Identification of Anticoagulant Properties of Dimethyl Fumarate and 4-Octyl Itaconate via Suppression of the Macrophage Type I Interferon-Tissue Factor Axis

Thesis submitted to the University of Dublin for the degree of Doctor of Philosophy

by

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Chapter 7 – Appendix
7.1 Oral and poster presentations

7.1.1 Oral Presentations

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<tr>
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<td>International Society of Thrombosis and Haemostasis 2023 Congress, Montréal, Canada (Early Career Award awardee)</td>
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<td>November 2022</td>
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7.1.2 Poster Presentations

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7.2 Record of Publications

7.2.1 Published


7.2.2 Accepted manuscripts

Tissue Factor in macrophages via inhibition of Type I Interferon. *Nature Communications (in press).*

### 7.2.3 Unpublished manuscripts

Modification of Proteins by Metabolites in Immunity

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SUMMARY

Immunometabolism has emerged as a key focus for immunologists, with metabolic change in immune cells becoming as important a determinant for specific immune effector responses as discrete signaling pathways. A key output for these changes involves post-translational modification (PTM) of proteins by metabolites. Products of glycolysis and Krebs cycle pathways can mediate these events, as can lipids, amino acids, and polyamines. A rich and diverse set of PTMs in macrophages and T cells has been uncovered, altering phenotype and modulating immunity and inflammation in different contexts. We review the recent findings in this area and speculate whether they could be of use in the effort to develop therapeutics for immune-related diseases.

The recent growth in the field of immunometabolism has revealed how specific changes in the concentrations of metabolites in immune cells can affect the immune response (Makowski et al., 2020; O’Neill et al., 2016). The consequences of these metabolic alterations go beyond the traditional role of metabolism in bioenergetics and biosynthesis. Immunologists have instead revealed roles for specific metabolites in targeting processes that have impact on immune cell function in more specific ways. Post-translational modification of proteins is emerging as a key means by which intracellular metabolites can modulate immunity. In this review, we focus on recent developments in this specific area and speculate on their importance as a critical functional readout for the metabolic changes that are happening in immune cells. A wealth of information is emerging that could prove useful in the search for immunomodulatory therapeutics for immune and inflammatory disease.

LACTYLATION CONTRIBUTES TO RESOLUTION OF INFLAMMATION

The renaissance of interest in metabolism in immune cells can be traced to the observation that inflammatory cells, such as classically activated macrophages and T helper-17 (Th17) cells, display the so-called Warburg effect (Krawczyk et al., 2010; Masters et al., 2010; Shi et al., 2011). This phenomenon was originally described in cancer, where glycolysis is potently upregulated, even when oxygen is readily available, and is a major generator of adenosine triphosphate (ATP) in tumors (Vander Heiden et al., 2009; Warburg, 1956). Blocking glycolysis in immune cells was shown to impact on interleukin-1β (IL-1β) production from macrophages (Mills et al., 2016; Tannahill et al., 2013), Th17 cell differentiation (Shi et al., 2011) and dendritic cell (DC) activation (Krawczyk et al., 2010). High rates of glycolysis mean that large amounts of lactate are produced and secreted. Previously, lactate was considered to be little more than a waste by-product of this metabolic process but emerging evidence indicates that it has a number of notable physiological roles, such as fueling Krebs cycle (Faubert et al., 2017; Hui et al., 2017).

A recent contribution to what Warburg metabolism might mean for macrophages has been made when lactate was shown to modify histones in a process termed lactylation. This post-translational modification (PTM) was shown to occur in several cancer cell lines under conditions of hypoxia and is highly prominent in proinflammatory classically activated macrophages (Zhang et al., 2019a). Lactylation of histones at promoter regions of genes increases their expression and genes associated with wound healing, such as Arg1, have been found to be particularly upregulated. Standard proinflammatory genes, such as those encoding Tnf and Il6, are unaffected. This study revealed a mechanism by which proinflammatory macrophages could adopt a more homeostatic alternatively activated phenotype at later time points, in order to facilitate the resolution of inflammation and was consistent with previous studies linking lactate to alternative macrophage polarization (Colegio et al., 2014). Importantly, the authors demonstrated that lactylation could be derived from both exogenous and glucose-dependent endogenous lactate. Histone lactylation may also contribute to the alternatively activated profile that is typical of tumor-associated macrophages (TAMs). As tumors are often hypoxic environments, this leads to lactate accumulation, which may alter the epigenetic landscape of the resident macrophages, giving rise to the typical TAM phenotype that is observed. High lactation in TAMs has also been observed in two mouse models of cancer (Zhang et al., 2019a). This study therefore reveals another important aspect of glycolysis in macrophages. At early time points, glycolysis is required for proinflammatory gene expression, but at later time points the main product of glycolysis in Warburg metabolism, lactate, promotes an anti-inflammatory phenotype by lactylating histones on specific target genes important for resolution of inflammation (see Figure 1). This PTM would therefore...
Acetylation of Histones and Other Immune Targets

Moving on from glycolysis, the next major metabolic pathway is Krebs cycle, the central hub of all metabolism. It takes place in the mitochondrial matrix and the main role ascribed to it is to generate nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂), which deliver electrons to the electron transport chain, leading to ATP production in the process called oxidative phosphorylation (OXPHOS). Krebs cycle is fueled by acetyl-CoA derived from glucose or fatty acids, which has been shown to lactate histones, thereby regulating the expression of genes encoding pro-resolving factors. The Krebs cycle metabolites succinyl-CoA, fumarate, and itaconate can also give rise to PTMs. In macrophages, the targets of such PTMs are often glycolytic enzymes, which represents a method of regulation between the two pathways. In this way Krebs cycle-derived metabolites can curb glycolysis, which might limit inflammation via effects on aldolase, GAPDH, and PKM2, or if lactate concentrations fall, control the resolution of inflammation.

Acetyl-CoA represents the entry point into Krebs cycle. The tricarboxylic acid (TCA) intermediate citrate can be exported from the mitochondria to the cytosol and converted to acetyl-CoA by ATP citrate lyase (ACLY), which leads to acetylation. Acetylation and deacetylation of lysine residues are the most broadly studied and extensively characterized epigenetic modifications of histones. Two types of key regulators, histone acetyltransferases (HATs) and histone deacetylases (HDACs), dynamically control the acetylation state of histones. The antagonistic actions of these enzymes on histones serve as an important mechanism for the epigenetic regulation of gene expression. HATs promote acetylation, which is related to transcriptional activity, whereas HDACs catalyze the removal of acetyl groups from histone tails, which is linked to transcriptional inhibition.

The acetylation state of proteins, which is reversible, is highly relevant to their stability and activity in cells. Defects in protein acetylation frequently result in severe abnormalities of development and physiology due to the dysregulation of gene expression and protein function in animal models, and are implicated in the pathogenesis of many human diseases. Histone acetylation is promoted by the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS), enhancing induction of specific gene sets associated with potent proinflammatory responses. Histone acetylation can also give rise to PTMs. In macrophages, the targets of such PTMs are often glycolytic enzymes, which represents a method of regulation between the two pathways. In this way Krebs cycle-derived metabolites can curb glycolysis, which might limit inflammation via effects on aldolase, GAPDH, and PKM2, or if lactate concentrations fall, control the resolution of inflammation.

In an LPS-stimulated macrophage, glycolysis is potently upregulated. This can generate lactate, which has been shown to lactate histones, thereby regulating the expression of genes encoding pro-resolving factors. The Krebs cycle metabolites succinyl-CoA, fumarate, and itaconate can also give rise to PTMs. In macrophages, the targets of such PTMs are often glycolytic enzymes, which represents a method of regulation between the two pathways. In this way Krebs cycle-derived metabolites can curb glycolysis, which might limit inflammation via effects on aldolase, GAPDH, and PKM2, or if lactate concentrations fall, control the resolution of inflammation.

Acetoacetic acid (SuccIno, fumarate, and itaconate...
HDACs play an important role in macrophage activation and have been shown to regulate production of multiple inflammatory genes (Das Gupta et al., 2016; Nguyen et al., 2018). Deacetylation, which is controlled by sirtuins, is important in M2 macrophage gene expression (Park et al., 2017). Moreover, the interplay between acetylation and deacetylation in macrophage activation is highlighted by the recent discovery that NLRP3, a pattern recognition receptor (PRR), is modified by the interplay between acetylation and deacetylation in macrophages, which leads to its activation. NLRP3 is deacetylated by SIRT2, which can be modulated to reverse aging-associated inflammation and insulin resistance (He et al., 2020).

The regulatory scope of lysine acetylation however is more broad. Recently, acetylation of the key DNA sensor cGAS has been shown to attenuate its activation and prevents self-DNA-induced autoimmunity (Dai et al., 2019). Furthermore, in 2020, further acetylations of cGAS were described, showing that acetyl-mimic mutants suppress the induction of cGAS-dependent apoptosis (Song et al., 2020). As cGAS plays an important role in autophagy, autoinflammatory diseases, and cancer (Gui et al., 2019; Li and Chen, 2018), its acetylation may have multiple downstream consequences. Acetate has also been found to be incorporated into CD8+ memory T cells, driving expansion of the cytoplasmic acetyl-CoA pool and acetylation of GAPDH (Peng et al., 2011). This modification enhances GAPDH activity, driving glycolysis and thus boosting rapid memory CD8+ T cell response. Consistently, acetate-exposed memory CD8+ T cells mediated superior protection in a murine Listeria monocytogenes infection model (Balmer et al., 2016).

Therefore, the acetylation of non-nuclear proteins has a prominent role in modulation of immune function and more protein targets of acetylation will likely emerge.

MALONYLATION DRIVES PROINFLAMMATORY MACROPHAGES

Acetyl-CoA can be converted to malonyl CoA by acetyl-CoA carboxylase (ACC) for the synthesis of fatty acids or cholesterol. Malonyl-CoA can also promote malonylation, a recently identified, evolutionarily conserved PTM (Malonyl-CoA can also promote malonylation, a recently identified, evolutionarily conserved PTM (Bowman et al., 2017; Colak et al., 2015), changing its charge from +1 to -1 (Galván-Peña et al., 2019). This change is predicted to disrupt electrostatic interactions with other amino acids and alter protein conformation and binding to targets (Peng et al., 2011). The only known regulator of malonylation is SIRT5, which acts not only as a deacetylase, but can also remove other acylations, such as succinylation and glutarylation (Du et al., 2011; Tan et al., 2014). GAPDH becomes malonylated (Figure 2) in pro-inflammatory macrophages, which enhances its glycolytic activity and reduces its RNA-binding capacity, allowing TNF-α to be translated, and enabling the production of further pro-inflammatory cytokines, including IL-1β and IL-6 (Gálvan-Peña et al., 2019). Malonylation was therefore proposed as a mechanism by which macrophages can control the production of proinflammatory cytokines through GAPDH.

mTOR has also been shown to undergo malonylation (Figure 2) when fatty acid synthase (FASN) is inhibited or silenced. This reduces mTORC1 activity, which has been found to contribute to defects in angiogenesis in endothelial cells (Buning et al., 2019a). Malonylation of mTOR may well play a notable role in macrophage, T cell, and B cell activation, given its central signaling role in these cell types, but this has yet to be examined.

SUCCEINYLATION, SUCCINATION, AND 2,3-DICARBOXYPROPYLATION TARGET GLYCOLYSIS IN IMMUNE CELLS

Succinate accumulation can give rise to a PTM termed succinylation, which involves the transfer of a succinyl-CoA moiety to a lysine residue (Zhang et al., 2011). Similar to malonylation, this modification alters the positively charged lysine to have a net charge of -1, and while no succinylating enzyme has been thus far identified, desuccinylation is catalyzed by SIRT5, as mentioned above (Du et al., 2011). A report of elevated succinate concentrations in proinflammatory macrophages also demonstrated an enrichment in global protein succinylation, including the identification of several metabolic enzymes that were succinylated, such as GAPDH and malate dehydrogenase (MDH), but the functional consequence of succinylation of these proteins has not been further investigated. There is also a decrease in the expression of SIRT5 when macrophages were stimulated with LPS (Tannaihi et al., 2013), which would skew proteins toward succinylation.

As shown in Figure 1, pyruvate kinase 2 (PKM2) is an important target for succinylation in macrophages. In LPS-activated macrophages, the glycolytic enzyme PKM2 has been shown to translocate to the nucleus, where it can form a complex with hypoxia inducible factor -1α (HIF-1α) and promote the transcription of HIF-dependent genes, including that encoding interleukin-1β (IL-1β) (Palsson-McDermott et al., 2015). Furthermore, nuclear translocation of PKM2 in T cells has been found to promote Th1 and Th17 cell polarization (Angiari et al., 2020), with nuclear PKM2 having been shown to induce STAT3 phosphorylation and thereby enhance IL-17 transcription (Pucino et al., 2019). Succinylation of PKM2 at K311 has been shown to impair its glycolytic activity and induce its nuclear translocation with a concomitant increase in IL-1β production (Wang et al., 2017). In addition, Sirt5-deficient mice exhibit increased susceptibility to dextran sulfate-induced colitis due to hypersuccinylation of PKM2, leading to elevated IL-1β (Wang et al., 2017). Interestingly, in the context of cancer, succinylation of PKM2 has been shown to increase its enzymatic activity but here a different succinylated cysteine 150 on GAPDH in murine macrophages and T cells, as well as the corresponding active site cysteine (C152) for...
human GAPDH in PBMCs from MS patients treated with DMF (Kornberg et al., 2018). The authors also show that GAPDH could be modified by endogenous fumarate in both murine and human immune cells. Succination of GAPDH has been shown to inhibit its enzymatic activity and curtail glycolytic flux. In macrophages, succination of GAPDH is required for DMF-induced inhibition of IL-1β and in CD4+ T cells for the ability of DMF to impair Th1 and Th17 cell differentiation and cytokine production. This study is crucial as it demonstrates that metabolic PTMs can be exploited as a clinical intervention.

Itaconate is another metabolite linked to Krebs cycle that can modify proteins. The accumulation of citrate can lead to the production of itaconate, via cis-aconitate, which is diverted away from Krebs cycle by the enzyme cis-aconitate decarboxylase (CAD), which is encoded by the gene termed Immune responsive gene 1 (IRG1). IRG1, or CAD, is induced in classically activated macrophages by LPS, via type I interferons (IFNs) (Naujoks et al., 2016). Prior to the functional characterization of IRG1, this gene is known to be potently upregulated in LPS-activated macrophages (Lee et al., 1995) before it was later identified as the enzyme responsible for the synthesis of itaconate (Michelucci et al., 2013). Itaconate is one of the most abundant metabolites in classically activated macrophages (Jha et al., 2015; Mills et al., 2018), and recent evidence indicates that it is an anti-inflammatory metabolite. Itaconate has been shown to exert some of these effects through succinate dehydrogenase (SDH) inhibition (Cordes et al., 2016; Daniels et al., 2019; Lampropoulou et al., 2016) and activating transcription factor 3 (ATF3) activation (Bambouskova et al., 2018). However, itaconate has also been identified as a cysteine modifier (Mills et al., 2018), as shown in Figure 1. The modification is called 2,3-dicarboxypropylation, which is a form of cysteine alkylation. It has been found to occur on several cysteine residues on KEAP1 in response to the cell-permeable itaconate derivative, 4-octyl itaconate (4-OI) (Mills et al., 2018), including C151, which has been previously reported to play a role in KEAP1-dependent regulation of NRF2 expression (Zhang and Hannink, 2003). Under basal conditions, KEAP1 associates with NRF2 and induces its degradation but modification of these key cysteine residues on KEAP1 by endogenous itaconate has not been demonstrated.
(Mills et al., 2018), possibly because modified KEAP1 is very unstable. Importantly however, endogenous itaconate in LPS-activated macrophages has also been shown to give rise to 2,3-dicarboxypropylation of a number of proteins (Mills et al., 2019). The functional consequences of 2,3-dicarboxypropylation on the other targets identified are still being elucidated but we can speculate that modification will impact on their function. These include lactate dehydrogenase A (LDHA), which catalyzes the last step of glycolysis, annexin A1 which regulates eicosanoid production and gamma-interferon-inducible lysosomal thiol reductase (GILT), which modulates antigen presentation.

Since the discovery of this modification, several other reports on 2,3-dicarboxypropylation have emerged. A thiol-reactive probe has been developed in order to reveal a more expansive profile of the itaconate-derived cysteine modifications occurring in macrophage lysates (Qin et al., 2019). In this screen, several of the proteins previously shown to be 2,3-dicarboxypropylated were confirmed, including LDHA and annexin A1, in addition to numerous additional protein targets being identified. The authors selected aldolase A, a glycolytic enzyme, as the focus for further validation and investigation. They showed that aldolase A is modified by endogenous itaconate on two cysteine residues and that this greatly impairs its glycolytic activity and contributes to the capacity of itaconate to downregulate IL-1β production, which is known to require glycolysis (Kim et al., 2019). It has also been shown that modification of LDHA by itaconate hinders its enzymatic activity but interestingly this does not affect IL-1β, suggesting that glycolysis per se is not required for IL-1β production. More recently, Qin et al. have developed a bioorthogonal itaconate-alkyne probe in order to assess targets of 2,3-dicarboxypropylation in living macrophages (Qin et al., 2020). This study identifies many additional immune targets that undergo 2,3-dicarboxypropylation in Raw264.7 macrophages, such as NLRP3, AIM2, IRAK4, and gasdermin D. Further functional characterization of one of the targets, RIPK3, has determined that modification of C360 on RIPK3 by itaconate promotes phosphorylation and activation of RIPK3 (Qin et al., 2020), suggesting that itaconate may play a role in necroptosis. Modification of another glycolytic enzyme, GAPDH, by itaconate has also been reported in this study (Qin et al., 2019), and this has been confirmed in another study, where cysteine 22 of GAPDH has been shown to be 2,3-dicarboxypropylated by 4-OI (Liao et al., 2019). This modification reduces enzymatic activity of GAPDH, decreasing aerobic glycolysis and lowering proinflammatory cytokine production (Liao et al., 2019). Interestingly, in another study, 2,3-dicarboxypropylation of GAPDH on cysteine 245 has been reported to have no effect on its glycolytic activity (Qin et al., 2019), again demonstrating that a metabolic PTM on different residues of the same protein can induce contrasting effects. However, it is important to recognize that metabolite derivatives may give rise to off-target effects. A recent study has shown that itaconate derivatives are not always converted to itaconate intracellularly and has also demonstrated that not all of the effects of 4-OI could be recapitulated by itaconate (Swain et al., 2020). Nonetheless, there is evidence that the same proteins, LDHA for example, can be modified by both derivatives and endogenous itaconate (Mills et al., 2018).

These studies indicate that glycolysis is another target for itaconate, acting via modification of aldolase A and GAPDH and limiting the inflammatory response. It is also quite striking that succinate, itaconate, and fumarate can target enzymes in glycolysis, which could be a broad method of negative feedback in activated macrophages in order to avoid excessive inflammation (see Figure 1). These findings point to a potentially important crosstalk from Krebs Cycle to glycolysis. The observation that the therapeutic agent DMF and itaconate can both modify GAPDH perhaps suggests that DMF has fortuitously been found as an anti-inflammatory agent that is carrying out the same natural process as itaconate to regulate inflammation. Certainly, the clinical use of DMF highlights the importance of PTMs for future therapeutic development.

**IMMUNE TARGETS OF PALMITOYLATION**

Similar to glycolysis and Krebs cycle, lipid metabolism also undergoes profound alteration during immune cell activation. Fatty acid oxidation is utilized for energy generation mainly by anti-inflammatory or tolerogenic immune cells such as M2 macrophages (Vats et al., 2006), memory T cells (van der Windt et al., 2012), and regulatory T cells (Michalek et al., 2011). Fatty acid synthesis, on the other hand, is generally associated with classically activated macrophages (Feingold et al., 2012), activated dendritic cells (Everts et al., 2014), effector CD4+ T cells (Berod et al., 2014), and activated B cells (Dufort et al., 2014). Fatty acid synthesis is considered to be essential for membrane biogenesis and the synthesis of some proinflammatory mediators. The fatty acids palmitate and myristate, however, can also modify proteins and thereby alter their function.

