). We next tested the capacity of human platelets to aggregate by using different concentrations of collagen, with 1 ug/ml being sufficient to reach maximum aggregation (Figure 4H). Similarly, to murine platelets, neither DNA nor cGAMP alone could induce platelets aggregation. However, when using a sub- 8 threshold concentration of collagen in combination with DNA or cGAMP, it led to a rapid aggregation of platelets (Figure 4G and 4H) clearly showing the capacity of cGAS and STING to enhance the aggregation of platelets. Taken together these results strongly support a new role for the cGAS-STING pathway in the biology of platelets.

Discussion

This study is, to our knowledge, the first to report that megakaryocytes possess a functional cGAS-STING pathway. This is also the first demonstration that stimulation of the cGAS-STING pathway activates platelets and potentiates their aggregation. Our findings define a novel and non-canonical function for the cGAS-STING pathway.

Using both a pharmacological inhibitor of STING and the genetic depletion of cGAS, we discovered that STING activation using DMXAA results in a potent IFN-I response. Similarly, we also found that DNA stimulation of megakaryocytes also results in IFN-I response, albeit to a lesser extent. This could be attributed to similar mechanisms to what was reported for the inhibition of cGAS during mitosis [28,29,38-40]. We observed a difference on the level of P-STING between *WT* and *cGAS*$^{-/-}$ megakaryocytes and measured a reduction of ISGs in megakaryocytes treated with the STING inhibitor C176 suggesting that there is a basal activation of STING signalling. The fact that we detected P-STING punctates in *cGAS*$^{-/-}$ megakaryocytes, suggests that other cGAS-independent mechanisms might be at play. In fact, in cancer cells treated with the DNA damage agent etoposide, nuclear STING activation requires the DNA repair proteins ataxia telangiectasia mutated (ATM) and poly-ADP-ribose polymerase 1 (PARP1) and interferon-inducible protein 16 (IFI16) and not cGAS [41]. Nonetheless, our results still suggest that cGAS is basally activated in megakaryocytes. Whereas the molecular causes of the increase in DNA damage remain to be defined, one possible explanation is an increase in reactive oxygen species (ROS). According to a recent study, megakaryocytes produce high levels of mitochondrial ROS during thrombopoiesis [42]. Whether these ROS cause DNA damage and contribute to the translocation of cGAS will need further investigations.

Megakaryocytes are also located in the lung vasculature where they contribute to the production of a pool of blood platelets [1]. In this study, we selectively characterised megakaryocytes from the bone marrow but lung-megakaryocytes have been suggested to act like antigen presenting cells [43,44]. As such, we could expect cGAS and STING proteins to share a more cytosolic localisation to rapidly alert the cells of invading pathogens [45]. In line with that, it was recently shown that SARS-CoV2 RNA has been detected in lung megakaryocytes [46] and that elevated levels of lung megakaryocytes

correlates with severe COVID-19 pathology [47]. Given that the cGAS-STING pathway contribute to the antiviral response against RNA viruses, including against SARS-COV2 [48-50], it would be interesting to determine whether cGAS is involved in the antiviral response of lung-megakaryocytes. Accordingly, a study reported that megakaryocytes infections by Dengue and Influenza viruses does induce a protective type-I IFN [25].

Besides their role in megakaryocytes, our study also reports for the first time that human and mouse platelets possess cGAS and STING proteins. In our image analysis, we could indeed detect cGAS and STING in proplatelets. How these proteins traffic from megakaryocytes to reach the sight of platelets generation is still not defined. STING is a transmembrane protein that localises at the endoplasmic reticulum and transit through the trans-golgi before being degraded in lysosomes or recycles after its activation [51,52]. Based on that, one could easily envision a model in which STING use the trans-golgi network and multivesicular bodies similarly to other membrane-associated receptors such as P-selectin, soluble fibrinogen (FGN), and secretory von Willebrand factor (vWF) to join the trafficking route of alpha granules [53]. Further imaging analysis using specific markers would be necessary to determine whether cGAS and STING are located in alpha granules or in any other type of granules (dense, lysosomal or T granules).

We have also discovered an unexpected role for cGAS and STING proteins in platelets. Our results show that cGAS and STING stimulation potentiates platelets activation and aggregation. Interestingly, aspirin an inhibitor of platelet functions also suppresses cGAS activity [54]. When activated, platelets release the content of their granules, which include inflammatory molecules, adhesion proteins, serotonin and small nucleotides [53]. A similar priming effect to sub-threshold concentrations of classical platelet agonists was reported for TLR9, another DNA receptor [30]. However, it was later reported that platelets from TLR9 KO mice still aggregate when stimulated with CpG-dinucleotides and collagen, suggesting that at least another receptor is involved in DNA sensing [30]. Our study suggest that this receptor is cGAS. Together these studies suggest that DNA stimulation of platelets contributes to both inflammation and clotting.

One of the important questions raised by our study is the biological contexts allowing cGAS and STING stimulation. To get activated, platelets-cGAS and -STING would need to be in direct contact with DNA or with cGAMP. Direct activation of STING

could occur during platelets infections of bacteria producing cyclic dinucleotides or by viruses that engage the cGAS-STING pathway such as Staphylococcus aureus or HIV [55,56]. Platelets are also known to endocytosed molecules from their extracellular environment. Whether the endocytosed molecules could reach cGAS or STING would however need to be investigated. If possible, inflammatory contexts leading to the release of cellular content such as infected cells that rupture [57] and circulating free DNA during sepsis [58] could lead to cGAS-STING activation in platelets and contribute to hypercoagulopathy. We also need to emphasise that STING activation in endothelial cells was reported in the context of severe COVID-19 in which high levels of activated platelets are present [59]. The outcome of activated platelets interacting with cGAMP-producing endothelial cells remains however to be determined. We can also foresee that platelets will be able to directly uptake extracellular cGAMP as shown for antigen presenting cells [60,61]. Conversely, a scenario in which cGAMP from platelets is transferred to interacting immune cells through protein channels like LRRC8 can also be envisioned [62]. In view of the many outcome resulting from STING activation, we could expect platelets cGAMP to modulate antigen presentation [63], autophagy [64], inflammatory response responses [65] and even cell death [66] in interacting cells.

Conclusion

In summary, we report for the first time that megakaryocytes and platelets possess a functional cGAS-STING pathway. We have identified a novel role for cGAS and STING in platelets, conserved in humans and mice. Taken together, these observations support a model in which STING activation in platelets potentiates activation and coagulation. This model could contribute to microbial clearance during acute immune responses but may also underpin tissue damage and hypercoagulopathy during uncontrolled immune responses as seen during sepsis. Given the growing contribution of megakaryocyte and platelets in immune responses, our findings pave the way to important research avenues.

Methods

Mice

The animal protocol was approved by the institutional review board protocol (*Université du Québec à Trois-Rivieres* (CBSA- ethic certificate 2021-G.P.1) and guidelines of the Canadian Council on Animal Care were followed. For our studies, we used male and female 8- to 14-week-old C57BL/6J wild type and *cGAS*$^{-/-}$ mice (B6(C)-Cgastm1d(EUCOMM)Hmgu/J; *Jackson Laboratories*). Mice were sex- and age- matched when directly compared. Blood was collected on mice anesthetized with isoflurane and bones were collected after cervical dislocation.