Palmitic acid is a common long-chain saturated fatty acid that is incorporated into phospholipids, sphingolipids, and triglycerides, but can also modify cysteine (or less frequently serine and threonine) residues in a process termed palmitylation. This lipid modification has been studied for around 40 years (Schmidt and Schlesinger, 1979). Palmitylation of proteins often facilitates docking to membranes and many transmembrane proteins also undergo palmitylation. Both palmitylation and depalmitylation are enzymatically controlled, with the DHHC protein family functioning as palmitoyltransferases (Korycka et al., 2012) and enzymes such as the acyl protein thioesterase and alpha beta hydrolase-domain containing protein 17 (ABHD17) protein family exhibiting depalmitylase activity (Duncan and Gilman, 1998; Lin and Conibear, 2015).

A number of proteins important for innate immune signaling pathways have been shown to undergo palmitylation. The lipopeptide-sensing pattern recognition receptor TLR2 undergoes palmitylation, which regulates its cell surface expression and ability to signal (Chesarino et al., 2014). The adaptor protein MyD88, which is involved in signaling by the IL-1 receptor and TLR family, has also been demonstrated to undergo palmitylation, which is required for IRAK4 kinase binding and downstream signal transduction in response to TLR4 stimulation with LPS (Kim et al., 2019). MyD88 palmitylation has been found to be mediated by the palmitoyltransferase ZDHHC6 but is also dependent on fatty acid synthase (FASN) activity.

The peptidoglycan-sensing PRRs nucleotide oligomerisation domain-like receptors 1 (NOD1) and NOD2 have been shown to undergo palmitylation in primary monocytes and macrophages and a variety of cell lines (Lu et al., 2019). This...
Palmitoylation has been found to be dependent on the palmitoyltransferase ZDHHC5, which is recruited to phagosomes in a model of *Salmonella typhimurium* infection. The palmitoylation of NOD1 and NOD2 is required for their membrane localization to bacteria-containing vacuoles, without which downstream NF-κB and MAPK activation was abrogated.

The P2X7 receptor also undergoes palmitoylation (McCarthy et al., 2019). P2X7 detects extracellular ATP, which acts as a danger-associated molecular pattern (DAMP) and can activate the NLRP3 inflammasome, promoting caspase-1 activation and leading to the production of IL-1β and IL-18, and driving a type of cell death termed pyroptosis via the cleavage of gasdermins (Piccini et al., 2008). P2X7, unlike the other P2X receptor family members, displays a lack of receptor desensitization, which has been found to be due to the palmitoylation of a cytoplasmic cysteine-rich site at the end of one of its transmembrane domains. Mutation or deletion of this site resulted in complete channel desensitization, indicating that palmitoylation protects P2X7 from desensitization. The TNF receptor TNF-R1 has also been found to be constitutively palmitoylated but rapidly depalmitoylated in response to ligand binding in a monocytic cell line. Mutation of the palmitoylated residue impairs TNF-R1 localization to the plasma membrane and downstream signaling (Zingler et al., 2019).

Stimulator of interferon genes (STING) is another important innate immune protein that undergoes palmitoylation (Mukai et al., 2016). STING is activated by the second messenger cGAMP, which is generated by the DNA sensor cGAS. STING in turn activates TBK1, which activates interferon regulatory factor 3 (IRF3), leading to the production of type I IFNs. Both pharmacological inhibition of palmitoylation and mutation of the palmitoylated cysteine residues (Cys88 and Cys91) on STING impaired its ability to induce type I IFNs during viral infection. Palmitoylation of STING may facilitate its anchoring to lipid rafts at the trans-Golgi network, as perturbing Golgi lipid composition also represses the type I IFN response.

All of these palmitoylations are therefore needed for these pathways to be triggered, pointing to palmitoylation as a key event in innate immunity. Figure 2 outlines the numerous immune effector proteins that are modulated by palmitoylation.

Palmitoylation has also been shown to play a role in adaptive immunity. It modulates the localization of linker of activation for T cells (LAT) during T cell receptor (TCR) engagement (Hundt et al., 2006; Zhang et al., 1998). The important T cell checkpoint regulator programmed death-ligand 1 (PD-L1) has been demonstrated to be palmitoylated, and this modification inhibits its ubiquitination and subsequent lysosomal degradation, thereby stabilizing PD-L1 (Yao et al., 2019). Inhibition of PD-L1 palmitoylation by 2-bromopalmitate or genetic silencing of the palmitoyltransferase ZDHHC5 augments T cell anti-tumor responses, both in vitro and in vivo. Blocking the palmitoylation of PD-L1 could potentially be explored as a therapeutic measure, since PD-L1 is commonly targeted by anti-PD-L1 antibodies for treatment of many cancers. However, one issue with this treatment is that the antibodies can only target PD-L1 on the cell surface. Intracellular PD-L1 can be stored and later redistributed to the plasma membrane to overcome immune checkpoint blockade therapy. However, Yao and colleagues have developed a peptide that specifically abolished PD-L1 palmitoylation, destabilizing it. This approach greatly enhances anti-tumor T cell immunity, which could have therapeutic potential.

**MYRISTOYLOYLATION MODULATES T CELL FUNCTION**

Another long-chain saturated fatty acid, myristic acid, can be attached to N-terminal glycine residues to give rise to another modification termed myristoylation. Similar to palmitoylation, myristoylation often induces trafficking to and association with membranes in the cell or lipid rafts. Myristoylation is catalyzed by the ubiquitously expressed N-myristoyltransferase 1 (NMT1) and NMT2 (Zha et al., 2000). Myristoylation has been shown to play a critical role in the initiation of the innate and adaptive immune responses.

In macrophages, myristoylation of TRIF-related adaptor molecule (TRAM) facilitates its association with TLR4 upon LPS stimulation and is required for downstream signaling and activation of NF-κB and IRF3 (Rowe et al., 2006). In T cells, in conjunction with palmitoylation, myristoylation contributes to the regulation of TCR signaling and in the absence of myristoylation the subcellular localization of myristoylated alanine-rich C-kinase substrate (MARCKS), Lck and Fyn is perturbed (Alland et al., 1994; Graff et al., 1988; Rampoldi et al., 2013). In CD4+ T cells isolated from PBMCs of rheumatoid arthritis (RA) patients a reduced expression of NMT1 has been observed, but not in CD4+ T cells of patients with other autoimmune diseases (Wen et al., 2019). This observation was somewhat surprising as a lack of myristoylation leading to T cell hyperactivation is unexpected. Restoring the expression of NMT1 in the RA patient CD4+ T cells diminishes Th1 and Th17 cell development, whereas genetic silencing of NMT1 in healthy donor CD4+ T cells augments Th1 and Th17 cell differentiation. NMT1 has also shown to protect against synovial inflammation in a murine model of arthritis. The authors found AMP-activated protein kinase (AMPK) was a key target here. It had previously been demonstrated that the b-subunit of AMPK undergoes myristoylation (Oakhill et al., 2010) and that this modification induces its activation and translocation to the lysosome, where it can inhibit mTORC1 activation (Zhang et al., 2014). The authors showed that NMT1 deficiency in RA CD4+ T cells resulted in impaired AMPK trafficking to the lysosome and hyperactivation of mTORC1, thereby ultimately promoting inflammation. In this case therefore, myristoylation is needed to limit T cell activation and control inflammation. Boosting myristoylation of AMPK would therefore have an anti-inflammatory effect. The role of both palmitoylation and myristoylation in immunity are summarized in Figure 2.

One aspect of lipid PTMs that has not been thoroughly examined is the source of the fatty acids used for palmitoylation and myristoylation. Several studies have used labeled palmitate or myristate to demonstrate the incorporation of exogenous fatty acids into lipid PTMs (Hundt et al., 2006; Kim et al., 2019; Mukai et al., 2016; Rowe et al., 2006; Wen et al., 2019) but few have determined whether endogenous processes such as fatty acid synthesis or triglyceride breakdown are utilized for these PTMs. One recent report has shown that inhibition or genetic silencing of FASN impaired MDY88 palmitoylation (Kim et al., 2019), in addition to demonstrating that exogenous palmitate contributes to this modification. An earlier study has found that while both exogenous and endogenous sources of palmitate...
and myristate are used for protein acylation, the ratio of endogenous to exogenous fatty acid incorporation varies between cell lines (Towler and Glaser, 1986). Therefore, the requirement of de novo lipogenesis for lipid-derived PTMs may well vary between immune cell type or depend on the activation state of the cell. It is also unclear if fatty acid binding proteins play a role in this process, although it was reported that the acyl-CoA binding protein ACBD6 sequesters palmitoyl-CoA to prevent it from binding and inhibiting NMT2, thereby allowing myristoylation to occur (Soupene et al., 2016). Although the evidence is limited, it is possible that fatty acid or acyl-CoA binding proteins are somehow involved in palmitoylation and myristoylation, as a large proportion of intracellular fatty acids are bound to these proteins (Resh, 2016).

**AMINO ACID PTMs REGULATE IMMUNE FUNCTION**

Amino acids can also modify proteins in immune cells. These PTMs occur at distinct amino acid side chains or peptide linkages and are most often mediated by enzymatic activity and can have various consequences.

Glutamate can modify proteins by glutamylation, also termed polyglutamylation. This PTM generates lateral acidic glutamate side chains onto the γ-carboxyl groups of glutamic acid residues in the sequence of target proteins (Gamnah et al., 2015; Rogowski et al., 2010; van Dijk et al., 2009). Glutamylation is highly conserved in all metazoans and protists, exerting critical roles in many physiological and pathological processes (Garnham and Roll-Mecak, 2012). Recent studies have highlighted the role of glutamylation in regulating immune effector mechanisms (see Figure 3).

Glutamylation of cGAS impairs its ability to bind cytosolic DNA and its capacity to synthesize cGAMP to initiate antiviral responses via STING (Xia et al., 2016). The α-chain of the IL-7 receptor undergoes glutamylation, which has been shown to increase the expression of transcription factor Stat3 via STAT5 activation. IL-7 is an important regulator of type 1 innate lymphoid cells (ILC) and promotes ILC3 differentiation (Liu et al., 2017). Thus, IL-7Ra glutamylation has a critical function in ILC3 development.

Glutamylation has been shown to occur on the deubiquitinase BAP1 and modulates hematopoietic stem cell (HSC) self-renewal and hematopoiesis (Xiong et al., 2020). Glutamylation of BAP1 promotes its ubiquitination and subsequent degradation, thereby hindering Hoxa1 expression, which is required for HSC self-renewal. BAP1 is a deubiquitinase involved in many cellular processes, including transcriptional regulation, cell cycle, proliferation, DNA damage, and cell death (Bononi et al., 2017). Further, BAP1 mutations have been reported to be implicated in oncogenesis of several malignancies (Pilarski et al., 2014).

Hypusination is another amino acid-based PTM which has recently emerged in immunity (see Figure 3). Hypusine (Ne-4-amino-2-hydroxybutyl(lysine)) is a modified lysine, which is made when lysine reacts with the polyamine spermidine. Remarkably, it occurs only in the protein eukaryotic translation factor 5A (eIF5A) (Park and Wolff, 2018). eIF5A is a small, highly conserved protein necessary for translational elongation of specific mRNA transcripts, especially of proteins involved in G1 to S cell cycle transition (Chatterjee et al., 2006) and cytotoxic stress response (Rahman-Roblick et al., 2007). It is a regulator of cell growth and tumor development (Nakanishi and Cleveland, 2019). Hypusinated eIF5A is essential for the expression of CD83 in DCs correlating with their maturation (Kruse et al., 2000), demonstrating its immunological role. eIF5A also participates in the inflammatory cascade leading to B cell dysfunction during the development of diabetes in NOD mice. Colvin et al. has demonstrated that eIF5A inhibition in NOD mice causes selective reduction of diabetogenic Th1 cells in the pancreatic lymph nodes (Colvin et al., 2013). Hypusination of eIF5A also regulates protein synthesis and autophagy in primary B cells (Zhang et al., 2019b). This step is required for the synthesis of the autophagosomal and lysosomal master regulator TFEB.

eIF5A is also important for alternative macrophage metabolism and activation. It has been demonstrated that the expression of several mitochondrial proteins involved in the TCA cycle and oxidative phosphorylation are dependent on eIF5A being hypusinated (Puleston et al., 2019). In macrophages, metabolic switching between oxidative phosphorylation and glycolysis supports divergent functional fates stimulated by activation signals (Puleston et al., 2019). Thus, in macrophages, hypusination of eIF5A appears to be dynamically regulated after activation, with hypusinated eIF5A being critical for IL-4-driven macrophages and being required for anti-parasitic responses to Helminthosomoides polygyrus. Puleston et al. further described a role for hypusination of eIF5A in T cells. T cells deficient in enzymes involved in hypusine synthesis failed to differentiate into functionally distinct subsets (Puleston et al., 2020). Further characterization of hypusination across different immune cell types is warranted.

Another amino acid-based PTM is citrullination. This modification occurs when peptidylarginine deiminases (PADs) catalyze the conversion of arginine residues to citrulline, an amino acid that is not conventionally present in proteins (Vossenaar et al., 2003). Autoantibodies against citrullinated proteins are a hallmark of rheumatoid arthritis, and dysregulated citrullination is implicated in the development of this disease (Schellekens et al., 1998). Interestingly, the presence of anti-citrullination antibodies can be used as a biomarker for rheumatoid arthritis and is associated with more severe disease (Lundberg et al., 2003).

**CONCLUDING REMARKS AND THERAPEUTIC IMPLICATIONS**

How metabolic PTMs regulate innate and adaptive immune function is a rapidly developing area of research. Our current knowledge already demonstrates the striking impact that the metabolic dynamics of an immune cell can have on the function of immune effector proteins. The PTMs described in this review are shown in Table 1. These modifications regulate immunological processes by a wide variety of mechanisms. They can alter enzyme activity, facilitate or disrupt protein-protein interactions, determine subcellular localization through membrane anchoring and govern gene expression, through modification of transcription factors or histones to alter chromatin accessibility. It is extremely likely that additional metabolic PTMs and further mechanisms by which these modifications shape the immune function of proteins are revealed in the near future.
A number of immune effector proteins undergo glutamylation. IL-7Rα glutamylation induces STAT5 activation and subsequent expression of Sall3, an important ILC3 transcription factor. Glutamylation of cGAS impairs both its DNA-sensing and cGAMP synthesis activity. BAP1 also undergoes glutamylation, which induces its ubiquitination and degradation, preventing the expression of genes necessary for HSC self-renewal. Hypusination mediated by spermidine occurs only on the translation initiation factor eIF5A, which impacts on protein expression in various immune cell types. eIF5A hypusination is required for the differentiation of CD4+ effector subsets. It is also required for CD83 expression, which is necessary for dendritic cell maturation. Hypusination of eIF5A is critical for TFEB expression in B cells, which induces autophagy and prevents B cell senescence. Furthermore, eIF5A hypusination is required for the mitochondrial metabolism associated with alternative macrophage activation.
response in health and disease will emerge over the course of the next few years. Recent advances in mass spectrometry techniques have made these discoveries possible but the ability to manipulate these modifications, in addition to their detection and identification by proteomics, is crucial for the understanding of the effects they have on the immune response. When an enzyme is known to catalyze the addition or removal of a modification, manipulation of the system is considerably simpler. For example, when investigating palmitoylation both genetic silencing and pharmacological inhibition of the ZDHHC enzymes using 2-bromopalmitate and cerulenin can be employed (Resh, 2006). While recent research has highlighted the importance of metabolic PTMs in the context of immunity, many more questions remain to be answered, as outlined in the Outstanding Questions box.

Metabolic PTMs can therefore potently modulate various immune functions and processes. Can these discoveries be harnessed therapeutically? One possibility is to further explore how these metabolic PTMs may influence drug binding, which could affect drug efficacy (Su et al., 2017). Monoclonal antibodies specific for a protein target with or without a PTM are already commonly used in research but the clinical implications of this have not yet been thoroughly explored. A recent study has shown that glycosylation of PD-L1 impairs antibody binding, which would lead to a poorer clinical outcome (Lee et al., 2019). Several PTMs have been reported to alter the activity of enzymes, several of which are discussed above. If it were possible to develop an inhibitor that bound the modified form of the enzyme this could potentially be an effective way to target enzymes only in certain cell types that undergo certain metabolic changes. For example, targeting modified glycolytic enzymes such as malonylated GAPDH (Galván-Peña et al., 2019) or succinylated PKM2 (Wang et al., 2017) could inhibit the hyperactivation of immune cells such as macrophages in inflammatory disease without affecting glycolysis in other cell types.

Table 1. Metabolic PTMs in Immunity

<table>
<thead>
<tr>
<th>Name of modification</th>
<th>Metabolite</th>
<th>Residue modified</th>
<th>Protein targets</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolysis-derived PTMs</td>
<td>Lactate</td>
<td>Lysine</td>
<td>Histones</td>
<td>(Zhang et al., 2019a)</td>
</tr>
<tr>
<td>Krebs cycle-derived PTMs</td>
<td>Acetyl CoA</td>
<td>Lysine</td>
<td>Histones, cGAS, NLRP3, GAPDH</td>
<td>(Chen et al., 2020), (Dai et al., 2019), (He et al., 2020), (Balmer et al., 2016)</td>
</tr>
<tr>
<td>Malonylation</td>
<td>Malonyl CoA</td>
<td>Lysine</td>
<td>GAPDH, mTOR</td>
<td>(Galván-Peña et al., 2019), (Bruning et al., 2018a)</td>
</tr>
<tr>
<td>Succinylation</td>
<td>Succinyl CoA</td>
<td>Lysine</td>
<td>PKM2</td>
<td></td>
</tr>
<tr>
<td>Succination</td>
<td>Fumarate</td>
<td>Cysteine</td>
<td>GAPDH</td>
<td>(Kornberg et al., 2018)</td>
</tr>
<tr>
<td>Dicarboxypropylation</td>
<td>Itaconate</td>
<td>Cysteine</td>
<td>NRF2, Aldolase, GAPDH, RIPK3</td>
<td>(Mills et al., 2018), (Qin et al., 2019), (Qin et al., 2020)</td>
</tr>
<tr>
<td>Lipid-derived PTMs</td>
<td>Palmitic acid</td>
<td>Cysteine, Serine, Threonine</td>
<td>TLR2, MYD88, NOD1/NOD2, P2X7, TNF-R1, STING, LAT, PD-L1</td>
<td>(Chesarino et al., 2014), (Kim et al., 2019), (Lu et al., 2019), (McCarthy et al., 2019), (Zingler et al., 2019), (Mukai et al., 2016), (Hundt et al., 2008), (Yao et al., 2019)</td>
</tr>
<tr>
<td>Myristoylation</td>
<td>Myristic acid</td>
<td>Glycine</td>
<td>TRAM, MARCKS, Lck, Fyn, AMPK</td>
<td>(Rowe et al., 2006), (Graff et al., 1989), (Rampoldi et al., 2015), (Alland et al., 1994), (Wen et al., 2019)</td>
</tr>
<tr>
<td>Amino acid-derived PTMs</td>
<td>Glutamate</td>
<td>Glutamate</td>
<td>cGAS, IL-7Rα, BAP1</td>
<td>(Xia et al., 2016), (Liu et al., 2017), (Xiong et al., 2020), (Xia et al., 2016)</td>
</tr>
<tr>
<td>Hypusination</td>
<td>Spermidine</td>
<td>Lysine</td>
<td>eIF5A</td>
<td>(Zhang et al., 2019b), (Zhang et al., 2019a)</td>
</tr>
</tbody>
</table>
Another prospect that could be explored is the pharmacological induction of metabolic modifications. We have already seen evidence of this approach from the study which showed that DMF mediates succination of GAPDH in immune cells, which contributes to its anti-inflammatory effects (Kornberg et al., 2018). This report indicates that it may be possible to use metabolite derivatives, including 4-OI or molecules based on it, to induce certain metabolic PTMs in the treatment of autoimmune disorders. It may also be possible to competitively inhibit the modification of a particular protein target. This was undertaken in the study that demonstrated that blocking PD-L1 palmitoylation improved anti-tumor T cell responses (Yao et al., 2019).