Megakaryocytes purification and culture

Bone marrow cells were obtained by flushing with DMEM the inside of bones (iliac crest, femurs, and tibiae from both legs of the mice) and centrifuged at 300g for 10 minutes at 4ºC and resuspended in 1mL of isolation buffer (phosphate-buffered saline 1x sterile (PBS), pH 7.2, 0.5% bovine serum albumin (BSA) (*BioTech*) and 2mM EDTA). The cells were centrifuged again at 300g for 10 minutes at 4ºC and resuspended in 90µL of isolation buffer. To isolate CD41+ cells, 10µL of FcR Blocking reagent (*Miltenyi Biotec*) was added to the cell suspension for 10 minutes at 4ºC. Cells were then labelled with 10 µL anti-CD41 antibody (coupled to Biotin or APC-*Miltenyi Biotec*) for 10 minutes at 4ºC, then washed with 2 mL of isolation buffer and centrifuged at 300g for 10 min. at 4ºC, twice prior to the addition of 20µL of Anti-Biotin/APC Microbeads (*Miltenyi Biotec*) and 70 µL of isolation buffer for 15 minutes at 4ºC. The cells were washed as described before and resuspended in 5 mL of isolation buffer solution. Then positive selection was carried out using large column cells according to the manufacturer protocol (*Miltenyi Biotec*). Finally, CD41+ cells were resuspended in growth medium (DMEM medium with 4.5g/L Glucose, L-glutamine supplementation, and sodium pyruvate additive accompanied with 10% (v/v) heat-inactivated foetal bovine serum and 1% antimycotic antimycin) supplemented with 50 ng/mL of thrombopoietin and grown for 3-5 days at 37 °C in a 5% CO2 and 95% air humidified incubator. When needed, cells were stimulated with 2µg/ml of DNA (unless mentioned otherwise) or 2µg/ml of cGAMP complexed with Lipofectamine 2000 (Invitrogen) with a ration of 1:0.5 in FBS free Medium (Optimem

(1x) with HEPES additive, 2.4g/L sodium bicarbonate and L-glutamine supplementation (*Gibco*)) or with the murine STING agonist DMXAA at 20 µg/ml.

Platelets isolation (mouse)

Blood was collected from the mice from the inferior vena cava (IVC) with a 25-gauge needle and a 1mL syringe containing 100 µl of 3.8% sodium citrate (3.22% citrate, 112.9 mM citrate, 123.6 mM glucose, 224.4 mM sodium and 114.2 mM hydrogen ions) as anticoagulant, and subsequently transferred to a 1.7ml microcentrifuge and gently inverted several times to ensure proper mixing. The blood solution was then diluted with 1:1 with PBS centrifuged at 130 g for 15 minutes at room temperature. All centrifugations were carried out without a brake and with low acceleration mode. Two thirds of the upper layer consisting of platelet rich plasma (PRP) was transferred to another 1.7ml microcentrifuge tube which was centrifuged at 130 g for 5 12 minutes to remove leucocyte contamination. This step was repeated and the supernatant was transferred to a new 1.7ml microcentrifuge and centrifuged at 1000g for 10 minutes with 100µl of 3.8% sodium citrate to pellet the platelets. Platelets were then resuspended in 90µL Hank's Balanced Salt Solution (HBSS) pH 6.4 and 1M EDTA or Tyrode's buffer pH 7.35-7.4 depending on the experiment. To deplete residual contaminating leucocytes, 10µL of Biotinylated CD45 anti-mouse antibody (*Miltenyi Biotec*) was added to the platelets solution and incubated for 10 minutes at room temperature. The platelet solution was then submitted to negative selection by placing the tube on a magnet to bind the CD45+ cells. The supernatant containing the platelets was then transferred to a new 1.7ml microcentrifuge tube and centrifuged for 1000g for 10 minutes to pellet the platelets without leucocyte contamination (platelets purity analysed by FACS was between 97-98%). Platelets were then resuspended in HBSS with 1M EDTA 6.4 pH or in Tyrode's buffer for experimentations. When needed, mouse platelets were stimulated with 5µg/ml DNA and with 0,25 to 1 µg/ml Type-IV collagen (*Chronolog inc*).

FACS analysis

To measure the surface level of CD62P (P-Selectin), washed platelets were stimulated at 37°C under agitation with the indicated agonist for 30 minutes prior to adding the collagen

for 15 minutes. Platelets were then stained with the anti-CD41-BV515 and anti-CD62P-R718 antibodies before being fixed for 15 minutes using 4% PFA, washed and analysed using a Beckman Cytoflex Cell analyser (4 lasers). 20 000 events were analysed per sample per condition. Results were analysed using the company software CytExpert (*Beckman*).

Platelets isolation (human)

This study was approved by the Research Ethics Committee of the *Montreal Heart Institute* (#2017-2154) and was conducted in accordance with the Declaration of Helsinki. Participants were considered healthy if they were aged 18 years or older, did not require long-term medical therapy, had refrained from drugs known to influence platelet function in the previous 2 weeks, had not undergone major surgery in the previous 6 months, did not have a history of bleeding symptoms, and had platelet counts and hemoglobin levels within the normal reference range. Informed consent was obtained from each participant. Blood was drawn by venipuncture into syringes containing acid citrate dextrose (ACD-A, DIN: 00788139) in a 1:5 volume ratio (ACD/blood). Blood was transferred to 50mL-tubes, 13 centrifuged at 200 g for 10 min without a brake, and PRP was collected. Prostaglandin E1 (1 µM) was added to PRP prior to centrifugation at 1000 g for 10 min. Plasma was removed and discarded. Pelleted platelets were resuspended in Tyrode's buffer (137 mM NaCl, 11.9 mM NaHCO3, 0.4 mM NaH2PO4, 2.7 mM KCl, 1.1 mM MgCl2, 5.6 mM glucose, pH 7.4). Platelet counts were determined using a Beckman Coulter hematology analyzer (*DxH 520*) and adjusted to a final concentration of 2.5 x 108 /mL. Platelets were allowed to rest at room temperature for 60 min prior to functional experiments.

Light transmission aggregometry

Platelet aggregation was measured using a Chronolog aggregometer (Model 700, Havertown, PA, USA) at 37 °C with continuous stirring at 1200 rpm. Platelet aggregation traces were recorded for 30 minutes using the AGGRO/LINK®8 Software package. Collagen (*Chronolog*) 0.06-1 µg/ml was used to stimulate platelet. When specified, washed platelets were preincubated with DNA (2µg/ml), cGAMP (2µg/ml) complexed

with Lipofectamine 2000 (Invitrogen) with a ratio of 1:0.5 in FBS-free Medium (Optimem (1x) with HEPES additive, 2.4g/L sodium bicarbonate and L-glutamine supplementation (*Gibco*)), or vehicle for 30 min at room temperature.

Western blot

To analyse proteins by western blotting, cells in 48 w/p were lysed using 100µL of RIPA lysis buffer (10mM Tris-HCl (pH 8.0), 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 140mM NaCl, diluted in dH2O) on ice and the cell lysates were cleared by centrifugation at 13 000 RPM for 10 minutes at 4ºC. Cell lysates were mixed with 6X loading blue and denatured by heating at 95ºC for 5 minutes. Proteins were separated by migration on a 10% SDS-PAGE and transferred on a nitrocellulose membrane prior to immunoblotting with specific antibodies. Bands were revealed using chemiluminescence.

Gene expression analysis

RNA was extracted from cell lysed with RLT lysis buffer using RNeasy Micro Kit (*Qiagen*) and according to the manufacturer protocol. RNA was reverse transcribed using Applied Biosystems High-Capacity cDNA Reverse. Quantitative PCR reaction was performed using SensiFAST SYBR No-ROX Kit (*Bioline*) and specific primers for genes of interest were used. 14 QPCR was performed in an iCycler (*Bio-Rad*) qPCR instrument. Results were normalized on the housekeeping gene TBP and 18S using the ddCT method.

ELISA

mIFNB was measured from the supernatant of cells stimulated for 5 hours with DNA or DMXAA using the mouse IFN-beta Quantikine ELISA kit (*R&D Sytems*) according to the manufacturer protocol. cGAMP ELISA was performed using specific cGAMP specific ELISA (*Cayman*) on either cell lysates or platelets supernatants following 1 hour of stimulation with DNA according to the manufacturer protocol. For platelets stimulation, C176 was added at a concentration of 1µM for 30 minutes prior to DNA stimulation.