We expect that more metabolic PTMs will emerge and provide insight into immunomodulation in health and disease. We anticipate that these metabolic PTMs may be exploited for the design of future therapeutics for immune and inflammatory disease.

OUTSTANDING QUESTIONS

- Several metabolite-derived PTMs are currently considered to occur non-enzymatically but will enzymes that regulate these modifications be discovered?
- Consensus motifs are known for some of the modifications discussed in this review but do all of these PTMs occur only at specific amino acid sequences?
- In studies where derivatives of metabolites are used, to what extent are these PTMs reflective of endogenous metabolic modifications?
- What is the stoichiometry of these PTMs and how much does the stoichiometry impact the immunomodulatory effects of the modifications?
- Do these PTMs differ in cell lines compared to primary cells due to differences in metabolism?
- What is the stoichiometry of these PTMs and how much does the stoichiometry impact the immunomodulatory effects of the modifications?
- To what extent do physiological environmental factors influence the frequency or control of these PTMs? For example, will la-clytated proteins be more abundant due to augmented glycolysis in all hypoxic tissues?

REFERENCES


Immunothrombosis and the molecular control of tissue factor by pyroptosis: prospects for new anticoagulants


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The interplay between innate immunity and coagulation after infection or injury, termed immunothrombosis, is the primary cause of disseminated intravascular coagulation (DIC), a condition that occurs in sepsis. Thrombosis associated with DIC is the leading cause of death worldwide. Interest in immunothrombosis has grown because of COVID-19, the respiratory disease caused by SARS-CoV-2, which has been termed a syndrome of dysregulated immunothrombosis. As the relatively new field of immunothrombosis expands at a rapid pace, the focus of academic and pharmacological research has shifted from generating treatments targeted at the traditional ‘waterfall’ model of coagulation to therapies better directed towards immune components that drive coagulopathies. Immunothrombosis can be initiated in macrophages by cleavage of the non-canonical inflammasome which contains caspase-11. This leads to release of tissue factor (TF), a membrane glycoprotein receptor that forms a high-affinity complex with coagulation factor VII/VIIa to proteolytically activate factors IX to IXa and X to Xa, generating thrombin and leading to fibrin formation and platelet activation. The mechanism involves the post-translational activation of TF, termed decryption, and release of decrypted TF via caspase-11-mediated pyroptosis. During aberrant immunothrombosis, decryption of TF leads to thromboinflammation, sepsis, and DIC. Therefore, developing therapies to target pyroptosis have emerged as an attractive concept to counteract dysregulated immunothrombosis. In this review, we detail the three mechanisms of TF control: concurrent induction of TF, caspase-11, and NLRP3 (signal 1); TF decryption, which increases its procoagulant activity (signal 2); and accelerated release of TF into the intravascular space via pyroptosis (signal 3). In this way, decryption of TF is analogous to the two signals of NLRP3 inflammasome activation, whereby induction of pro-IL-1β and NLRP3 (signal 1) is followed by activation of NLRP3 (signal 2). We describe in detail TF decryption, which involves pathogen-induced alterations in the composition of the plasma membrane and modification of key cysteines on TF, particularly at the location of the critical, allosterically regulated disulfide bond of TF in its 219-residue extracellular domain. In addition, we speculate towards the importance of identifying new therapeutics to block immunothrombotic triggering of TF, which can involve inhibition of pyroptosis to limit TF release, or the direct targeting of TF decryption using cysteine-modifying therapeutics.

The traditional coagulation cascade model was first described in two seminal papers in 1964 and drastically modernized the field of haematology. Robert Gwyn Macfarlane’s Nature manuscript, closely followed by Earl Davie’s and Oscar Davis Ratnoff’s Science publication, heralded the now-familiar ‘waterfall’ model of coagulation [1,2]. They described how surface contact causes factor (F)XII activation, triggering a chain reaction of coagulation factor activation events, resulting in
the clotting enzyme thrombin cleaving fibrinogen into a fibrin clot. However, it was not until later that tissue factor (TF) (previously known as factor III/thromboplastin/CD142) was identified as the key trigger of coagulation [3]. We now know that haemostatic clot formation is initiated via exposure of sub-endothelial TF to blood upon vessel injury. Blood-borne FVII/FVIIa binds TF, and this complex activates the coagulation cascade [4]. Activation of FX by TF promotes prothrombins complex formation, driving sufficient thrombin generation to promote fibrin deposition. Alternatively, coagulation activation can be achieved via FXII activation when plasma comes into contact with a diverse range of artificial or pathological surfaces [5–7], although this pathway is not linked to haemostasis and instead contributes predominantly to pathological clot formation [8].

**Immunothrombosis: the link between innate immunity and coagulation**

Blood coagulation is a potent defensive mechanism that maintains haemostasis by curtailing bleeding following insult or injury. However in pathophysiological circumstances, overactivation of the coagulation cascade takes place, leading to sepsis and disseminated intravascular coagulation (DIC), the leading cause of death in intensive care units, making sepsis a global health priority [9]. This process by which innate immune pathways and aberrant coagulation are intrinsically connected is termed immunothrombosis [10]. This process can be activated by bacterial and viral infection. One well-described bacterial trigger for immunothrombosis is the endotoxin, lipopolysaccharide (LPS), which is found in the outer membrane of gram-negative bacteria [11]. Activation of toll-like receptor 4 (TLR4) by LPS triggers multiple downstream signalling events, including induction of transcription factors such as NF-κB and interferon regulatory factors (IRFs) [12]. The IRFs in turn induce type I interferons (IFNs) which propagate expression of interferon-stimulated genes (ISGs). If LPS penetrates the cytosol, however, it can be sensed by an ISG product, caspase-11, which is part of the non-canonical inflammasome. Inflammasomes are intracellular multi-protein signalling platforms that regulate the inflammatory response and drive antimicrobial host defences [13]. They are assembled in the cytosol following recognition of pathogenic microorganisms and danger signals, and activate inflammatory caspases to produce cytokines and to induce pyroptotic cell death via cleavage of gasdermins [13–16]. Pyroptosis is a programmed, pro-inflammatory form of cell death, characterized by cell swelling, lysis, and release of cytoplasmic content [17–19]. NLRP3, which cleaves and activates caspase-1, is the best-characterized inflammasome, sensing multiple noxious stimuli phagocytosed by macrophages. Caspase-11, however, can also form an inflammasome upon sensing of cytosolic LPS, and its activation drives pyroptosis. Mouse caspase-11, or caspase-4 and -5 in humans, is expressed in various cell types, such as macrophages and endothelial cells (ECs), and is required for non-canonical inflammasome-triggered macrophage cell death and endothelial dysfunction [17,20,21]. Detection of cytosolic LPS by caspase-11 and the interferon-induced guanylate-binding protein 1, assemblies a platform for caspase-11 recruitment and activation [22–24]. This promotes the exposure of phosphatidylserine (PS) on the cell surface. Importantly, this in turn has been shown to post-translationally activate TF, in a process termed decryption, increasing its procoagulant activity (PCA) (Figure 1), as we will discuss in detail later in this review. These studies identify caspase-11 as a key driver of immunothrombosis occurring in response to LPS. Concurrently, this process cleaves gasdermin D (GSDMD), a member of the family of gasdermin proteins that execute cell death and inflammation. GSDMD is highly conserved in mammals, and is expressed in a broad range of different tissues and cell types [25]. Structurally, GSDMD comprises a C-terminal repressor domain and a cytotoxic N-terminal domain, connected by a flexible linker. Proteolytic cleavage by active caspase-11 releases the 31 kDa cytotoxic N-terminal fragment, which induces NLRP3 inflammasome-dependent IL-1β release in addition to pyroptosis [17–19,26,27]. This results in GSDMD being the conduit for NLRP3-dependent IL-1β processing but also lethal septic shock as plasma levels of IL-1β, a biomarker of GSDMD activation, are correlated with DIC scores in septic patients [28]. This process of cytosolic LPS detection activating caspase-11 and cleaving GSDMD has also been shown to take place in neutrophils, contributing to the formation of neutrophil extracellular traps (NETs) [29–31], which can also drive thrombosis by capturing TF and TF-positive extracellular vesicles for amplification of the coagulation cascade [32–35]. Therefore, inflammasome activation, which can occur by activation of NLRP3 or caspase-11 can induce systemic thrombosis and blood clotting [36].

Apart from gram-negative sepsis, COVID-19 causes thrombotic complications similar to sepsis including platelet aggregation, intravascular inflammation, and vascular endothelial damage, affecting multiple vital organs and increasing mortality burden in SARS-CoV-2 patients [37]. As such, COVID-19 represents an
Figure 1. Tissue factor drives immunothrombosis and the coagulation cascade via three key signals.

An inflammatory stimulus induces a pro-immunothrombotic phenotype in immune cells, such as macrophages. For example, extracellular LPS from gram-negative bacteria activates TLR4 in the plasma membrane, initiating the first step of tissue factor (TF) control. Signal 1 is induction of expression of pro-immunothrombotic genes. Upon binding of LPS to TLR4, TF expression is induced by the transcription factor NF-κB, whilst concurrently the type I interferon response to extracellular LPS triggers the JAK-STAT pathway to induce interferon-stimulated genes such as pro-caspase-11. Once HMGB1 transports LPS to the cytosol, pro-caspase-11 is cleaved into its active form termed the non-canonical inamasome. Caspase-11 in turn cleaves and activates gasdermin D (GSDMD) to form a pore in the cell membrane, initiating pyroptosis, a form of programmed, pro-inflammatory, lytic cell death. NLRP3 inamasome and caspase-1 activation can also be triggered by potassium (K⁺) efflux via pyroptosis in a feedback manner. TF then undergoes post-translational activation, termed decryption, increasing its procoagulant activity (signal 2 — see Figure 4 for more detail). Decrypted TF is released via the pyroptic pore in the outer membrane of extracellular microvesicles and forms a cell-surface complex with FVII, triggering the traditional extrinsic pathway of coagulation (signal 3). This highlights how, in the absence of FVIIa, TF is unable to contribute to immunothrombosis-induced coagulation. TF:FVIIa then converges with the FXIIa-initiated intrinsic pathway of coagulation to activate FX. The intrinsic pathway is initiated via injury to blood vessels upon exposure of plasma to a diverse range of artificial or pathological surfaces, including negatively charged endogenous activating surfaces such as RNA, DNA, polyphosphate, and/or components of atherosclerotic plaques. FXa cleaves prothrombin to thrombin, which activates FXI in a feedback manner to generate sufficient FXa to promote further thrombin generation. Thrombin also cleaves fibrinogen to fibrin to form a mesh-like clot, and activates PARs, which regulates the neutrophil-
exemplar model of immunothrombotic disease [38]. For example, LDH and D-dimer levels correlate with markers of SARS-CoV-2 severity [39]. It was also reported early in the pandemic that histological analysis of pulmonary vessels in patients with COVID-19 demonstrated widespread thrombosis with microangiopathies such as structurally deformed capillaries, with alveolar capillary microthrombi nine times as prevalent in COVID-19 compared with influenza [40]. Furthermore, systemic fibrinolysis is considerably suppressed in SARS-CoV-2, as it is in sepsis-associated DIC [41]. While activation of NLRP3 and caspase-11 have been correlated with disease severity in SARS-CoV-2 patients in inflammatory monocytes, macrophages, and neutrophils [42,43], it remains unknown whether pyroptosis-triggered TF activity is directly involved in SARS-CoV-2-mediated coagulation.

Inflammasome activation therefore provides an important link between inflammation and TF-driven immunothrombosis, the major cause of host death following inflammasome activation [36]. Inhibition of NLRP3 has been shown to be protective in septic models. For example, the NLRP3 inhibitor MCC950 attenuates platelet activation and multi-organ injuries in rats in a caecal ligation and puncture (CLP) model of sepsis [44]. Therefore, blocking inflammasome activation has emerged as an attractive target to block aberrant immunothrombosis.

**Tissue factor: the key trigger of immunothrombosis**

As stated above, caspase-11 promotes coagulation by driving TF release during pyroptosis. First described in 1965 [3], TF is a 47 kDa membrane glycoprotein receptor and the primary initiator of haemostatic clot formation (Figure 2) [5,45]. TF is expressed by manifold cell types, but can be readily induced upon exposure to inflammatory stimuli [46–49]. TF exerts its role in maintaining haemostasis by forming a high-affinity cell-surface complex with FVII/VIIa, which is the initiating step in fibrin formation (Table 1) [1–3,5,50]. TF activity via the TF:FVIIa complex is then rapidly inhibited by TF pathway inhibitor (TFPI) [5,6,51]. However, under pathological conditions, increased expression and decryption of TF triggers aberrant coagulation and subsequent thromboinflammation, which exceeds TFPI regulation (Figure 3 and Table 1) [38]. In addition to increased innate immune cell TF expression and activity, TF is incorporated and released from damaged cells as part of extracellular vesicles. This greatly increases the PCA of TF compared with soluble TF [52,53]. Moreover, increased TF expression is associated with malignancy in breast cancer [54], gliomas [55], and angiogenesis in colorectal cancer [56]. Therefore, TF plays a central role in triggering both arterial and venous thrombosis, and initiating DIC in systemic inflammatory and cardiovascular disorders, including sepsis, diabetes mellitus, atherosclerosis, and acute coronary syndromes [5,36,49,57].

Regulated by the transcription factor protein complex NF-kB, TF is normally expressed in adventitial tissues such as perivascular and epithelial cells at organ and body surfaces where it forms a haemostatic barrier [5,26,45,51,58]. LPS can induce TF gene expression via NF-kB in innate immune cells such as monocytes and ECs [59]. This induction of TF mRNA occurs rapidly and peaks at 2 h post-LPS stimulation, with TF protein levels on the cell surface peaking at 4 h, and remaining high for up to 24 h after LPS stimulation [51,60–63]. In vivo, inhibiting TF expression, decryption, and the subsequent increase in PCA attenuates endotoxemia-induced DIC [45,57]. Thus, decryption of TF couples inflammation with thrombosis. In summary, there are three mechanisms that control TF: up-regulation upon exposure to inflammatory stimuli via NF-kB, increased decryption and PCA, and accelerated release into the intravascular space via pyroptosis (Figure 1).

**TF in monocytes and macrophages**

Recent studies, however, have highlighted the importance of monocytes and macrophages in immunothrombosis, with depletion of these cells significantly attenuating inflammasome-driven TF-dependent coagulation. Using clodronate-containing liposomes, which deplete monocytes and macrophages, Wu et al. [36] significantly attenuated caspase-1- and TF-dependent DIC and lethality induced by the *Escherichia coli* (*E. coli*) type III secretion system rod protein EprJ. Similarly, in a venous thrombosis model, which is driven by caspase-1,
GSDMD, and TF (released from pyroptotic monocytes and macrophages), Zhang et al. depleted peripheral monocytes and macrophages using gadolinium chloride, resulting in protection against venous thrombosis, one of the most common causes of cardiovascular death [64]. This is particularly important in the lung, where alveolar macrophage (AM) death leads to the progression of lung inflammation through its influence on neutrophils and ECs [65], cell types which are particularly prevalent in the lung and which contain high levels of TF during inflammation. In addition, Vega-Pérez et al. [66] found that monocytes, resident peritoneal macrophages, and neutrophils contribute to formation of a dynamic multicellular aggregate supporting the fibrin clot that forms upon exposure to intraperitoneal E. coli. Depletion of macrophages with clodronate again suppressed clot formation, highlighting their importance.

Neutrophils, platelets, and immunothrombosis
In addition to monocytes and macrophages, neutrophils are a major leukocyte population that contribute to thrombus development [67] and there is a well-characterized link between neutrophils and TF decryption in human immunothrombosis [33]. In a healthy lung, neutrophils patrol the blood vessels surrounding the alveoli while resting macrophages reside within the alveoli. However, systemic exposure to LPS promotes lung
inflammation as the AMs undergo pyroptosis, initiating an immunothrombotic response [65]. This AM pyroptosis promotes neutrophil migration and infiltration into the lungs, increasing alveolar concentrations of the pro-inflammatory cytokines IL-6, TNF, and IL-1β, and worsening overall lung injury [65]. This occurs via

| Table 1. The contrasting states of tissue factor during haemostasis and immunothrombosis (related to Figure 3) |
|-------------------------------------------------|-------------------------------------------------|
| Tissue Factor during haemostasis | Tissue Factor during immunothrombosis |
| Expressed basally on adventitial tissues (e.g. perivascular and epithelial cells) at organ and body surfaces [5,6,45,51,58] | Rapidly induced at the mRNA and protein levels via NF-κB upon exposure of innate immune cells to a pathogen [60–63] |
| Pre-loaded with FVII/FVIIa on cells surrounding blood vessels [4], but TF remains encrypted in a neutral phospholipid environment [52,59,85–87,97,98,105,110] | Allosteric alterations to negatively charged phospholipids (PS, SM) in the cell membrane, and/or oxidation of Cys186–209 in the extracellular domain of TF, decrypts and activates TF [52,59,86,87,97,98,105,107–109] |
| TF has low procoagulant activity in its cryptic form [85,170] | Once decrypted, procoagulant activity of TF increases up to 100-fold [52,84] |
| Following vascular injury, TF:FVIIa is exposed to the circulation and a low basal level of TF triggers local blood clotting via continuous low level activation of coagulation [171,172]. TF activity is then rapidly inhibited by TFPI [5,6,51] | Decrypted, procoagulant TF is released from cells in the membrane of extracellular vesicles after inflammasome-driven pyroptosis [28,36,57,88,89] to trigger the coagulation cascade and thromboinflammation via interactions with platelets, endothelial cells, and neutrophils (which degrade TFPI) [10,38,173,174] |

Figure 3. Haemostasis and immunothrombosis differ via pyroptosis-triggered decryption of tissue factor (related to Table 1).

During normal conditions, tissue factor (TF) is pre-bound with FVII and is expressed exclusively by extravascular cells. In this state, TF has a low PCA, and TF:FVIIa maintains haemostasis upon blood vessel injury via low basal activation of the clotting cascade. However, in the context of immunothrombosis, exposure to a pathogen triggers transcriptional induction of TF and inflammasome-induced pyroptosis of innate immune cells, resulting in the release of TF from immune cells into the circulation via extracellular vesicles. The key cysteines on TF (Cys186–Cys209) are oxidized and decrypted (see Figure 4), increasing the PCA of TF up to 100-fold. This procoagulant phenotype characterizes TF-triggered thromboinflammation. Repurposing existing anti-inflammatory drugs, or generating new therapeutics, to inhibit the inflammatory events that lead to pyroptosis or TF decryption is an attractive concept to prevent thromboinflammatory diseases. For example, cysteine-modifying therapeutics could be employed to inhibit oxidation of the critical Cys186–209 bond on TF, and thus block decryption of TF into its procoagulant form.
caspase-11 in mice or caspase-4 and -5 in humans, as described earlier, which triggers extensive EC pyroptosis (generating a ‘leaky’ endothelium), in addition to pyroptosis in macrophages and neutrophils [68,69].