Microscopy

For live cell imaging of megakaryocytes, an Optika inverted trinocular LED fluorescence microscope was used. For fixed cells analysis, cells were prepared as followed. Platelets were isolated, fixed with PFA 4% for 10 minutes and added on coverslips. For megakaryocytes confocal imaging, CD41+ cells were grown and differentiated directly on coverslip for 1 to 4 days depending on the experiment. Cells were fixed with 4% paraformaldehyde. Cells (not platelets) were permeabilized using 0.2% Triton in PBS 1X. Permeabilized cells or platelets were incubated in blocking solution (0.1% Triton with 1% BSA in PBS 1X) for 15 minutes prior to their incubation with specific antibodies. Coverslips were mounted on slides with ProLong™ Gold Antifade mounting reagent (*ThermoFisher*). DAPI was used to stain the DNA of megakaryocytes. Images were taken using a Leica SP8 laser scanning fluorescence confocal and analysed with the LAS X software from Leica.

List of primers

mInfb1 (F: CCCTATGGAGATGACGGAGA, R: CCCAGTGCTGGAGAAATTGT); mRsad2 (F: CTGTGCGCTGGAAGGTTT, R: ATTCAGGCACCAAACAGGAC); mIfit1(F: GAGAGTCAAGGCAGGTTTCT, R: TCTCACTTCCAAATCAGGTATGT); mIsg15 (F: CAATGGCCTGGGACCTAAAG, R: TAAGACCGTCCTGGAGCACT); mIfitm3 (F: GGT CTGGTCCCTGTTCAATAC, R: GTCACATCACCCACCATCTT); mCcl5 (F: GCTGCTTTGCCTACCTCTCC, R: TCGAGTGACAAACACGACTGC); m18s (F: 15 GTAACCCGTTGAACCCCATT, R: CCATCCAATCGGTAGTAGCG); mTbp (F: AATAAGAGAGCCACGGACAAC, R: TCTGGATTGTTCTTCACTCTTGG)

List of antibodies

Anti-cGAS D3080, Cell Signaling (NEB 31659S); Anti-STING D1V5L, (NEB 50494S); AntiP-STING D8F4W, (NEB 72971S) ; Anti- IRF-3 (D83B9), (NEB 4302S); Anti-P-IRF3 D6O1M, (NEB 29047S); Anti-P-yH2AX 647 20E3, (NEB 9720S) Anti-β-Actin (8H10D10) (NEB 3700); Anti-CD41-APC, (*Miltenyi Biotec* 130-122-761); Anti-CD41-BV515, (*Miltenyi Biotec* 130-122-768); Anti-CD45-PE, (*Miltenyi Biotec* 130-117-498);

Anti-Mouse CD62P R718 (BD 751855); Anti-rabbit Alexa Fluor 555 (*ThermoFisher* A21428); Anti-rabbit Alexa Fluor 488 (*ThermoFisher* A32723); Anti-β-Tubulin (9F3) 488 (NEB 3623S); Anti-Lamin A/C (4C11) 488 (NEB 8617S)

Statistical analyses. Statistical analyses were carried out using Prism 7 (*GraphPad Software Inc.*). Two-tailed unpaired T- tests were used to compare pairs of conditions. Symbols used: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

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AUTHOR CONTRIBUTIONS

FEM, KL, SBP performed experiments and analyzed data. AP, FAA and GJ helped with specific experiments. JH, BK and MPG supported initial experiments of this project. ML supported the experiments with human platelets. GP conceived and coordinated the study, 16 designed and analysed experiments, and prepared the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Chapter IV

4. Discussion

4.1 Bone marrow CD41+ isolation showed a high degree of MK purity

In this study we wanted to characterize the inflammation pathway of cGAS-STING in bone marrow MKs. We chose bone marrow MKs for their specific characteristic of high ploidy, a characteristic very common in cancer cells which have genetic instability proportional to their degree of aneuploidy. Also, bone marrow MKs were studied over lung MKs because bone marrow MKs have on average a ploidy of 16N while lung MKs have on average a ploidy of 2N [34]. Our observations of differentiated BM MKs showed a high degree of ploidy which concorded with what's been previously found in the literature [27].

We then wanted to ensure that our MK isolation is pure. To do so, we imaged the isolated cells in the wells of our cell culture plates with a brightfield microscope during a 3-day incubation time and visually assessed MK purity and differentiation stages (see Fig. 1A). We also observed in confocal microscopy the CD41 antigen expression on the isolated cells membrane which is a marker for mature MKs (see Fig. 1B). Although our method of isolation seemed to result in a high proportion of MKs, assessing the purity of our cells by observations alone is a limitation to our study. On top of that, a CD41+ isolation gave us different population of MKs at the same time which could have affected our results since different population of MKs could have different immune responses and differentiation time periods. An access to a fluorescence-activated cell sorting (FACS) would have been preferred to isolate our MKs since we could have had better purity and could have divided the MKs by ploidy and study these populations of MKs separately.

4.2 STING stimulation in megakaryocytes induces a type-I Interferon response

In this study, we wanted to show that cGAS-STING pathway was functional in MKs, which is important for these cells to defend themselves against foreign pathogens in case of an infection. This pathway also ensures MKs can detect dsDNA damage and since we hypothesize that they have genetic instability we wanted to see if there was a basal activation of the pathway. Also, we compared cGAS KO mice to *WT* mice to ensure that the dsDNA immune response was cGAS specific and not coming from another PRRs like AIM2 or TLR9 [7]. Collectively, our results showed that MKs have all the necessary proteins to initiation a normal dsDNA immune response going through the cGAS-STING pathway (see fig 2C). They also showed that MKs can express IFN-b induced genes production (see Fig. 2D) and produce IFN-b and CCL5 (see Fig. 2E-F) which tells us that they can defend themselves against pathogens. Additionally, this dsDNA immune response is not going through another receptor as cGAS KO MKs that have a functional pathway downstream of STING did not respond to a dsDNA transfection (Fig. 2A-B-C). A study has shown that MKs express TLR9 and is distributed to PLTs during pro-PLT production [48]. Unmethylated CpG sequences of bacterial DNA are the only known ligands for TLR9 [49]. It has also been shown that cGAS can bind to CpG DNA [50]. Knowing MKs express both TLR9 and cGAS, it would certainly be interesting to see which pathway is more important in a CpG DNA induced inflammatory response.

Although there is a cGAS driven immune response to DNA in MKs (Fig. 2A), the immune response is fairly low compared to other cellular types to this same DNA. Indeed, in cell like MEFS and macrophage IFN-b1 related gene are induced at a higher level in cells transfected with dsDNA [51]. Since MKs are going through endomitosis and since cGAS is inactive during the G2/M to M Phase[13], and since the Golgi is dispersed during mitosis and STING cannot translocate to the Golgi when it's activated [52], this could explain the reason for this cGAS-STING inhibited immune response. To test this, it would be interesting to look at difference of dsDNA immune response between cGAS KO mice and *WT* mice in other cellular types than MKs like lung fibroblasts or macrophage to verify that it is a cellular related inhibition. An augmented immune response in other cell types could suggest that endomitosis is affecting cGAS activity.

4.3 cGAS and STING localization in mouse megakaryocytes

Micronuclei are extranuclear DNA-containing structures that are the result of chromosomal mis segregation [26]. During endomitosis, MKs at a later stage have been characterized to form micronuclei complexes [28] which are known to colocalize with and activate cGAS. Our results showed that in about a third of MKs there are micronuclei. Interestingly, high level of cGAS is detected in 90.7% of those micronuclei (Fig. 1G). cGAS was also seen in the nucleus which would potentially be tethered to chromatin to prevent activation with genomic DNA and in the cytosol, where it can sense DNA that is abnormally present [13].