The life-span of neutrophils is significantly increased during inflammatory reactions and is coupled to neutrophil activation to promote the inflammatory response [70]. This may occur when activated platelets and coagulation factors (such as FXa, FXIa, thrombin, and fibrinogen [71,72]) bind to neutrophils via CD40L–CD40, platelet P-selectin, or high mobility group protein B1 (HMGB1), inducing TF via the alternative activation pathway of the complement system [38,70,73,74]. Furthermore, LPS in the blood may be detected by platelet TLR4, resulting in the binding of platelets with adherent neutrophils, which drives neutrophil activation [75,76]. For example, a recent study identified that the transcription factor NFAT is activated in platelets upon stimulation of the thrombin receptor (protease activated receptor 1; PAR1) to regulate platelet response to coagulopathy [77]. In a mouse model of LPS-induced septic shock, inhibition of NFAT in platelets boosted platelet aggregation and neutrophil activation, increasing disseminated coagulation [77]. This highlights a new, important interaction in the crucial interplay between neutrophils and platelets in driving immunothrombosis.

Once activated, neutrophils expel their DNA in conjunction with histones and granule-derived enzymes, such as myeloperoxidase and elastase, to form networks of extracellular fibres termed neutrophil extracellular traps (NETs), in a process called NETosis [70,78]. This often occurs in the process of neutrophil death, whereby bacteria and other toxins are captured and immobilized in the bloodstream. In this way, NETs provide host defence against extracellular pathogens, unlike pyroptosis of macrophages, which provides host defence against intracellular pathogens by killing the cell and therefore eliminating the pathogen. NETs have been described as important mediators of tissue damage in inflammatory diseases, particularly in the context of immunothrombosis [79]. Thus, NETs contribute to intravascular immunity by exerting thrombogenic activity by providing a structural platform for activation of contact pathway-mediated thrombin generation and microthrombosis [32–35,70,80]. However, NET formation can be toxic to the surrounding vasculature. For example, NET release occurs in acute respiratory distress syndrome (ARDS), a severe form of acute lung injury, which results from sepsis-induced direct pulmonary insults and indirect systemic inflammatory responses [65]. ARDS is a devastating clinical complication of bacterial sepsis, during which macrophages and neutrophils rapidly migrate into lung tissue, resulting in widespread inflammation and a mortality rate greater than 40% [68]. TF-enriched NETs have been observed in ARDS patients and mice [34]. Consequently, exposure of TF drives immunothrombosis in ARDS patients, resulting in a vicious feedback amplification of thrombin-activated platelets interacting with neutrophils [34], augmenting ARDS-associated thromboinflammation.

More recently, increased neutrophil counts and extensive neutrophil infiltration of pulmonary capillaries have been associated with decreased clinical severity and poor prognosis in SARS-CoV-2-infected patients [33]. Neutrophils of SARS-CoV-2 patients yield high TF expression and release NETs carrying active/decrypted TF, with increased levels of NETs and TF activity present in the plasma of SARS-CoV-2 patients [33]. SARS-CoV-2 can also directly induce the release of NETs by healthy neutrophils via inflammasome activation, with NETs released by SARS-CoV-2–activated neutrophils promoting lung epithelial cell death in vitro [79]. Thus, NET-induced endothelial injury is strongly associated with vascular dysfunction [80,81], contributing to the amplified immune response and immunothrombosis that drives hyperinflammation and subsequent mortality in SARS-CoV-2 [79]. Inhibition of lung neutrophil infiltration — the most abundant leukocyte population found within venous thromb — and subsequent TF-driven NETosis has been shown to attenuate thrombus formation in mice [80], making it a target with potential therapeutic value.

### Endothelial cells and immunothrombosis

LPS and TNF have also been shown to induce TF in ECs in vitro and in vivo [5,45,49]. For example, LPS increases EC TF expression in the lungs of mice and humans, triggering immunothrombosis via a chain reaction of responses from macrophages, neutrophils, and ECs in the lung, leading to acute lung injury [5,65,68]. In mice, this occurs via the caspase-11-GSDMD axis, which leads to widespread pyroptotic EC death and subsequent breakdown of the lung epithelial barrier. Deletion of caspase-11 in ECs has been shown to reduce endotoxemia-induced lung oedema, neutrophil accumulation, and death [68]. This is key because during haemostasis, an intact endothelial barrier tightly regulates lung vascular permeability and leukocyte recruitment. However, immunothrombosis disrupts the endothelial barrier resulting in tissue oedema, neutrophil influx, TF activity, and release of proinflammatory cytokines including IL-1β via activation of the NLRP3 inflammasome [68,82]. It is this endothelial barrier breakdown that is critical for the mortality rate associated
with septic acute lung injury. Furthermore, this highlights the fundamental contribution of the endothelium to the regulation of haemostasis and immunothrombosis by acting as a selective barrier between the bloodstream and surrounding tissue [68,70]. For example, ECs maintain haemostasis and counteract coagulation by expressing heparan sulfates, to support antithrombin anticoagulant activity, and TFPI, to attenuate extrinsic tenase complex formation. ECs also promote anticoagulant and anti-inflammatory protein C pathway activity by expression of the EC protein C receptor and thrombomodulin [70]. In addition, ECs synthesize and release tissue plasminogen activator, which is required for plasmin generation. Together, this provides a non-thrombogenic surface area that enables maintenance of blood fluidity in healthy blood vessels [49]. However, in septic conditions, these anti-thrombotic roles are often attenuated due to bacterial infection, which drives a pro-thrombotic state in ECs via NF-κB activation. As ECs become activated, their expression pattern is dramatically altered such that anticoagulant pathways are attenuated and pro-immunothrombotic activity is increased [49,70,83]. Thus, NF-κB serves as a survival factor for ECs upon LPS exposure [70]. Targeting aberrant TF expression in the endothelium may therefore represent a desirable approach to limit immunothrombosis and subsequent thromboinflammation.

**Decrypting the regulation of TF activation**

So far, we have considered two of the three points of TF control: induction of TF expression by NF-κB and inflammasome-driven pyroptosis which releases TF. The critical, post-translational activation step of TF control is termed decrypation. Decryption of TF is proposed to represent a crucial step for induction of TF-mediated immunothrombotic activity (Figure 3), and structural and local membrane changes on TF-expressing cells can increase TF PCA up to 100-fold [52,84]. To date, a number of processes have been proposed to regulate TF activity, as we will outline below (Figure 4 and Table 1).

**Phospholipids in the cell membrane regulate TF decryption**

Perturbations to the phospholipid environment on the plasma membrane of leukocytes have been shown to modulate TF activity. TF remains encrypted in a neutral phospholipid environment [85], while a negatively charged phospholipid surface is required for TF decryption [86,87]. One such negatively charged phospholipid is phosphatidylserine (PS). Under normal, haemostatic conditions, PS localizes exclusively to the inner leaftlet of the plasma membrane and is therefore not in contact with the TF extracellular domain. However, cell activation caused by injury, stress, or infection prompts PS membrane externalization to the outer leaftlet of the plasma membrane and this has been proposed to represent a key step in the promotion of TF PCA [28,36,52,88–90], and furthermore, could contribute to TF-rich extracellular vesicle release. For example, TF-positive extracellular vesicles are a marker of severity of COVID-19 in patients [91,92]. This occurs when caspase-11, which is activated and cleaved by cytosolic LPS, triggers GSDMD cleavage which in turn mediates a rapid influx of calcium into the cytosol. This calcium influx induces perturbations to the phospholipid environment on the plasma membrane by mediating PS externalization. A recent study suggests this exposure of PS may be due to activation of transmembrane protein 173 (also known as STING), which regulates the increase in cytosolic calcium to trigger TF release via pyroptosis [93]. Another study showed that the calcium-dependent phospholipid scramblase, transmembrane protein 16F (TMEM16F; also called anoctamin 6), is required for both PS exposure and subsequent TF activity induced by cholera toxin B (which acts as a vehicle for delivery of cytosolic LPS) [57]. In this study by Yang et al., the use of TMEM16F small interfering RNA, or deletion of Casp11- or GSDMD-, attenuated PS exposure and TF activity in macrophages in contrast with wild-type controls, and protected from LPS-induced DIC in vivo. Notably, GSDMD deletion alone did not affect TF expression, suggesting a key synergistic role for the calcium-triggered GSDMD-PS process in regulating TF activity. Furthermore, PS presentation on the surface of activated cells contributes to coagulation by recruiting vitamin K-dependent proteases such as FVII, FIX, FX, and prothrombin, that are necessary for procoagulant complex formation and subsequent fibrin deposition [94,95].

Unlike PS, which is constitutively found in the inner leaftlet of quiescent cells, sphingomyelin (SM) is an important phospholipid situated in the outer leaftlet of the plasma membrane [96,97]. SM maintains TF in an encrypted state [97], highlighting haemostatic regulation of TF activity at both the inner- and outer-plasma membranes. This has been shown by blocking TF activity by reconstituting human TF into liposomes, whilst not affecting the interaction or active site function of TF:FVIIa [98]. Hydrolysis of SM by acid sphingomyelinase (ASMase) has been shown to increase TF activity at the cell surface on macrophages and subsequent
release of TF-positive extracellular vesicles [97]. Although the mechanism by which SM maintains TF in a quiescent, non-procoagulant state is not known, SM hydrolysis does not increase PS externalization, suggesting inter-dependent mechanisms that contribute to membrane TF PCA. ASMase, which is constitutively expressed in monocytes [59] and secreted by platelets upon thrombin stimulation [99], is translocated from the cytoplasm to the plasma membrane upon stimulation of macrophages with ATP and LPS, increasing SM hydrolysis [97]. Recently, it was discovered that SARS-CoV-2 latches onto this mechanism by activating ASMase to decrypt TF and trigger immunothrombosis [98]. Therefore, inhibition of ASMase expression and activity is an attractive target as an anti-immunothrombotic therapy, especially considering the ASMase inhibitors desipramine and imipramine are FDA-approved tricyclic antidepressants. Treatment with these inhibitors has been shown to block ATP-induced SM hydrolysis in addition to ATP-induced TF decryption and release of TF-positive microvesicles [97]. Furthermore, a prophylactic dose of desipramine and imipramine blocks LPS-induced TF PCA.

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and thrombin generation in human monocyte-derived macrophages, mouse PBMCs, and an LPS-in vivo model followed by intraperitoneal injection of ATP, which induces translocation of ASMase to the plasma membrane [59]. ASMase inhibition has also previously been shown to attenuate liver damage (oxidative stress levels, hepatobiliary function, macrophage infiltration, and hepatic stellate cell activation) in a sepsis mouse model [100]. Thus, follow-up experiments in a therapeutic context are warranted to further decipher the potential of ASMase inhibitors as anti-immunothrombotic therapies.

**TF allostERIC disulfide bond formation**

Three regions make up the structure of TF: the extracellular (residues 1–219), transmembrane (220–244), and intracellular (245–263) regions [101]. It is the 219-residue extracellular, membrane-proximal region, consisting of two fibronectin type III domains made of two antiparallel β-sheets anchored into the cell membrane [51,101,102], that has been of greatest interest for TF decryption. Within the C-terminal domain of the extracellular region exists a Cys186–Cys209 disulfide bond in an -RHS Staple configuration, which is characteristic of allostERIC disulfide bonds that link cysteines between two adjacent strands within a β-sheet [103]. The redox potential (~278 mV) and spacing of these unbonded Cys186–Cys209 sulfur atoms (3–6 Å) implies that the cysteines are primed for disulfide bond formation [103,104]. It is hypothesized that this disulfide bond — which controls TF function in a redox-dependent manner [105] — is exposed and kept in cryptic form until triggered to induce increased TF PCA. This occurs when Cys186–Cys209 on cell surface-expressed cryptic TF undergoes selective covalent post-translational modifications via S-nitrosylation or S-glutathionylation [105–108]. Thus, reduction in this allostERIC disulfide bond indicates cryptic TF. Decryption of TF occurs when the Cys186–Cys209 disulfide bond is oxidized, indicating procoagulant, active TF which can bind FVII with high affinity [108]. This has been demonstrated by mutating TF at the allostERIC disulfide site. One study found that substitutions at the Cys186–Cys209 region resulted in decreased expression of TF on the cell surface, and could even lead to intracellular TF accumulation [106]. Furthermore, this mutation leads to binding of TF with FVIIa with weakened affinity, at a level comparable with cryptic TF, and therefore is incapable of activating FX to generate thrombin downstream [104,106,109]. Notably, a Cys209Ala mutant retained TF:FVIIa signalling indistinguishable from that of wild-type TF [106]. There is also evidence for Cys209 reacting with nitric oxide and/or glutathione, with alkylation by one or both of these compounds restricting formation of the disulfide bond [104,106,107]. In addition, a number of studies have verified that removal of the Cys186–Cys209 disulfide bond in full-length or soluble TF eliminates PCA [104,106,109–111], further highlighting its importance in regulating TF activity.

**Protein disulfide isomerase-dependent TF decryption**

AllostERIC structural re-arrangements within TF have been proposed to be mediated by protein disulfide isomerase (PDI)-dependent modulation and isomerization of the Cys186–Cys209 disulfide bond [108]. PDI is an oxidoreductase enzyme constitutively expressed in most tissues and organs and found abundantly in the endoplasmic reticulum of eukaryotic cells where it catalyzes disulfide bond formation [103]. It is hypothesized that this disulfide bond is oxidized, indicating procoagulant, active TF which can bind FVII with high affinity [108]. Once cleaved, pannexin-1 facilitates release of ATP from TF-expressing macrophages and smooth muscle cells injured as a result of mechanical stress, inflammation, or ischemia, and this ATP activates P2X7 receptor-mediated pyroptosis [115]. This ATP-triggered stimulation of P2X7 decrypts and activates TF, resulting in the release of procoagulant TF-positive extracellular vesicles [114]. This is critical for LPS-induced lethality in vivo [115]. This process also
occurs in neutrophils [116]. Further evidence for Cys186–Cys209-dependent TF decryption is provided by the thioredoxin/thioredoxin reductase/NADPH system, for which PDI is a substrate [117]. In haemostasis, this system maintains the disulfide bond in a reduced, anticoagulant form, with inhibition of thioredoxin expression in human breast cancer cells markedly increasing surface TF PCA via oxidation of Cys186–209 [110]. Therefore this PDI-induced pathway directly associates tissue damage with cellular TF decryption [101].

Complement mediates TF decryption

Furthermore, complement activation has been shown to trigger rapid TF decryption. Studies using antithymocyte globulin (ATG) — a polyclonal horse or rabbit IgG with pleiotropic cellular effects employed as an immunosuppressive agent in the treatment or prevention of allograft rejection and graft-versus-host disease [118] — have shown that ATG activates TF to induce low-grade DIC in patients undergoing hematopoietic stem cell transplantation in a complement-dependent feedback process [119]. The complement factor C3 is essential for platelet activation and platelet deposition in vivo, and in turn, thrombin can activate C3 and C5 [120]. This leads to increased expression of TF on ECs via interactions of C5a and the membrane attack complex (C5b-9) [121–124], highlighted by deficiency of C5 significantly reducing TF- and myeloid cell-dependent fibrin formation in models of venous thrombosis [125] and Staphylococcus aureus-induced coagulation [126]. C5a also induces clotting by disturbing the endothelial glycocalyx (a glycoprotein-polysaccharide layer that coats the exterior of the endothelium) [127]. Together, complement and coagulation activation correlates with COVID-19 disease severity [128]. Release of TF-positive extracellular vesicles has been blocked by administration of an antithrombotic anti-PDI antibody, while ATG- and complement-induced decryption of TF is blocked by the thiol alkylators 3,3′-dithio-bis(6- nitrobenzoic acid) (DTNB) and nitric oxide, for which maximal PS membrane exposure is not required [114,129,130]. This finding suggests a synergistic role of PS & PDI in the regulation of TF decryption.

Decryption of TF and subsequent TF activity drives thrombin generation

TF PCA is therefore regulated at multiple levels that include genetic up-regulation by NF-κB, activation via post-translational modifications, and extracellular release via pyroptotic innate immune cells. The above findings have mostly been demonstrated in vitro, so development of specific tools to analyze the cysteine modifications involved in TF decryption and activation in vivo is much needed to clarify the exact physiological mechanisms that take place during this critical immunothrombotic event. What the above does emphasize, however, is that multiple steps regulate TF, and each combine for maximal cellular TF decryption.

Anti-immunothrombotic therapies

Therapeutic targeting of TF is therefore a potential treatment strategy for immunothrombotic diseases (Figure 4). A recent innovation in anti-inflammatory therapy development has been the identification of specific, covalent inhibitors that modify key cysteines. Given the relative importance of decryption of TF via its key cysteines, this may present a more clinically relevant solution of preventing aberrant immunothrombosis versus existing treatments such as broad spectrum anticoagulants and direct oral anticoagulants. There is a range of therapies currently employed clinically to treat and modulate immunothrombosis including heparin and direct oral anticoagulants (which inhibit coagulation) and low-dose aspirin and P2Y12 receptor inhibitors (which inhibit platelet activation). Heparin is a widely used anticoagulant medicine to treat thrombotic disorders, and acts by activating antithrombin which inactivates thrombin, FXa, and FIXa, to improve patient outcomes in sepsis. A recent study showed that heparin, or a chemically modified form of heparin without anticoagulant function, prevented caspase-11-dependent immune responses and lethality in sepsis by inhibiting the HMGB1-LPS interaction, which is important for delivering LPS to the cytosol [69,131]. As a result, heparin (administered as full-dose or low-dose unfractionated heparin, or low molecular weight heparin) blocks the cytosolic delivery of LPS in macrophages and the subsequent activation of caspase-11 leading to immunothrombosis. This dual role of heparin in blocking both inflammasome-driven immunothrombosis and activating antithrombin to inhibit thrombosis may contribute to the contrasting effects of its use in patients with COVID-19. Recent trials examining heparin in noncritically ill patients with COVID-19 found that it increases the probability of survival to hospital discharge [132], whereas heparin does not significantly improve the
likelihood of survival to hospital discharge when administered to critically ill COVID-19 patients [133]. This highlights the importance of timing when administering anticoagulation medication and the need for broader spectrum anti-immunothrombotic treatments. Another anticoagulant commonly used is the vitamin K antagonist warfarin, First sold as rat poison in 1948, warfarin was the most widely used anticoagulant worldwide until 2013 [134,135]. Warfarin decreases availability of active vitamin K$_1$ leading to a reduction in blood coagulation [134]. However, by decreasing activity of clotting factors, anticoagulant therapies such as heparin and

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Year</th>
<th>Clinically approved</th>
<th>Immunotheromotic target(s)</th>
<th>References</th>
</tr>
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<tr>
<td>Aspirin</td>
<td>1897 (first synthesized)</td>
<td>Yes</td>
<td>NF-κB, Platelet activation</td>
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<td>Disulfiram</td>
<td>1961</td>
<td>Yes</td>
<td>NF-κB, GSDMD</td>
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<td>Ruxolitinib</td>
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<td>Yes</td>
<td>JAK1/JAK2/JAK3</td>
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<td>Tofacitinib</td>
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<td>Dimethyl fumarate (DMF)</td>
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<td>Yes</td>
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<td>Bay 11-7082</td>
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<td>Itaconate</td>
<td>2016</td>
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<td>4-octyl itaconate (4-OI)</td>
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<td>[156]</td>
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<td>P2X7R signalling Complement (C3a, C5a)</td>
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<td>No</td>
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<td>No</td>
<td></td>
<td>[147]</td>
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<td>No</td>
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<td>[144,145]</td>
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<td>1990</td>
<td>No</td>
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<td>HMGB1-LPS, caspase-11, Fxa, thrombin</td>
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warfarin are associated with a bleeding risk for patients [41,69,136–138]. This bleeding is significantly enhanced in septic patients and can result in life-threatening bleeds such as haemorrhagic stroke.

A step towards overcoming this was the development of direct oral anticoagulants (DOACs). First approved by the US Food and Drug Administration in 2010, DOACs are categorized by two main classes: oral direct factor Xa inhibitors, including rivaroxaban, apixaban, edoxaban, and betrixaban, and direct thrombin inhibitors, including dabigatran [135,139]. DOACs demonstrate similar or reduced bleeding risk compared with existing treatments, including heparin and warfarin [135]. Whilst the development of DOACs represents a considerable step forward in the management of patients at risk of coagulopathies such as stroke, deep vein thrombosis, and pulmonary embolism [135], the risk of bleeding with their administration persists and there remains scope for the generation of improved treatments that are not associated with a risk of bleeding, particularly during acute inflammatory disease where current anticoagulant strategies remain sub-optimal.

Given the shared anticoagulant and anti-inflammatory properties of the three primary endogenous anticoagulant pathways (antithrombin, protein C, and TFPI), previous efforts have sought to harness these pathways for therapeutic benefit during sepsis. This approach has, however, been met with mixed results, with promising preclinical studies failing in larger clinical trials. For example, recombinant TFPI reduced lethality in an E. coli-induced model of septic shock in baboons [140], but was not successful in clinical trials. Administration of recombinant TFPI, termed tifacogin, had no effect on all-cause mortality in severe septic patients and actually increased bleeding risk [141].

**Inhibition of the TF:FVIIa complex**

As indicated above, a novel approach to limit immune-mediated TF expression and activity could represent a useful strategy to treat immunothrombotic disease. A modified TFPI hybrid, termed X,Lc:LACI,K1, inhibited TF:FVIIa independently of FXa [142] and prevented thrombotic arterial re-occlusion in vessels of dogs following extensive vascular injury [143]. As a further proof of concept, studies employing inhibitory antibodies directed at the TF:FVIIa complex have been performed. In rabbits, an anti TF:FVIIa antibody (AP-1) inhibited thrombus formation [144] and showed reduced bleeding tendency when compared with heparin and a direct thrombin inhibitor [145]. In macaque monkeys, the pyrimidinones PHA-796 [146] and PHA-927 [147] potently reduced TF activity to inhibit thrombosis. A number of peptidomimetics and small molecular weight inhibitors have also been synthesized to target the TF:FVIIa complex as a result of these initial studies [148–152]. Furthermore, a soluble human recombinant TF (hTFAA) was generated by substituting two lysine residues with alanines (K165A/K166A) in the 219-residue extracellular domain of TF. hTFAA displayed greater antithrombotic effects when compared with heparin in a rabbit model [153] and inhibited the activity of FVIIa in guinea pigs to prevent recurrent arterial thrombosis without disturbing normal haemostasis, bleeding time, or activated partial thromboplastin time [154]. In addition, modification of 10 amino acids to hTFAA increased its binding affinity for FVIIa and increased its antithrombotic activity 20-fold [155], highlighting the potential benefits of targeting TF as the key trigger of immunothrombosis. However, despite the promising results shown by TF:FVIIa inhibitors in vivo, no direct TF inhibitor has been clinically approved to treat coagulopathies due to the critical role TF plays in maintaining haemostasis and the associated bleeding risk of directly blocking TF. Thus, targeting inflammasome activation, in addition to TF, may be a more effective method of treating immunothrombotic diseases.

**Targeting NLRP3 and caspase-11**

It has been demonstrated that inhibition of the expression of type I IFNs and their receptor IFNAR or downstream effectors (such as the increased PCA of TF by the promotion of PS externalization to the outer cell surface) decreases gram-negative bacteria-induced DIC [26]. This presents inhibition of the pathway prior to TF decscription as an attractive concept. One way to target coagulation during immunothrombosis while preserving normal haemostasis would be to block NLRP3 or caspase-11, or downstream components they regulate. MCC950 is a highly selective inhibitor of NLRP3 and blocks immunothrombosis in a CLP model [44]. There are also a number of cysteine-modifying compounds that react with NLRP3 and inhibit it, including MNS [156], oridonin [157,158], parthenolide [159,160], and Bay 11-7082 [160]. The Krebs cycle-derived metabolite, itaconate, and its cell-permeable derivative, 4-octyl itaconate (4-OI), also modify NLRP3 [161] and 4-OI has been shown to protect against LPS-induced acute lung injury in mice [162]. Finally, two clinically approved compounds which target GSDMD and subsequent pyroptosis may be redeployed in an anti-
immunothrombotic context. Disulfiram, which is approved for the treatment of alcohol addiction, covalently modifies human Cys191 and mouse Cys192 in GSDMD, which are critical for oligomerization of the N-terminal, blocking pyroptosis and LPS-induced sepsis [163]. Inhibition of GSDMD by disulfiram also blocks NET formation in neutrophils, reducing sepsis-induced lethality [164]. Dimethyl fumarate (DMF), which is a clinically approved immunomodulatory drug for the treatment of multiple sclerosis and psoriasis, succinates five cysteines on human GSDMD and ten cysteines on mouse GSDMD, including human Cys191 and mouse Cys192, protecting against LPS-induced lethal endotoxemia in vivo [165,166]. In addition, DMF blocks activation of the NLRP3 inflammasome in a murine experimental colitis model [167]. DMF also blocks type I IFN in humans by targeting IRAK4-MyD88 interactions and IRAK4-mediated cytokine production in a Cys13-dependent manner [168]. As these interactions drive NF-κB, this hints at deploying DMF to inhibit TF-induced immunothrombosis. Furthermore, a recent study showed that LPS stimulation and caspase-11-driven pyroptosis induces human and mouse macrophages and monocytes to secrete SQSTM1, or p62, a key autophagy receptor, which signals to polarize macrophages in an insulin receptor- and NF-κB-dependent manner [169]. Mice treated with anti-SQSTM1-neutralizing monoclonal antibodies, or myeloid-specific deletion of the insulin receptor, were protected from TF-driven lethal sepsis in CLP, E. coli, and Streptococcus pneumoniae models of lethal sepsis. This provides further evidence that inhibition of NLRP3, caspase-11, or the downstream components that they regulate is an attractive target for generating anti-immunothrombotic therapies (Figure 4 and Table 2). Indeed, further studies are warranted to identify if these cysteine-modifying therapeutics may also target the key cysteines on TF, which are critical for TF degradation.

Concluding remarks
Since the term immunothrombosis was first coined in 2013, myriad new elements of this pathway have been described, revealing many new interactions of the coagulation cascade with inflammasome-driven innate immunity. Regardless of the inflammatory stimuli, immunothrombosis-driven coagulation can involve the three signals of TF control: increased expression via NF-κB, subsequent decryption of TF (which occurs at the membrane proximal, extracellular domain of TF), and release of TF via pyroptosis. This is a critical feature of a range of diseases, from sepsis and DIC to cancer, venous thrombosis, diabetes mellitus, atherosclerosis, and acute coronary syndromes. Current anticoagulants are associated with bleeding risks especially in septic patients. Therefore, new approaches to regulate TF decryption represent an attractive target for urgently needed anti-immunothrombotic therapies. Thus, as we emerge from the COVID-19 pandemic, and with the renewed focus on strategies to mitigate dysregulated immunothrombosis, we believe that repurposing existing anti-inflammatory drugs which block elements of the immunothrombotic pathway, in addition to generating new therapies which block TF decryption or its release via pyroptosis, warrant further investigation. Ultimately, new and effective approaches to safely block immunothrombosis without the associated increased risk of bleeding represents a critical unmet clinical need for the treatment of multiple thromboinflammatory diseases.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

Author Contributions
T.A.J.R. wrote the original draft. R.J.S.P. and L.A.J.O’N. critically reviewed and edited the manuscript.

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Abbreviations
4-OI, 4-octyl itaconate; AM, alveolar macrophage; ARDS, acute respiratory distress syndrome; ASMase, acid sphingomyelinase; ATG, antithymocyte globulin; CLP, caecal ligation and puncture; COVID-19, coronavirus disease 2019; DIC, disseminated intravascular coagulation; DMF, dimethyl fumarate; DOAC, direct oral anticoagulant; DTNB, 3,3’-dithio-bis(6- nitrobenzoic acid); E. coli, Escherichia coli; EC, endothelial cell; GSDMD, gasdermin D; HMGB1, high mobility group protein B1; hTFAA, human recombinant tissue factor; IFN, interferon; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; LACI, lipoprotein-associated coagulation inhibitor; LPS, lipopolysaccharide; NET, neutrophil extracellular trap; NLRP3, NOD-, LRR- and pyrin
domain-containing protein 3; PAR, protease activated receptor; PBMC, peripheral blood mononuclear cell; PCA, procoagulant activity; PDI, protein disulfide isomerase; PS, phosphatidylserine; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SM, sphingomyelin; STING, stimulator of interferon genes; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TLR4, toll-like receptor 4; TMEM16F, transmembrane protein 16F.

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Basic HIGHLIGHTS

REVIEW

Innate immune signaling and immunothrombosis: New insights and therapeutic opportunities


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Activation of the coagulation cascade is a critical, evolutionarily conserved mechanism that maintains hemostasis by rapidly forming blood clots in response to blood-borne infections and damaged blood vessels. Coagulation is a key component of innate immunity since it prevents bacterial dissemination and can provoke inflammation. The term immunothrombosis describes the process by which the innate immune response drives aberrant coagulation, which can result in a lethal condition termed disseminated intravascular coagulation, often seen in sepsis. In this review, we describe the recently uncovered molecular mechanisms underlying inflammasome- and STING-driven immunothrombosis induced by bacterial and viral infections, culminating in tissue factor (TF) activation and release. Current anticoagulant therapeutics, while effective, are associated with a life-threatening bleeding risk, requiring the urgent development of new treatments. Targeting immunothrombosis may provide a safer option. Thus, we highlight preclinical tools which target TF and/or block canonical (NLRP3) or noncanonical (caspase-11) inflammasome activation as well as STING-driven TF release and discuss clinically approved drugs which block key immunothrombotic processes and, therefore, may be redeployed as safer anticoagulants.

Keywords: coagulation · immunothrombosis · inflammasomes · STING · tissue factor

Introduction

Coagulation is a core component in maintaining physiological hemostasis and the host response to infection. The coagulation cascade is defined by two major pathways—the intrinsic and extrinsic pathways—which culminate in a common pathway which ultimately results in formation of a thrombus and fibrin clot, stopping bleeding. The intrinsic pathway, which primarily contributes to pathological clot formation [1], is initiated via injury to blood vessels by autoactivation of coagulation factor (F)XII upon exposure of plasma to a diverse range of blood-borne artificial or pathological surfaces, including negatively charged endogenous activating surfaces such as RNA, DNA, polyphosphate, and/or components of atherosclerotic plaques [2]. The extrinsic pathway is initiated by coagulation FIII, also called tissue factor (TF) or CD142, which is expressed at low, basal levels in a complex with FVII on the membrane of circulating immune cells and cells in the blood vessel wall [3–5]. Blood clotting is controlled by endogenous anticoagulants such as tissue factor pathway inhibitor (TFPI), activated protein C, or antithrombin [6]. However, under pathogenic circumstances, exposure to, and detection of, microbes by innate immune cells amplifies the procoagulant activity of TF up to 100-fold, resulting in clot formation with the dual role of preventing bleeding but also inhibiting the dissemination of the provoking pathogen [7, 8]. Exposure to bacteria or viruses is detected by pattern recognition receptors (PRRs) on immune cells, such as monocytes, macrophages, endothelial cells (ECs), neutrophils, and platelets, triggering TF production and release. TF is released from macrophages, ECs, and neutrophils via inflammasome-mediated pyroptosis [9, 10]. This activates the coagulation cascade, restoring, and maintaining
Two major pathways of coagulation converge during hemostasis to form a blood clot. The original “waterfall” model of the coagulation cascade comprises the intrinsic and extrinsic pathways which converge into a common pathway to generate thrombin and form a fibrin clot. The intrinsic pathway primarily contributes to pathological clot formation and is activated when FXII encounters blood-borne, negatively charged surfaces such as RNA, DNA, and components of atherosclerotic plaques. The extrinsic pathway is activated when subvascular TF is exposed to plasma, or released into the bloodstream via innate immune cell pyroptosis, where TF forms a cell-surface complex with FVIIa. The intrinsic and extrinsic pathways combine to activate FX, which drives thrombin generation and ultimately blood clot formation. Endogenous inhibitors of the coagulation cascade include TFPI, activated protein C, and antithrombin.

TF is a 47-kDa membrane glycoprotein and receptor and the key trigger of infection- and injury-induced coagulation [5, 22–24]. TF is critical for survival, as deletion in mice leads to universal embryonic death [25–27], and defects in TF gene expression are associated with differing clinical outcomes in patients with severe sepsis [28]. TF is expressed by adventitial tissues, such as ECs, and blood-borne circulating immune cells such as monocytes, macrophages, and neutrophils [5]. During hemostasis, blood vessel injury triggers exposure and release of extravascular TF into the bloodstream, where it forms a complex with FVII and contributes to blood clotting via low-level activation of the extrinsic pathway of the coagulation cascade, before rapid inhibition by TFPI (Fig. 1) [4]. However, detection of pathogen-associated molecular patterns (PAMPs), such as LPS by PRRs such as TLR4, triggers immunothrombosis via rapid induction of TF at the mRNA level. This occurs via PAMP-induced activation of the transcription factor NF-κB both in vitro and in vivo [29], in monocytes and macrophages [29–31], neutrophils [32, 33], ECs [34, 35], and epithelial cells [36], the primary sources of TF [37]. TF is modified in a process termed decryption, which occurs in-part via changes in the lipid composition in the outer leaflet of the cell membrane [8, 10, 38], increasing the procoagulant activity of TF [7, 8]. Decrypted TF is then released from immune cells.
through inflammasome-induced pyroptotic pores, via activation of the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3, via caspase-1) or noncanonical (via caspase-11) inflammasomes [9, 10]. The molecular mechanisms underlying this process have recently been studied in detail in monocytes and macrophages, as they are the main source of circulating TF [23, 37]. For example, deletion of monocytes and macrophages using clodronate or gadolinium chloride significantly attenuates thrombin generation and septic shock-induced mortality in mice in vivo [9, 10].

Following its release via pyroptotic pores, decrypted TF is expressed in the circulation on outer membrane vesicles [39–42] and forms a high-affinity cell-surface complex with FVII/VIIa to proteolytically activate factors IX to IXa and X to Xa, resulting in thrombin generation [5, 43]. Thrombin then activates PARs which are critical for the interplay between inflammation and coagulation, boosting proinflammatory cytokine secretion but also activating platelets [44, 45]. Thrombin also cleaves fibrinogen to fibrin which generates a clot by forming a mesh at the site of infection, in conjunction with activated platelets and neutrophils which expel their DNA, histones, and granule-derived enzymes to form networks of extracellular fibres called neutrophil extracellular traps (NETs), in a process termed NETosis [46–50]. NETs then propagate thrombosis by capturing TF and TF-positive extracellular vesicles from the circulation, further driving coagulation [51, 52]. Thus, detection of PAMPs by PRRs triggers induction and decryption of TF, increasing its procoagulant activity, which is the key initiating step in coagulopathy associated with immunothrombosis and thromboinflammation (Fig. 2).

**Inflammasomes and TF**

Caspase-11 (in mice; caspase-4/5 in humans) is a member of the evolutionarily conserved family of caspases that mediate cell death [53]. It is induced and activated in response to Gram-negative bacteria, but not Gram-positive bacteria [54]. The response of caspase-11 to Gram-negative bacteria forms what has been termed as a noncanonical inflammasome. LPS induces transcriptional upregulation of caspase-11 in a range of immune and nonimmune cells including macrophages, neutrophils, and ECs [14, 53, 55–59]. Activation, and subsequent cleavage, of caspase-11 occurs upon detection of cytosolic LPS [56, 60, 61], triggering proteolytic cleavage of gasdermin D (GSDMD), a member of the family of gasdermin proteins that cause cell death [57, 62]. The pore-forming, N-terminal fragment of GSDMD is released, inserting into the cell membrane to form large oligomeric pores [63]. This leads to a proinflammatory, lytic form of cell death, termed pyroptosis, as first identified by Kayagaki et al. in a seminal paper in 2011 [55]. Pyroptosis, therefore, provides a critical step in the evolutionarily conserved family of caspases that mediate cell death [53]. It is induced and activated in response to Gram-negative bacteria, but not Gram-positive bacteria [54]. The response of caspase-11 to LPS triggers induction and decryption of TF, increasing its procoagulant activity, which is the key initiating step in coagulopathy associated with immunothrombosis and thromboinflammation (Fig. 2).

**cGAS-STING and immunothrombosis**

Recently, activation of the DNA sensor cyclic GMP–AMP synthase (cGAS)-STING has been implicated as a driver of sepsis in models of human and mouse coagulopathies. In 2014, mutations in transmembrane protein 173 (TMEM173) (the gene which encodes STING) were found to increase production of IFN-β in PBMGs and fibroblasts from pediatric patients presenting with recurrent fevers, ulcerative skin lesions, vasculitis, and interstitial lung disease, in addition to systemic inflammation, cutaneous vasculopathy, and pulmonary inflammation [70, 71]. ECs, which express STING, were also found to increase IFN-β production when stimulated with the second messenger cyclic guanosine monophosphate–adenosine monophosphate [70]. Furthermore, TF expression was upregulated in vascular ECs from patients with...
Figure 2. Inflammasome- and STING-mediated TF release drives thrombosis. Detection of a diverse range of microbes (such as viruses and Gram-negative and Gram-positive bacteria) by PRRs triggers innate immune signalling cascades which converge to activate IRF3/7 and NF-κB. IRF3/7 stimulates expression of type I IFNs. This leads to IFN-β release, which acts via the JAK-STAT signalling complex to drive transcription of hundreds of ISGs including caspase-11. Activation of STING can also drive this process. Caspase-11 is then cleaved and activated upon recognition of cytosolic LPS (which occurs via HMGB1 and RAGE), triggering cleavage and activation of GSDMD, resulting in pyroptosis. GSDMD cleavage can also be triggered by caspase-1 or caspase-8 activation. Simultaneously, TF is induced by NF-κB, before TF is post-translationally activated, in a process termed decryption. Procoagulant TF is then released through the pyroptotic pores to drive thrombosis, which can result in thromboinflammation, sepsis, and disseminated intravascular coagulation. These signalling cascades have been shown to be blocked by a number of immunomodulatory compounds including DMF, heparin, STING inhibitors (C-176, C-178, H-151), JAK inhibitors (Baricitinib, Ruxolitinib, Tofacitinib), and NLRP3 inflammasome inhibitors (4-OI, Itaconate, MCC950). Thus, innate immune signalling can trigger TF-mediated thrombosis via activation of the inflammasome and STING.
a mutation in TMEM173 [70]. These reports, describing a severe autoimmune inflammatory syndrome termed STING-associated vasculopathy with onset in infancy, were the first to link STING with a coagulopathy.