Similar, to cGAS, STING is observed in the nucleus and the cytosol (Fig.1G). Cytosolic STING was previously observed in mitotic arrested cells which could explain the abundance of cytosolic STING [52]. An abundance of cGAS and STING in the nucleus raises the possibility of nuclear cGAS-STING sensing nucleic acids from infectious agent from within the nucleus along with cytoplasmic activation and in consequence activating an innate immune response from inside the nucleus [18]. Our results showed that there is a lot of genomic instability in MKs, the basal response of cGAS-STING pathway could potentially be a result of nuclear cGAS and STING recognizing damaged dsDNA as a DAMP and initiating an immune response. Since cGAS is probably inactivated in the nucleus, this immune response could come from cGAS activation to micronuclei.

Furthermore, using confocal microscopy, we observed P-STING, which is the activated form of STING, in *WT* bone marrow MKs (Fig. 2E) which suggests that the inflammation pathway is fundamentally active during endomitosis most likely due to the replication stress mediated by the endomitosis process. P-STING localization near the middle of the cells next to the nucleus is an expected finding since P-STING has been noted to localize in the Golgi and the RE [9], it would be good to do colocalizing experiments for P-STING with Golgi proteins or P-STING with RE proteins. Levels of P-STING were also more elevated in *WT* than $cGAS^{-/-}$ and so was the average number of foci per cell (Fig. 2F). Additionally, finding foci in $cGAS^{-/-}$ mice was unexpected but there has been a study that shows that STING could be activated independently of cGAS,

partnering with SYNCRIP, MEN1, DDX5, snRNP70, RPS27a, and AATF as novel modulators of dsDNA-triggered IIRs [18]. Also, to solidifies that STING plays an active role in innate immune response during the endomitosis of MKs, we treated our cells with C-176 STING inhibitor and our results demonstrated a reduction in IFN-b1 genes expression which support STING basal activity during endomitosis (Fig. 3A). To find if this response is cGAS-dependant or STING-dependant, we could introduce a cGAS inhibitor in WT and *cGAS*$^{-/-}$ MKs from littermates mice bone marrow MKs and see if there is also a reduction in IFN-b1 genes expression. If there isn't a reduction it would mean this is a STING-dependant response.

4.4 Mouse bone marrow MKs exhibit difference in genomic instability depending on the stage of their differentiation

Next, we studied the levels of Phospho-γH2AX, which marks damaged double stranded DNA, in MKs at different stages of differentiation. Our results showed that levels of Phospho-γH2AX were increasing or decreasing depending on the differentiation stage of the cell. In accordance with our first interpretation that the more DNA replicates the more DNA damage is present, we noticed that MKs with increased ploidies have higher levels of DNA damage, but this suddenly decrease in the latest stage of differentiation (Fig. 3B-C).

This sudden decrease in DNA damage in the latest stage of differentiation MKs, while unexpected and could be explained by ROS production during the different stages of the MKs. As shown previously [39], ROS initiate thrombopoiesis from mature MKs. These ROS are at first increased from round MK to intermediate MK and then sharply decreased in terminal MK producing proplatelets (Fig. 1.9). ROS have also been proven to induce DNA damage, so these changes in levels of ROS could suggest a reason for the different levels of DNA damage depending on the stage of the MKs [53]. Another reason is that in a DNA damaged nucleus, one role of cGAS is to bind to dsDNA breaks and inhibits homologous recombination-derived DNA repair, leading to genomic instability and tumorigenesis [54]. A recent study showed that DNA damage change localisation of cGAS which in turn could be activated. This means that the basal immune

activity could be caused by a mechanism to send cGAS into the cytosol due to MKs genomic instability.

4.5 Platelets inherit cGAS from megakaryocytes and respond to foreign DNA

During the imaging of MKs, we observed small buds near the cytoplasmic membrane that were expressing high levels of cGAS and STING (Fig. 4A). Knowing pro-platelets formation can come from extension of the MKs or from a biological phenomenon called MK budding [36], we studied the transfer of cGAS and STING proteins from MKs to platelets and if they can be activated.

Our results showed that platelets expressed STING using confocal microscopy (Fig. 4B). We also saw a production of cGAMP in *WT* platelets but not *cGAS*$^{-/-}$ platelets after dsDNA transfection (Fig. 4C) which confirm that platelets interaction with dsDNA leads to a cGAS-dependent activation. This is an important finding since we know platelet can interact with macrophage and other blood cells [43]. It would certainly be interesting to study if cGAMP can be transferred from platelets to other cells to induce an immune response. It has also been shown that platelets can be phagocytosed by macrophage in cases of chilling or sepsis in the liver [55]. This new concept of platelet clearance by macrophage could potentially be a way for macrophage to initiate the cGAS-STING pathway by phagocytosis of platelet intracellular cGAMP, which could contribute to inflammation.

4.6 Human platelets in contact with collagen stimulate a synergic aggregation with DNA and cGAMP

After confirming that human platelets have both cGAS and STING proteins inside them with western blot (Fig. 4D), we wanted to study the role of proteins in platelets in a context of synergy with collagen to simulate a vascular injury [44]. Our aggregation experiments points to a cGAS and STING mediated platelet activation and aggregation. Although, alone, cGAS and cGAMP did not induce an aggregation in platelets, when in synergy with collagen, DNA and cGAMP initiate platelet aggregation (Fig. 4E-F). This

synergy is still not well understood but some molecular mechanisms could explain this phenomenon. A study of GTP and ATP thrombin-induced aggregation on washed human platelets showed these molecules have an inhibitory effect on platelet aggregation. cGAS modulating the available pool of GTP and ATP to produce cGAMP [9] using these 2 molecules could potentially lead to an adverse effect in which platelet are more susceptible to activation due to the reduced amount of ATP and GTP in them [56]. In another matter, our results showed that different individuals had platelets that were more activatable than others. We also saw that there were different STING bands in the western blot depending on each person, these could either be variants in the population, or post-translational modification (Fig.4D). It would certainly be interesting to study the differences of humans STING variants and see if some variants are more activatable than others.

An important insight of our research is better understanding how platelet aid in cancer cells development. A recent study has shown that cancer cells can activate platelet and trigger platelet Toll-like receptor 4 (TLR4) related NETosis [57]. The release of histone 3 and 4 as well as the extracellular DNA in NETs can activate platelets directly which initiate continuous loop of activation [57]. NET formation has been shown to aid in cancer progression and some studies even described elevated levels of plasma cell-free DNA in patients with different cancer types [58]. Studying platelet activation with this plasma cell-free DNA in relation to the cGAS-STING pathway could help us understand how to prevent this continuous activation loop and prevent metastatic progression.

Conclusion and insights

In conclusion, our results suggest that both MKs and platelets express the cGAS-STING pathway and can activate it to produce an immune response. Our findings also suggests that cGAS-STING activity is normally expressed during endomitosis. With this research, we pave the way to understanding important insight in the biology of MKs and other polyploid cell populations such as trophoblast cells. A better understanding of MKs will improve our understanding of platelet biology; platelets are now recognized as key players in the immune response. Learning how cGAS modulates the MK transcriptome will also help decipher the physiological condition that shapes basal platelet reactivity. Moreover, understanding the many aspects regulating MK senescence will have a direct impact on our ability to produce functional platelets in vitro which would be a breakthrough for medicine. Our findings on the role of cGAS and STING in MK will be of interest to other areas of immunology and infectiology since cGAS is also an important pathogen sensor. Even more promising, the results of this research will open new questions such as the importance of cGAMP transfer from MKs to neighboring cells in modulating bone marrow microenvironment, or the impact of cGAS activation on platelet content and reactivity.

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