STING has been shown to sustain the host procoagulant response at later timepoints by regulating calcium release from macrophages and monocytes to drive GSDMD cleavage, facilitating the release of TF [72]. Notably, however, Zhang et al. found that this occurs in a type I IFN-independent manner [72]. This occurs in monocytes and macrophages via binding of STING with inositol 1,4,5-trisphosphate receptor type 1 (ITPR1), the primary calcium release channel from the ER. The authors found that a STING-ITPR1 complex forms after infection with the Gram-negative bacterium E. coli, or the Gram-positive bacterium Streptococcus pneumoniae (S. pneumoniae), which activates caspase-8. STING-ITPR1 binding boosts release of calcium from the ER into the cytosol, triggering cleavage of GSDMD via activation of caspase-1/11 (after E. coli infection) or caspase-8 (after S. pneumoniae infection). This facilitates pyroptosis and subsequent release of TF, resulting in sepsis and DIC [72]. The authors concluded that this process was type I IFN-independent as deletion of IFNAR, the type I IFN receptor, did not significantly alter mouse blood coagulation markers, such as platelets, fibrinogen, D-dimer, and TF when assayed 48 h after caecal ligation and puncture (CLP)-induced sepsis [72]. Furthermore, stimulation of human and mouse monocytes and macrophages with IFN-α and IFN-β did not induce TF release, whereas stimulation with E. coli and S. pneumoniae both induced TF release [72]. This highlights the specificity of pathways that drive coagulation within certain contexts. Two key signals are required for inflammasome-mediated coagulation: the first signal is infection- or injury-associated induction of TF at the mRNA and protein levels; the second signal is activation and cleavage of inflammatory caspases to trigger pyroptosis and release of procoagulant TF. After infection with E. coli or S. pneumoniae, TF is induced rapidly at the mRNA level via NF-κB, in addition to activation of caspase-1/11/8-mediated pyroptosis, representing the two key signals of inflammasome-mediated coagulation. However, when cells are stimulated with IFN-β, there is no known direct induction of TF mRNA via NF-κB.

Contrastingly, Yang and Cheng et al. showed a critical role for type I IFN signaling as a driver of coagulation in mouse models of LPS- and CLP-induced septic shock. In this study, the authors assessed coagulation markers between 6 and 16 h after infection, and found that the deletion of IFNAR significantly reduced LPS-induced plasma levels of thrombin-antithrombin and D-dimer, in addition to increasing survival of mice [73]. This was verified using TIR-domain-containing adaptor-inducing interferon-β (TRIF) KO mice, which were also protected against LPS-induced septic shock [73]. The different timepoints used in these two studies may explain their differing conclusions, but may also point toward type I IFNs driving a procoagulant phenotype at the onset of infection or injury, while STING may directly trigger coagulation at later timepoints after the type I IFN response has peaked.

**HMGB1 and immunothrombosis**

The danger-associated molecular pattern, high-mobility group box protein 1 (HMGB1), has been linked with coagulation as it is increased in the serum of LPS-infected mice and septic patients [74]. In addition, HMGB1 expression on circulating platelets is increased in trauma patients [75]. Recent studies have found that HMGB1 derived from platelets, hepatocytes, and myeloid cells mediates LPS-induced thrombosis in mice in a TLR4- and MyD88-dependent manner [75–77]. HMGB1 contributes to Gram-negative sepsis by binding to LPS [78], and it has been shown that hepatocyte-released HMGB1 transports extracellular LPS into the cytosol of macrophages and ECs [79]. This occurs via endocytosis of HMGB1-LPS, mediated by the receptor for advanced glycation endproducts (RAGE), and subsequent HMGB1-induced rupture of the endolysosomal membrane, releasing LPS into the cytosol. Cytosolic LPS is then detected by caspase-11, triggering noncanonical inflammasome-induced pyroptosis, releasing TF to drive coagulation [79].

HMGB1 has also been shown to stimulate expression of TF in vitro at the mRNA and protein levels in vascular ECs and macrophages via activation of the transcription factors NF-κB and Egr-1 [80]. However, Yang and Cheng et al. did not see an effect on LPS-induced TF protein levels in vivo after deletion of IFNAR, TRIF, or hepatocyte HMGB1 [73]. Using KO mice, they surmised that type I IFN and extracellular HMGB1 drive procoagulant TF activation and coagulation post-transcriptionally via caspase-11- and GSDMD-triggered pyroptosis and subsequent exposure of PS (which decrypts TF to trigger coagulation) [73]. In addition, a recent study assessing the role ninjurin1 (Ninj1) in lytic cell death found that deletion of Ninj1 in macrophages impaired pyroptosis and release of HMGB1, highlighting the importance of cell membrane rupture in driving inflammation and coagulation via release of HMGB1, and likely, TF [81].

Therefore, it is possible that extracellular LPS stimulates caspase-11-TF-induced coagulation initially by activating NF-κB (and inducing TF at the mRNA level), while simultaneously, extracellular LPS also drives type I IFN-mediated induction of IFN-stimulated genes (ISGs) such as caspase-11. LPS is then delivered to the cytosol via HMGB1, cleaving and activating caspase-1 (inducing sublytic pores in the cell membrane) and caspase-11, which triggers lytic pyroptosis and TF release. HMGB1 might then feedback to induce further TF expression, amplifying the available procoagulant TF. Furthermore, as the type I IFN response subsides, STING might then sense bacterial or host-derived DNA, driving HMGB1-induced pyroptosis. Further in vivo studies are required to unravel the differing roles of these key players in immunothrombosis.

**Virally-induced immunothrombosis**

Induction and decryption of TF has been shown to occur in vitro and in vivo in response to a range of viruses and the viral ds RNA
mimic polyinosinic:polycytidylic acid (poly[I:C]) [82–84]. TF procoagulant activity is increased in ECs infected with Herpes simplex virus (HSV) [85]. HSV infection in ECs also stimulates increased thrombin generation and platelet activity [86]. Ebola virus infection is also associated with severe hemorrhagic complications, manifesting as DIC which is driven by TF activity [87]. Geisbert et al. showed that TF is increased at the mRNA and protein levels in PBMCs from macaque monkeys infected with Ebola virus, with TF-positive microvesicles also increased in plasma from infected macaques [87]. A follow-up study from Geisbert et al. found that inhibition of TF:FVIIa, using recombinant nematode anticoagulant, is associated with increased risk of bleeding because coagulopathies: could targeting both PRR-mediated induction of TF and/or inflammasome activation within immune cells, rather than clotting factors themselves, prevent coagulopathy while also eliminating the associated bleeding risk?

Might inhibition of the transcriptional processes that lead to inflammasome activation and pyroptosis be particularly attractive targets in this context? PAMP-induced type I IFN and JAK-STAT signalling is required for expression of ISGs such as caspase-11. Baricitinib, ruxolitinib, and tofacitinib are clinically approved JAK inhibitors for the treatment of rheumatoid arthritis and myeloproliferative neoplasms [113], and thus, potentially could be reemployed as inhibitors of inflammasome. Recently identified STING inhibitors, such as the nitrofurans (C-176 and C-178) [114, 115], indole ureas (H-151) [114], and the acrylamides (BPK-21 and BPK-25) [116], which covalently modify STING, might also be useful. Notably, a recent study showed that ex vivo treatment with H-151 blocked induction of TF mRNA in primary human ECs infected with SARS-CoV-2 [99]. In addition, H-151 reduced lung SARS-CoV-2-induced TF mRNA levels in a mouse model of COVID-19 [99].

Directly targeting inflammasome activation is another strategy that has been shown to reduce immunothrombosis in several models. MCC950 is a highly selective inhibitor of NLRP3 [117, 118] and attenuates platelet activation and multiorgan injuries in a rat model of CLP-induced sepsis [119]. Similarly, the endogenous, Krebs cycle-derived metabolite itaconate, and its potently anti-inflammatory cell-permeable derivative, 4-octyl itaconate (4-OI), also block NLRP3 activation [120], with 4-OI attenuating lung injury in a murine model of LPS-induced coagulopathy [121]. This warrants further testing of these preclinical inhibitors of the canonical (NLRP3) and noncanonical (caspase-11) inflammasomes as potential treatments for inflammasome-driven immunothrombosis. Inhibition of GSDMD activation and pyroptosis occurs following treatment with dimethyl fumarate (DMF) [122, 123]. DMF is a clinically approved drug for the treatment of multiple sclerosis and psoriasis, and it exerts its immunomodulatory effects in-part by blocking induction of type I IFN [124] and inhibiting NLRP3 activation in a murine experimental colitis model via activation of the regulatory transcription factor Nrf2 [125]. Activation of Nrf2 is protective in a model of LPS- and NF-κB-induced sepsis [126], which would further support the

Targeting immunothrombosis to prevent coagulopathies

Current clinically approved anticoagulant therapies, while highly effective, are associated with increased risk of bleeding because blood clotting, platelet aggregation, and fibrin cross-linking are essential during normal hemostasis [105–109]. This life-threatening bleeding risk is significantly increased with treatment of sepsis and DIC [110]. Anticoagulant therapies exert their function by decreasing activity of clotting factors in the common pathway of the coagulation cascade. The widely used anticoagulant heparin exerts its anticoagulant function by activating antithrombin, which in turn inactivates thrombin, FXa, and FIXa [111]. Intriguingly, it has recently been shown that heparin, or a chemically modified form of heparin without anticoagulant function, also blocks HMGB1-mediated cytosolic delivery of LPS, thus, inhibiting caspase-11-driven pyroptosis to prevent aberrant immunothrombosis and subsequent sepsis-induced lethality in mice [112]. This hints at a potential solution to the bleeding risk associated with existing anticoagulant drugs and an exciting prospect for the development of new anticoagulant therapies: could targeting both PRR-mediated induction of TF and/or inflammasome activation within immune cells, rather than clotting factors themselves, prevent coagulopathy while also eliminating the associated bleeding risk?
testing of DMF as an anti-immunothrombotic agent, as TF-driven thrombosis occurs via activation of NF-κB. As such, DMF is currently being investigated as a potential broad spectrum anti-inflammatory therapy for COVID-19 in the ongoing RECOVERY trial [127].

Clinically approved anti-inflammatory therapies as potential anticoagulants?

Recent clinical trials have also studied the effects of anti-inflammatory therapies on thrombosis (discussed in detail in Refs. [18, 109]). The anti-inflammatory drug, colchicine, utilized for the treatment of gout and pericarditis, significantly lowered the risk of ischemic events in the COLCOT trial when administered to patients after myocardial infarction [128]. Colchicine blocks immunothrombosis by inhibiting NET formation and can also attenuate NLRP3 activation [129, 130]. A follow-up trial, LoDoCo2, using low-dose colchicine, found that IL-18 and myeloperoxidase (an enzyme released during neutrophil activation) were markedly decreased when administered to patients with chronic coronary disease [131, 132], highlighting the importance of drug dosing in anticoagulation treatment. However, a limitation of colchicine is that it is renally excreted, and thus, can be toxic in patients with chronic kidney disease [133], restricting its use as a treatment for cardiovascular diseases.

Concluding remarks

The past decade has seen a flurry of research in the area of immunothrombosis. As targeting mediators of the coagulation cascade downstream of inflammasome activation and pyroptosis has not yielded any new, safer anticoagulant drugs [134], developing therapeutics that inhibit immunothrombosis during activation of the innate immune response to infection, for example, by blocking TF expression and/or inflammasome or STING activation and subsequent pyroptosis, presents an exciting prospect. As this occurs prior to the activation of the coagulation cascade and generation of thrombin, the anti-inflammatory agents described above may in turn provide a safer method of anticoagulation by preventing any risk of unwanted bleeding, which has been termed the Holy Grail of identifying new treatments for immunothrombosis [135]. In the interim, redeployment of clinically approved anti-inflammatory drugs for the safer treatment of aberrant coagulation might well be a highly effective way to prevent the coagulopathies associated with immunothrombosis.

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SARS-CoV-2: severe acute respiratory syndrome coronavirus 2 · STING:
stimulator of interferon genes · S. pneumoniae: Streptococcus pneumoniae · TF: tissue factor · TFPi: tissue factor pathway inhibitor · TMEM173: transmembrane protein 173 · TRIF: TIR-domain-containing adaptor-inducing interferon-β

Abbreviations: 4-OI: 4-octyl itaconate · gGAMP: cyclic guanosine monophosphate–adenosine monophosphate · eGAS: cyclic GMP–AMP synthase · CKD: chronic kidney disease · CLP: caecal ligation and puncture · COVID-19: coronavirus disease 2019 · DAMP: danger-associated molecular pattern · DIC: disseminated intravascular coagulation · DMF: dimethyl fumarate · EC: endothelial cell · E. coli: Escherichia coli · GSDMD: gasdermin D · HMGB1: high-mobility group box protein 1 · HSV: Herpes simplex virus · IFN-α/β receptor · ISG: IFN-stimulated gene · ITTP1: inositol 1,4,5-trisphosphate receptor type 1 · NET: neutrophil extracellular trap · NINJ1: ninjurin1 · NLRP3: NOD-, LRR- and pyrin domain-containing protein 3 · PAR: protease activated receptor · poly(I:C): polyinosinic-polycytidylic acid · PS: phosphatidyserine · RAGE: receptor for advanced glycation endproducts · SARS-CoV-2: severe acute respiratory syndrome coronavirus 2 · STING: stimulator of interferon genes · TLR: Toll-like receptor · TRAF2: TNF receptor associated factor 2 · TRIF: TIR-domain-containing adaptor-inducing interferon-β

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An Emerging Role for Type I Interferons as Critical Regulators of Blood Coagulation


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Abstract: Type I interferons (IFNs) are central mediators of anti-viral and anti-bacterial host defence. Detection of microbes by innate immune cells via pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and cGAS-STING, induces the expression of type I IFN-stimulated genes. Primarily comprising the cytokines IFN-α and IFN-β, type I IFNs act via the type I IFN receptor in an autocrine or exocrine manner to orchestrate rapid and diverse innate immune responses. Growing evidence pinpoints type I IFN signalling as a fulcrum that not only induces blood coagulation as a core feature of the inflammatory response but is also activated by components of the coagulation cascade. In this review, we describe in detail recent studies identifying the type I IFN pathway as a modulator of vascular function and thrombosis. In addition, we profile discoveries showing that thrombin signalling via protease-activated receptors (PARs), which can synergize with TLRs, regulates the host response to infection via induction of type I IFN signalling. Thus, type I IFNs can have both protective (via maintenance of haemostasis) and pathological (facilitating thrombosis) effects on inflammation and coagulation signalling. These can manifest as an increased risk of thrombotic complications in infection and in type I interferonopathies such as systemic lupus erythematosus (SLE) and STING-associated vasculopathy with onset in infancy (SAVI). We also consider the effects on coagulation of recombinant type I IFN therapies in the clinic and discuss pharmacological regulation of type I IFN signalling as a potential mechanism by which aberrant coagulation and thrombosis may be treated therapeutically.

Keywords: type I interferons; blood coagulation; IFN-α; IFN-β; thrombin; PARs; haemostasis; thrombosis; tissue factor; SLE; APS; COVID-19; cGAS-STING; neutrophil extracellular traps; FXII

1. Introduction

The type I interferon (IFN) family is expressed by most cells in humans and mice and is a critical host defence mechanism for mounting anti-viral responses. The best characterized members are IFN-α (of which there are 13 subtypes in humans and 14 in mice) and IFN-β. Type I IFNs are induced upon detection of infiltrating microbes in the bloodstream via a diverse range of interactions between pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) [1], depending on the stimulus. PRRs that induce type I IFNs include Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors, and cyclic GMP-AMP synthase (cGAS) [2]. In particular, type I IFN induction can be induced via detection of pathogen-derived dsRNA by endosomal TLR3; ssRNA by TLR7, RIG-I, and MDA-5; and cytosolic dsDNA by cGAS-STING. These are the main receptors that drive type I IFNs in response to viruses, attesting to the importance of type I IFNs in anti-viral immunity.

In addition to viral-mediated type I IFN induction, exposure to bacteria can also trigger type I IFN signalling. One well-described system of type I IFN induction in myeloid cells occurs via activation of the TLR4-mediated immune signalling pathways upon detection of endotoxin, also called lipopolysaccharide (LPS), which is found in the outer membrane of
Gram-negative bacteria. Host recognition of LPS by TLR4 induces increased expression of proinflammatory cytokines in a process regulated by the nuclear factor (NF)-κB and interferon-regulatory factor (IRF) transcription factors [3]. This occurs via recruitment of myeloid differentiation primary-response gene 88 (MyD88) by MyD88-adaptor-like protein (MAL), with MyD88 forming a complex with members of the IL-1R-associated kinase (IRAK) family, in particular IRAK4, to activate NF-κB. A second signalling cascade triggered by the LPS–TLR4 interaction involves TRIF-related adaptor molecule (TRAM), which recruits TIR-domain-containing adaptor-inducing interferon-β (TRIF) to induce transcriptional upregulation of IRFs, including IRF3/7 [4]. IRFs in turn stimulate the expression of type I IFNs. This leads to IFN-α and IFN-β release, which act in an autocrine or paracrine manner via their ubiquitously expressed heterodimeric IFN-α/β receptor (IFNAR), which comprises two subunits, IFNAR1 and IFNAR2.

IFNs drive the transcription of hundreds of IFN-stimulated genes (ISGs) via the transcription factors signal transducer and activator of transcription (STAT)1, STAT2, and IRF9 of the Janus kinase (JAK)-STAT signalling pathway [5]. The precise context-dependent regulation of type I IFN induction has been reviewed in great detail previously [6–9]. Whilst type I IFNs are required early in the mounting of a host response to infection and the maintenance of homeostasis in human health and disease, overamplification of this response, or prolonged type I IFN signalling, can be detrimental [10,11]. Emerging evidence indicates that dysregulated type I IFN signalling can manifest as being a critical mediator of pathological blood coagulation in both viral and bacterial infection. Activation of coagulation may also feed-forward to amplify type I IFN production, which can have detrimental consequences. We will describe these key studies in this review and speculate on the future therapeutic implications of treating dysregulated type I IFN signalling for the management of coagulopathy.

2. Type I Interferons as Drivers of Blood Coagulation

Blood coagulation maintains physiological haemostasis following blood vessel injury or infection via formation of a haemostatic plug primarily comprising platelets, before activation of coagulation upon exposure of tissue factor (TF), the initiator of the extrinsic pathway of coagulation, from leukocytes or sub-endothelial tissue. TF forms a complex with coagulation factor (F)VIIa, initiating thrombin generation. Thrombin generation is then amplified and propagated in concert with activated platelets and leukocytes [12], as well as via formation of the tenase FVIIIa:FIXa and prothrombinase FXa:FVa complexes, which feed-forward to further generate thrombin. Endogenous anticoagulants such as antithrombin and tissue factor pathway inhibitor (TFPI) maintain haemostasis by rapidly inhibiting further thrombin generation, before the blood clot is dissolved via fibrinolysis [13].

Sepsis, a lethal inflammatory condition accompanied by multi-organ dysfunction, is often amplified by inflammation-induced blood vessel injury which exposes TF to be released into the bloodstream. In addition, activation of innate immune signalling pathways, such as the NLRP3 inflammasome, can also trigger TF release on extracellular vesicles from innate immune cells such as macrophages. This can occur during pyroptotic cell death during sepsis [14]. This can result in excessive thrombin generation and blood clot formation during sepsis. As a result, sepsis is often associated with coagulopathy.

Recently, numerous studies have directly implicated excessive type I IFN induction and signalling as a critical driver of blood coagulation, particularly in the context of Gram-negative bacterial-induced sepsis. This has been demonstrated using genetic mouse models lacking in core components of the type I IFN pathway. A recent study showed that Trif−/− mice are protected from the thrombotic complications associated with LPS-induced sepsis. Following intraperitoneal LPS administration, Trif−/− mice exhibited decreased thrombin generation and fibrin deposition in the livers and lungs compared with wild-type mice [15]. As a result, Trif−/− mice were protected from LPS-induced lethality. Deletion of TRIF also reduced Ifnb1 expression in the liver, spleen, and gut [15]. In the same study, investigators found that IFnar−/− mice, which do not express the IFN-α/β receptor, displayed decreased
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D-dimer levels as well as thrombin-antithrombin complex formation compared with wild-type mice following intraperitoneal LPS injection [15]. Ifnar−/− mice also had reduced fibrin deposition in their livers and lungs compared with wild-type mice. After cecal ligation and puncture (CLP), a clinically relevant model of polymicrobial sepsis, both Trif−/− and Ifnar−/− mice were protected from excessive thrombin generation and coagulopathy [15]. This indicates that the TLR4-TRIF-type I IFN pathway is a critical mediator of thrombosis following Gram-negative bacterial infection.

In another study, Dejager et al. showed that serum IFN-α is significantly elevated in mice as late as 48 h post CLP-induced sepsis [16]. Treatment of wild-type mice with an anti-IFNAR1 antibody, or deletion of IFNAR, protected mice from LPS-induced lethality and CLP-induced sepsis. In addition, this also significantly reduced serum interleukin (IL)-6 levels [16]. This is notable as IL-6 (formerly known as Ifnb2) contributes to coagulation via the rapid induction and synthesis of fibrinogen in the liver [17]. In another model of CLP-induced sepsis in mice, investigators found reduced endothelial damage in Ifnar−/− mice, with an associated suppression of inflammation-related gene expression in endothelial cells [18]. Consistent with a previous study [15], Ifnar−/− mice also displayed decreased aortic mRNA expression of Pai-1, an inhibitor of fibrinolysis [18], indicating that type I IFNs drive dysregulated fibrinolysis, which can lead to a prothrombotic state.

This evidence of IFNAR playing a critical role in type I IFN-mediated sepsis and coagulation is intriguing, as there is a significant similarity between IFNAR and TF, which is itself a membrane glycoprotein receptor. Global alignment of the sequence and structural patterns of IFN receptors indicates that transmembrane TF is structurally homologous to IFNAR [19]. In particular, both TF and IFNAR share evolutionarily conserved fibronectin type III domains comprising antiparallel β-sheets [20] which, in the case of TF, are proposed to be essential for the post-translational activation of TF. This process, termed decryption, occurs in the extracellular domain of TF and leads to a significant increase in TF procoagulant activity [21–23]. One intriguing possibility therefore is that TF might bind type I IFNs, acting as a decoy receptor or possibly a facilitator of IFNAR signalling.

2.1. Type I Interferon, the Caspase-11 Non-Canonical Inflammasome, and Blood Coagulation

Activation of type I IFN signalling can induce the ISG caspase-11 (in mice; caspase-4 and -5 in humans) [24], which is essential for the formation of a non-canonical inflammasome [25]. Type I IFN-mediated induction of caspase-11 occurs in response to Gram-negative—but not Gram-positive—bacteria [26]. Detection of cytosolic LPS in macrophages activates caspase-11 [27,28], which can then cleave and activate gasdermin D (GSDMD) to form pyroptotic pores in the cell membrane [29,30], through which proinflammatory and prothrombotic mediators are released. During Gram-negative bacterial sepsis, macrophage pyroptosis leads to TF release into the circulation on extracellular vesicles [31,32]. This results in excessive thrombin generation, leading to disseminated intravascular coagulation in mice [31,32]. Deletion of caspase-11 or GSDMD, or administration of an anti-TF antibody, protects mice from LPS-induced coagulation and lethality [31,32]. Caspase-11-mediated GSDMD cleavage also triggers the exposure of phosphatidylserine onto the outer membrane of macrophages [32], and this potentiates TF procoagulant activity [21,22]. Furthermore, CASPASE-5 expression is significantly increased in primary human macrophages from sepsis patients [33]. At the transcriptional level, induction of Gsdmd at the mRNA level is governed by the transcription factor IRF2 [34], which lies downstream of STAT1 and STAT2. IRF2 is also essential for induction of caspase-11 in macrophages [35], and thus IRF2 is a critical mediator of non-canonical inflammasome-induced pyroptosis. This indicates a critical role for type I IFN induction in the regulation of two key mediators of pyroptosis, which drives aberrant coagulation, and suggests that therapeutic targeting of IRF2 or the downstream non-canonical inflammasome may be a prospect for the inhibition of type I IFN-induced thrombosis.

Another key player in IFN-related coagulation is the high-mobility group box (HMGB) protein family, which is critical for host defence during both sterile and infectious injury
such as sepsis, and there is an intricate interplay between type I IFN and HMGB signalling. One of these mechanisms is via cytosolic detection of DNA or RNA. In particular, HMGB1 binds to the multivalent receptor for advanced glycation end-products (RAGE) to activate the endosomal TLRs 3, 7, and 9, and thus HMGB1 is essential for cytosolic nucleic acid-mediated induction of type I IFNs [36]. As such, elevated serum HMGB1 is a biomarker of inflammatory diseases [37]. Upon innate immune cell activation, HMGB1 translocates from the nucleus to the cytosol. This occurs upon activation of JAK/STAT1 via type I IFN signalling [37]. HMGB1 is then released from immune cells via inflammasome-mediated pyroptosis, which is induced upon autophosphorylation of the dsRNA-dependent protein kinase R (PKR), which is itself an ISG [38]. This results in physical interaction between PKR and the inflammasomes (NLRP1, NLRP3, NLRC4, and AIM2) [39]. Extracellular HMGB1 can then feed-back to further amplify the type I IFN signalling cascade in macrophages via activation of TLR4 [40]. In addition, HMGB1 physically binds extracellular LPS and is internalized into macrophage lysosomes via RAGE [41]. Destabilization of the lysosomal membrane by HMGB1 then releases LPS into the cytosol of macrophages, where it cleaves and activates caspase-11 to mediate pyroptosis, resulting in the release of proinflammatory and prothrombotic cytokines including IL-1β, IL-18, TF, and further HMGB1, amplifying and exacerbating the resulting inflammation [41]. Therefore, HMGB1 activity is critical for feed-forward inflammation and aberrant blood coagulation, possibly via type I IFNs. In addition, HMGB1 may contribute to blood coagulation by inducing F3 (TF) mRNA and TF protein, as well as TF procoagulant activity, in macrophages and endothelial cells [42]. This is likely due in part to HMGB1 being able to induce phosphatidylserine exposure from macrophages in a pyroptosis-dependent manner [15]. Induction of F3 by HMGB1 may also occur via activation of the transcription factor NF-κB [42], which is a transcriptional regulator of F3 [43]. Furthermore, platelet-derived HMGB1 has been implicated as an important contributor to neutrophil extracellular trap (NET) formation and subsequent deep vein thrombosis in mice [44]. These studies describing type I IFNs as drivers of blood coagulation are depicted in Figure 1.

A recent study also implicated interferon-inducible transmembrane (IFITM) proteins, which block the early stages of viral replication, as drivers of platelet activation in bacterial sepsis. In vitro stimulation of megakaryocytes with IFN-α induced IFITM3 protein expression via STAT1 phosphorylation, as well as mTOR [45]. This increased fibrinogen endocytosis in megakaryocytes via localization of the integrin αIIbβ3 and clathrin into lipid rafts. This translated to an increase in platelet aggregation in mice in vivo. Furthermore, IFITM3 expression and fibrinogen endocytosis were increased in platelets from humans with non-viral sepsis [45]. Therefore, IFITMs are required for IFN-α-induced thrombosis.

2.2. cGAS-STING and Blood Coagulation

Mounting evidence indicates a critical role for cGAS-STING signalling in the regulation of blood coagulation following infection. cGAS senses cytosolic dsDNA, generating the second messenger cyclic guanosine monophosphate–adenosine monophosphate (cGAMP), which activates STING. This promotes activation of TANK-binding kinase 1 (TBK1), which in turn phosphorylates IRF3 [46], resulting in type I IFN production [47]. The severe autoinflammatory syndrome, STING-associated vasculopathy with onset in infancy (SAVI), is a rare clinical condition where patients with gain-of-function mutations in TMEM173 (the gene that encodes for STING) present with cutaneous vasculopathy and vasculitis [48,49]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can also induce cGAS-STING activation. This occurs via the release of mitochondrial DNA (mtDNA) into the cytosol, which is detected by cGAS [50]. Following SARS-CoV-2 infection, cGAS-STING activates NF-κB-mediated inflammatory cytokine production as well as TBK1-IRF3-mediated type I IFN induction [51] to sustain the anti-viral response. In the context of blood coagulation, SARS-CoV-2-infected human endothelial cells exhibit increased IFNB1 and F3 mRNA and decreased TFPI mRNA expression, which are restored to basal levels upon treatment with the STING inhibitor H-151 [50]. H-151 also significantly reduced the SARS-
CoV-2-induced expression of IL6 [50], which is prothrombotic [17]. Therefore, cGAS-STING likely contributes to type I IFN induction and COVID-associated pathology, including coagulopathy. However, the potent induction of type I IFNs by cGAS-STING can be harnessed to promote the host anti-viral response to SARS-CoV-2 infection. Humphries et al. found that a pharmacological STING activator, diABZI-4, potently induced type I IFNs and suppressed SARS-CoV-2 replication in human A549 lung epithelial cells [52]. Therapeutic administration of the STING agonist protected mice from SARS-CoV-2-induced pulmonary damage and mortality, with direct STING activation proving more effective at eliminating weight loss and reducing mortality than the administration of IFNs in mice after SARS-CoV-2 infection [52]. This raises the possibility of targeting STING for the treatment of COVID-19-associated inflammation and coagulopathy.

**Figure 1.** Type I interferons as drivers of blood coagulation. Activation of innate immune cells occurs upon detection of PAMPs (such as LPS) by PRRs (such as TLR4). This rapidly induces type I IFNs via TRIF or NF-κB. The cytosolic PRR cGAS-STING can also induce type I IFNs upon detection of dsDNA, which can be released from hyperpolarized mitochondria. This results in the release of IFN-α and IFN-β, which act via their receptor IFNAR to induce hundreds of ISGs. Whilst this is a potent and necessary host anti-viral response, excessive type I IFN production can trigger excessive blood coagulation, particularly following Gram-negative bacterial infection. Caspase-11 and PKR, which are ISGs, trigger pyroptosis via inflammasome activation. This releases proinflammatory and procoagulant cytokines, including TF, the initiator of the extrinsic pathway of coagulation. HMGB1 is also released and can physically bind with LPS to activate RAGE. Internalization of HMGB1-LPS may trigger further induction of type I IFNs via activation of endosomal TLRs, potentially amplifying the associated inflammation and coagulation.

STING may also drive blood coagulation following infection in a type I IFN-independent manner. In one study, STING increased GSDMD-mediated TF release in monocytes and macrophages [53]. The authors, however, noted that this occurred in a type I IFN-
independent manner as there were no differences in disseminated intravascular coagulation (DIC) markers, including fibrinogen, D-dimer, fibrin, and TF release in the plasma of Ifnar−/− mice compared with wild-type mice 48 h after CLP-induced sepsis. The acute nature of type I IFN production in the innate immune response and in driving blood coagulation might explain why the authors attributed the role of STING in these models to be type I IFN-independent. It may also be because CGAS-STING can trigger pyroptosis in a type I IFN-independent manner. Gaidt et al. showed that the detection of cytosolic DNA results in pyroptosis via activation of TBK1 and IKKε to induce type I IFN-independent NLRP3 activation, as well as simultaneous type I IFN-dependent induction of caspase-11 [54]. Mechanistically, STING promoted DIC via binding to ITPR1 on the endoplasmic reticulum (ER) to promote ER calcium release, which decrypts TF [22,55] and triggers TF release via GSDMD-induced pyroptotic pores [53]. Therefore, the therapeutic targeting of STING may have beneficial effects for the treatment of inflammation-associated coagulopathy, regardless of the extent of the contribution of type I IFNs in those conditions.

2.3. Type I Interferon, COVID-19, and Blood Coagulation

The host IFN response to viral infection has been the focal point of much research in the past three years, as it is an essential defence mechanism following SARS-CoV-2 infection. In particular, rapid type I IFN induction is necessary for the mounting of an effective antiviral response against COVID-19. However, aberrant type I IFN signalling in COVID-19 is detrimental either due to overactivation of type I IFNs, or via SARS-CoV-2-mediated disruption of cellular RNA splicing and translation, as well as degradation of host mRNAs, which limits ISG production and enables further propagation of SARS-CoV-2 [56,57]. Thus, COVID-19 pathology is associated with dysregulated type I IFN signalling [58–60]. COVID-19 is also characterized by systemic coagulopathy [61]. This includes elevated plasma levels of D-dimer in patients [62] and increased pulmonary deposition of the thrombotic marker fibrinogen/fibrin [63], as well as elevated pulmonary von Willebrand Factor (vWF) deposition, which is a clinical marker of both acute and sustained endothelial cell activation, following SARS-CoV-2 infection [64]. Innate immune cells are core contributors to this pathology, with a recent report identifying a transcriptional shift in monocytes to a more prothrombotic genotype following SARS-CoV-2 infection [65]. Expression of F3 and TF-positive microvesicles are also increased in monocytes, macrophages, and platelets [66], as well as in endothelial cells and epithelial cells from patients with severe COVID-19 [50,67], propagating the coagulopathy associated with COVID-19. TF-positive microvesicles then drive excessive thrombin generation and coagulopathy following infection with SARS-CoV-2 [68,69]. In addition, thrombin and FXa have recently been shown to directly cleave the SARS-CoV-2 spike protein, augmenting viral entry into human airway epithelial cells and human pluripotent stem cell-derived lung organoids [70]. This suggests a broader anti-viral effect of deploying anticoagulants, in particular direct thrombin inhibitors and direct FXa inhibitors, as well as inhibitors of type I IFNs, in the treatment of COVID-19, as this will likely suppress pathological type I IFN production (as a result of reduced viral uptake) as well as thromboinflammation.

Assessment of whole blood from COVID-19 patients found a correlative relationship between defective type I IFN signalling, elevated coagulation markers, and increasing disease severity [60]. In addition, macrophage type I IFN- and caspase-11-dependent pyroptosis have been implicated in mediating TF-dependent coagulopathy in a mouse model of COVID-19 [71]. Therefore, investigators have attempted to target this type I IFN-caspase-11–TF axis as a means of limiting inflammation and coagulopathy in COVID-19. Heparin, the clinically approved antithrombin activator, can also block caspase-11-mediated pyroptosis at concentrations lower than those required for heparin to activate antithrombin [72]. Notably, anticoagulation treatment with heparin has been shown to decrease mortality in non-severe COVID-19 patients [73] and patients with elevated D-dimer levels [74]. However, a caveat of using heparin for the treatment of COVID-19 is that it is not effective in severe patients when administered therapeutically versus standard care pharmacologic
thromboprophylaxis [75]. This indicates a correlation between the rapid type I IFN response and the efficacy of heparin therapy following SARS-CoV-2 infection, with heparin proving most effective when administered prior to the onset of severe pathology. Therefore, inhibition of type I IFNs and the non-canonical inflammasome is an attractive target for anticoagulation treatment in COVID-19 associated coagulopathy, as well as during bacterial infections.

Emerging evidence indicates that the presence of persistent fibrin amyloid microclots may provide a mechanistic basis for the long-term effects of long COVID (also termed post-acute sequelae of COVID-19), which include fatigue and vertigo, as fibrin amyloid microclots block capillaries and the transport of oxygen to tissues [76,77]. Type I IFNs may contribute to the development of microclots in long COVID as elevated type I IFN expression has been detected for at least 8 months after infection [78]. Thus, new studies are urgently required to study the effect of IFN-mediated coagulation in long COVID.

2.4. Type I Interferonopathies and Blood Coagulation

Type I interferonopathies, an umbrella term first coined in 2011 by Yanick Crow [79], describes a group of clinical conditions associated with sustained, elevated type I IFN production. These related syndromes include systemic lupus erythematosus (SLE), an autoimmune disease characterized by persistent type I IFN upregulation [10]. Patients with SLE are at significantly greater risk of developing atherothrombotic cardiovascular disease [80]. PBMCs from patients with SLE have elevated mRNA expression of F3 [81]. SLE patients also have a greater number of activated platelets compared with healthy controls, and these platelets exhibit an elevated type I IFN mRNA and protein signature [82]. In addition, SLE patients with a history of vascular disease have increased type I IFN-regulated protein levels compared with SLE patients without a history of vascular disease [82]. Furthermore, in SLE patients, IFN-α rapidly triggers apoptosis of endothelial progenitor cells (EPCs) and myelomonocytic circulating angiogenic cells (CACs), which are required for blood vessel repair [83]. SLE EPCs and CACs exhibit increased IFN-α expression and an elevated type I IFN signature [83]. This indicates that sustained type I IFN production in SLE is closely intertwined with vascular damage and coagulopathy. This was demonstrated in one study whereby deletion of IFNAR in lupus-prone mice improved endothelial function and decreased atherosclerosis severity [80]. One primary mechanism of type I IFN induction in lupus occurs via the release of oxidized mtDNA, which is elevated in skin lesions from lupus patients and is associated with increased type I IFN production [84]. As cGAS-STING detects dsDNA and is a potent inducer of type I IFNs, cGAS-STING likely plays a key role in the pathogenesis of SLE. This is demonstrated by elevated cGAS expression in PBMCs from SLE patients [85]. Furthermore, expression of apoptosis-derived membrane vesicles, which are associated with elevated dsDNA levels, have been shown to activate cGAS-STING to induce type I IFNs in serum from SLE patients [86].

In addition to the thrombotic complications associated with SLE and SAVI, the importance of a functional type I IFN signalling pathway for regulating innate immune pathways and control of blood coagulation is demonstrated by the mutation of core signalling components which can predispose to increased risk of mortality. For example, a mutation in JAK2 first identified in 2005, JAK2V617F, is present in >80% patients with polycythaemia vera, a myeloproliferative leukaemia whereby excessive erythrocyte production can lead to excessive blood clotting and is associated with increased mortality [87]. In addition, the JAK2V617F mutation can also lead to essential thrombocythemia, a neoplasm whereby increased platelet production may further propagate the risk of excessive blood clotting [88]. Although the exact mechanisms underlying the contribution of type I IFNs to the increased thrombosis risk in these clinical conditions remains to be elucidated, this further indicates that dysregulated type I IFN signalling is a critical signal that drives aberrant blood coagulation.
2.5. Pharmacological Targeting of Type I Interferons to Treat Coagulopathies

The association between dysregulated type I IFN signalling and coagulopathy was highlighted during the COVID-19 pandemic, with pharmacological inhibition of excessive type I IFN production being associated with a concomitant reduction in SARS-CoV-2-induced coagulopathy. A number of studies reported the beneficial effects of inhibition of type I IFN signalling, particularly using JAK inhibitors, which are clinically approved for the treatment of conditions such as rheumatoid arthritis and myeloproliferative neoplasms (MPNs). For example, the JAK1/3 inhibitor tofacitinib lowered the SARS-CoV-2-induced risk of death or respiratory failure over 28 days versus placebo [89]. Another clinical trial found a decrease in D-dimer and C-reactive protein levels in the blood of hospitalized SARS-CoV-2 patients when treated with the JAK1/2 inhibitor baricitinib plus corticosteroids, versus treatment with corticosteroids alone [90]. JAK inhibitors have been especially effective in COVID-19 patients receiving high-flow oxygen or non-invasive ventilation [91], indicating that pharmacological inhibition of type I IFN signalling is most beneficial prior to the onset of severe disease. JAK inhibition will also block IL-6 signalling, further limiting inflammation and coagulation. Increased TYK2 expression is also associated with mortality in COVID-19 patients [92]. Therefore, targeting JAKs with specific inhibitors and therefore downstream JAK-TYK signalling may confer protection on COVID-19 patients. In addition, JAK inhibition may also suppress thromboinflammation in COVID-19.

3. The Effect of Blood Clotting on Type I Interferons

There is also a growing body of evidence which suggests that the key mediators of blood clotting, the coagulation factors themselves, can act on innate immune cells to induce proinflammatory cytokines and type I IFNs. This can amplify IFN production to combat bacterial or viral infection and restore haemostasis via the rapid resolution of inflammation, but it can also trigger a detrimental, pathological inflammatory cycle, as we will describe below.

The heterotrimeric GTP-binding protein-coupled protease-activated receptors (PARs) are expressed by a range of immune cells, and whilst PAR signalling is essential for the maintenance of haemostasis, it can also lead to the induction of proinflammatory cytokines [93]. PARs are the main substrate for thrombin and therefore numerous studies have assessed the induction of type I IFNs via thrombin-PAR signalling. The core procoagulant role of thrombin is the cleavage of fibrinogen into fibrin to generate a thrombus by forming a mesh at the sites of infection and vascular damage, in conjunction with activated platelets and neutrophils which expel their DNA, histones, and granule-derived enzymes during NETosis. Thrombin generation can occur as the endpoint of the intrinsic/contact (FXII-mediated) or extrinsic (TF-mediated) pathways of the coagulation cascade. Moreover, it has recently emerged that extracellular vesicles on erythrocytes may also contribute to the generation of thrombin [94,95]. However, excess thrombin generation can be pathological in a range of clinical conditions, resulting in tissue ischaemia by microvascular and macrovascular thrombosis.

**PAR-Mediated Induction of Type I Interferons**

Thrombin can trigger inflammatory signalling through PARs, which can result in a process termed thromboinflammation. PARs 1, 3, and 4 recognize and are cleaved and activated by thrombin [93]. In addition, a recent study suggests that thrombin, when bound to the endogenous anticoagulant thrombomodulin, can also cleave PAR2 [96]. Thrombin-PAR signalling is critical for the interplay between inflammation and coagulation, boosting proinflammatory cytokine secretion, as PARs can physically interact and therefore synergize with TLRs on innate immune cells. For example, Subramaniam et al. found that although stimulation of human umbilical vein endothelial cells (HUVECs) with thrombin did not directly induce the mRNA expression of ISGs or F3, co-stimulation of HUVECs with both thrombin and the dsRNA polyinosinic:polycytidylic acid (poly(IC)) resulted in significantly increased expression of F3 and TF procoagulant activity compared with
poly(I:C) stimulation alone [97]. This provides evidence of PAR1/2 and TLR3 synergy and suggests that in the context of an innate immune response, thrombin may positively feed-back to amplify its own production.

Recent studies have employed PAR knockout mice to examine further the effects of thrombin signalling on the innate immune system. Macrophages and splenocytes from Par1\(^{-/-}\) mice exhibited decreased type I IFN signalling after administration of poly(I:C) [98]. Furthermore, mRNA expression of Ifnb1, Irf7, and Cxcl10 were reduced in Par1\(^{-/-}\) mice after infection with Coxsackievirus group B (CVB), suggesting that PAR1, and therefore thrombin signalling, contributes to type I IFN-mediated anti-viral responses [98].

Furthermore, after stimulation of mouse cardiac fibroblasts with poly(I:C), PAR1 and TLR3 synergized to drive an anti-viral but proinflammatory response via induction of IFN-\(\gamma\) and CXCL10 via increased phosphorylation of the MAPK p38 [99]. In vivo, poly(I:C) administration increased expression of F3 in the heart and liver as well as thrombin generation in mouse plasma. Administration of an anti-TF monoclonal antibody or the thrombin inhibitor dabigatran etexilate significantly increased CVB3-induced myocarditis [99], indicating that haemostatic coagulation contributes to the innate, anti-viral response. Moreover, the chemotherapeutic drug, doxorubicin, has recently been found to increase thrombin generation in a TF-dependent manner, driving thromboinflammation in mice via PAR1 activation in cardiomyocytes and cardiac fibroblasts [100]. This prothrombotic phenotype may explain the underlying basis for the cardiotoxicity associated with doxorubicin.

In addition, PAR2 has also been shown to synergize and physically interact with TLRs during inflammation [101]. PAR2 synergized with TLRs 2, 3, and 4 in mucosal epithelial cells following poly(I:C) stimulation, which activated NF-\(\kappa\)B via degradation of IкB\(\alpha\) and phosphorylation of p65 [102]. However, in contrast to PAR1, PAR2 negatively regulated the TLR3 signalling pathway, resulting in decreased phosphorylation of IRF3 & STAT1, and therefore suppressing the type I IFN-mediated anti-viral response. Thus, Par2\(^{-/-}\) and Tlr4\(^{-/-}\) mice were protected from lethality induced by infection with H1N1 influenza A virus [102]. Additionally, in LPS-stimulated bone marrow-derived macrophages, PAR2 activation resulted in increased IL-10 secretion, possibly via increased STAT3 phosphorylation, but decreased secretion of the proinflammatory cytokines IL-6, TNF, and IL-12p40 [103]. This suggests that PAR2 counteracts LPS-induced proinflammatory cytokine production.

Moreover, PAR2 restrained type I IFN signalling in fibroblasts via the binding of TLR3 in a mouse model of CVB3-induced myocarditis [104]. Higher cardiac PAR2 mRNA expression correlated with low IFNB1 expression in patients with non-ischaemic cardiomyopathy, resulting in increased expression of inflammatory markers, including CD3+ and CD45+ T cells [104]. Thus, modulation of the host response by PAR2 differs in response to activation of different TLRs. Therefore, synergy of individual PARs with TLRs can have contrasting effects on downstream innate immune signalling, particularly with regards to type I IFN signalling.

In addition to the induction of type I IFNs by thrombin-PAR-TLR signalling, thrombin can also induce the proinflammatory cytokines IL-6, TNF, and IL-12p40 [105] in human monocytes [105] and vascular smooth muscle cells [106]. Intraperitoneal injection of thrombin in mice increased IL-6 secretion from peritoneal macrophages into the peritoneum in a fibrinogen-dependent manner [107]. Thrombin can also cleave and activate pro-IL-1\(\alpha\) (p33) into its active form (p18) when expressed on the surface of macrophages, platelets, and keratinocytes [108]. Thrombin-activated IL-1\(\alpha\) was found to be important for rapid thrombopoiesis and wound healing. Furthermore, thrombin-cleaved IL-1\(\alpha\) is elevated in the plasma of ARDS patients versus healthy controls [108], and thus can be considered a biomarker of thromboinflammatory conditions.

Furthermore, the formation of TF:FVIIa rapidly induces FXa to drive cytokine production via PAR2 [109]. TF:FVIIa:FXa can also form a complex with EPCR to trigger TLR4/PAR2-mediated type I IFN signalling. This occurs via induction of pellino-1, the TLR3/4 adaptor protein, in addition to IRF8 [110]. Deficiency of EPCR, PAR2, or TF in mice attenuates LPS-induced expression of IRF8 and subsequent type I IFN induction in vitro.
and in vivo [110]. This indicates a role for TF:FVIIa as a DAMP by activating innate immune signalling pathways. The role of PARs in the induction of type I IFNs and downstream blood coagulation is summarized in Figure 2.

Figure 2. Induction of type I IFNs and downstream blood coagulation by PARs. In addition to thrombin’s procoagulant (cleavage of fibrinogen) and proinflammatory (cleavage of IL-1α) roles, thrombin can also activate PARs 1, 3, and 4, which can synergize with TLRs to induce type I IFNs. This can occur either via the NF-κB or TRIF signalling cascades, resulting in the production of IFN-α and IFN-β, which act via IFNAR to induce anti-viral but proinflammatory ISGs. PAR2, however, which is activated by FXa via TF:FVIIa complex formation, suppresses type I IFN induction whilst activating NF-κB. This results in the induction of F3, leading to TF release and the further feed-forward amplification of PAR2-TLR signalling. Thus, activation of PARs can trigger type I IFN production, which can amplify the inflammatory and procoagulant response during infection.
4. Interferon Therapy and Coagulopathy

In the clinic, type I IFNs are administered therapeutically. Recombinant IFN-α is approved for the treatment of chronic hepatitis B and C viral infections as well as various cancers, including MPNs, whilst recombinant IFN-β is approved for multiple sclerosis (MS) treatment to regulate the persistent inflammation associated with the condition. Although these therapies are effective, there have been indications that IFN therapy can lead to an increased risk of thrombosis.

Elevated vWF antigen expression and activity has been detected in the plasma of MPN patients receiving IFN-α compared with healthy controls [111]. Plasma from IFN-α-treated MPN patients also displayed significantly increased activity of fibrinogen and the coagulation factor FVIII, as well as reduced protein S activity, indicating a shift in MPN patients to a more procoagulant phenotype. Functionally, this resulted in elevated thrombin generation in MPN patient plasma. The investigators tracked the patients for 6 months and found that haemostasis was restored in patients when IFN-α treatment was discontinued, as demonstrated by a significant reduction in vWF and fibrinogen levels, as well as increased protein S activity [111]. Thus, IFN-α therapy increases prothrombotic biomarkers in the plasma of MPN patients. Recombinant type I IFN therapies have also been linked with a dose-dependent increase in the risk of thrombotic microangiopathies in MS patients [112]. A study by Jia et al. suggested a potential mechanism by which type I IFNs might drive thrombotic microangiopathies. They compared the effects of recombinant IFN-α and IFN-β on endothelial cell function and found that IFN-β suppressed proliferation and survival of HUVECs, but IFN-α did not affect these parameters [113]. Meanwhile, both IFN-α and IFN-β blocked angiogenesis via activation of IFN-inducible CXCL10 when HUVECs and human dermal fibroblasts were co-incubated in vitro. Endothelial cell activity was impaired by IFN-α and IFN-β via inhibition of endothelial cell-produced nitric oxide and prostacyclin. Intriguingly, IFN-β significantly increased PAI-1 and downregulated uPA in HUVECs, which is indicative of decreased fibrinolysis [113]. Thus, these studies indicate that type I interferonopathies or administration of type I IFNs may be associated with increased risk of pathological blood clotting.

5. The Interrelationship between Type I Interferons and Thrombosis in Disease

In addition to the emerging evidence linking aberrant type I IFN signalling with coagulopathy in conditions such as Gram-negative bacterial infection and COVID-19, type I interferonopathies such as SLE have been associated with an increased risk of thrombosis, as discussed above. Emerging evidence indicates a key role for excessive neutrophil activation and NET formation in the pathogenesis of SLE. Activation of neutrophils with ribonucleoprotein immune complexes, which are highly expressed in lupus, induce hyperpolarization of mitochondria, followed by the translocation of mitochondria to the cell surface and subsequent release of mtDNA [114]. Oxidized mtDNA drives NETosis in SLE and lupus-like diseases [114], leading to increased deposition of dsDNA, IL-17, HMGB1, and the anti-microbial peptide LL-37 in NETs from SLE patients [115,116]. LL-37 induces type I IFNs in plasmacytoid dendritic cells (pDCs) by binding extracellular dsDNA and transporting it into endosomal compartments of pDCs, triggering TLR9-mediated IFN production [117]. LL-37 may also transport dsDNA into monocytes to induce type I IFN signalling via cytosolic STING-TBK1 activation [118]. Interestingly, this occurs independently of TLR9 in monocytes [118]. This is supported by the fact that stimulation of human PBMCs with NETs induces IFNA1 and IFNB1 expression [119]. Thus, NETs are interferogenic. However, NETs are also prothrombotic, particularly in COVID-19 [120], in part by capturing TF and TF-positive extracellular vesicles from the circulation [121,122], thereby facilitating activation of the extrinsic pathway of coagulation. Furthermore, whilst neutrophil-released DNA and histones are prothrombotic, intact NETs are not directly thrombogenic themselves [123]. This is notable as DNA released from NETs can drive thrombin generation in a FXII-dependent manner [124], with a FXII-NETs cross-talk implicated in the pathogenesis of COVID-19 [125] as well as deep vein thrombosis [126].
In addition, in a feed-forward manner, FXII has been shown to drive NETs in a baboon model of *Escherichia coli*-induced sepsis [127]. Increased levels of autoantibodies to FXII have also been associated with thrombosis in SLE [128], suggesting the possibility that FXII might drive type I IFN signalling in SLE via NET formation. Future studies should assess this hypothesis to potentially unravel FXII-mediated thrombosis as a novel target for SLE therapy.

The autoimmune disease antiphospholipid syndrome (APS) is also associated with an increased risk of aberrant type I IFN signalling and thromboinflammation. In APS, endocytosis of antiphospholipid antibodies (aPLs) into pDCs induces TLR7/8-dependent type I IFN production [129], as characterized by increased expression of TLR7 [130] and TLR8 [131] in PBMCs from patients with APS. This leads to IFN-α release from pDCs, which stimulates the production of B1a cells, a type of B cell associated with autoimmune diseases such as APS and SLE. B1a cells then produce further lipid-reactive aPLs, propagating APS [129]. aPLs also hijack haemostatic control of coagulation by inhibiting TFPI [132]. This results in increased thrombosis via TF:FVIIa-dependent thrombin generation, highlighted by elevated F3 expression in monocytes and PBMCs in patients with APS [133,134], demonstrating the prothrombotic genotype associated with APS. Furthermore, monocytes from patients with APS and SLE exhibit increased expression of PLSCR1 [135], which is involved in monocyte phosphatidylserine externalization and TF decryption [23]. In the presence of an aPL, stimulation of macrophages with IFN-α significantly increased F3 expression [135], indicating that type I IFN signalling drives TF-dependent thrombin generation in APS. Soluble TF levels are elevated in plasma from patients with APS [136], and there is an increase in TF-dependent procoagulant activity in the PBMCs of patients with APS [137] as well as in the carotid artery homogenates of mice injected intraperitoneally with serum IgG isolated from APS patients [138]. This suggests a mechanism (and potential therapeutic target) for the innate immune-mediated microthrombosis associated with APS. In addition, identifying the molecular mechanisms underlying a prothrombotic type I IFN–TF axis in APS would contribute greater understanding of the complex processes driving thrombosis in APS.

Furthermore, excessive type I IFN production via cGAS-STING activation has been implicated in further models of inflammation-associated coagulopathy, including cerebral venous sinus thrombosis [139] and acute lung injury [140], indicating a broad spectrum of conditions where there is a strong correlation between dysregulated type I IFN production and aberrant coagulation. It would be intriguing to hypothesize that these two events are interrelated, and thus future studies should assess the extent of the cross-talk between type I IFN and thrombosis in the pathogenesis of these clinical conditions.

6. Conclusions

Mounting evidence indicates that dysregulated host type I IFN production can manifest as being a critical driver of pathological blood coagulation during infection but also in such conditions as SLE and SAVI, and possibly during IFN therapy. Type I IFNs can trigger pathological coagulation via both the intrinsic/contact and extrinsic pathways of blood clotting. Coagulation factors can feed-forward to induce proinflammatory cytokines and type I IFNs, for example via thrombin-PAR-TLR signalling. Therefore, type I IFNs may be critical drivers of thrombosis. With this review, we hope to inspire greater focus on the precise mechanisms by which type I IFNs mediate blood coagulation and thrombosis, and vice versa, which may prove that type I IFNs lie at the fulcrum of inflammation and coagulation. Targeting IFNs may therefore present therapeutic opportunities for the treatment of aberrant coagulation in infection and inflammatory diseases, as well as providing new avenues for the prevention or treatment of DIC in Gram-negative bacterial sepsis.

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Abbreviations
aPL: antiphospholipid antibody; APS, antiphospholipid syndrome; CAC, circulating angiogenic cell; cGAMP, cyclic guanosine monophosphate–adenosine monophosphate; cGAS, cyclic GMP-AMP synthase; CLP, cecal ligation and puncture; CVB, Coxsackievirus group B; DAMP, danger-associated molecular pattern; DIC, disseminated intravascular coagulation; ER, endoplasmic reticulum; GSDMD, gasdermin D; HMGB, high-mobility group box; HUVEC, human umbilical vein endothelial cell; IFITM, interferon-inducible transmembrane; IFN, interferon; IFNAR, IFN-α/β receptor; IL, interleukin; IRAK, IL-1R-associated kinase; IRF, interferon-regulatory factor; ISG, IFN-stimulated gene; JAK, Janus kinase; LPS, lipopolysaccharide; MAL, MyD88-adaptor-like protein; MPN, myeloproliferative neoplasm; MS, multiple sclerosis; mtDNA, mitochondrial DNA; MyD88, myeloid differentiation primary-response gene 88; NET, neutrophil extracellular trap; NF-κB, nuclear factor (NF)-κB; PAMP, pathogen-associated molecular pattern; PAR, protease-activated receptor; pDC, plasmacytoid dendritic cell; PKR, protein kinase R; poly(IC), polyinosinic-polycytidylic acid; PRR, pattern recognition receptor; RAGE, receptor for advanced glycation end-products; RIG-I, retinoic acid-inducible gene I; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SAVI, STING-associated vasculopathy with onset in infancy; SLE, systemic lupus erythematosus; STAT, signal transducer and activator of transcription; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1; TF, tissue factor; TFPL, tissue factor pathway inhibitor; TLR, Toll-like receptor; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adaptor-inducing interferon-β; TYK, tyrosine kinase; vWF, von Willebrand Factor.

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Macrophage fumarate hydratase restrains mtRNA-mediated interferon production

Metabolic rewiring underlies the effector functions of macrophages1–3, but the mechanisms involved remain incompletely defined. Here, using unbiased metabolomics and stable isotope-assisted tracing, we show that an inflammatory aspartate–argininosuccinate shunt is induced following lipopolysaccharide stimulation. The shunt, supported by increased argininosuccinate synthase (ASS1) expression, also leads to increased cytosolic fumarate levels and fumarate-mediated protein succination. Pharmacological inhibition and genetic ablation of the tricarboxylic acid cycle enzyme fumarate hydratase (FH) further increases intracellular fumarate levels. Mitochondrial respiration is also suppressed and mitochondrial membrane potential increased. RNA sequencing and proteomics analyses demonstrate that there are strong inflammatory effects resulting from FH inhibition. Notably, acute FH inhibition suppresses interleukin-10 expression, which leads to increased tumour necrosis factor secretion, an effect recapitulated by fumarate esters. Moreover, FH inhibition, but not fumarate esters, increases interferon-β production through mechanisms that are driven by mitochondrial RNA (mtRNA) release and activation of the RNA sensors TLR7, RIG-I and MDA5. This effect is recapitulated endogenously when FH is suppressed following prolonged lipopolysaccharide stimulation. Furthermore, cells from patients with systemic lupus erythematosus also exhibit FH suppression, which indicates a potential pathogenic role for this process in human disease. We therefore identify a protective role for FH in maintaining appropriate macrophage cytokine and interferon responses.

Stimulation of macrophages with the TLR4 ligand lipopolysaccharide (LPS) leads to reprogramming of central metabolic pathways involved in bioenergetics, which can facilitate cytokine production. Changes in macrophage metabolism have emerged as a major regulator of inflammation4–7. Although metabolic reprogramming is crucial for macrophage activation the players involved and how they regulate cytokine production remain incompletely characterized.

Accumulation of fumarate in macrophages

To evaluate metabolic alterations that occur during LPS stimulation, we used an unbiased metabolomics approach based on liquid chromatography–mass spectrometry to characterize the metabolome of inflammatory bone-marrow-derived macrophages (BMDMs). The TCA cycle metabolite fumarate stood out as one of the most significantly upregulated metabolites following exposure to acute LPS stimulation, joining previously identified metabolites such as itaconate6 (Fig. 1a). We also observed a significant increase in fumarate–mediated protein succination8–10, which resulted in the formation of the fumarate-cysteine adduct (S)-2-succinocysteine (2SC) (Extended Data Fig. 1a–c). As acute LPS stimulation failed to impair respiration (Fig. 1b,c), TCA cycle disruption is unlikely to be sufficient for fumarate accumulation. Increased flux through the aspartate–argininosuccinate shunt has been reported to support nitric oxide production11. As fumarate...
is a by-product of argininosuccinate cleavage by argininosuccinate lyase (ASL) in the cytosol, we proposed that argininosuccinate may be a source of fumarate. In support of this hypothesis, we observed decreased aspartate, the precursor to argininosuccinate, and increased argininosuccinate, fumarate and malate levels (Fig. 1d), a result consistent with increased flux through the shunt. This rewiring also occurred during prolonged LPS stimulation (Extended Data Fig. 1d).

Argininosuccinate synthase (ASS1) and FH (encoded by Fhl1 in mice and FH in humans) expression increased and decreased, respectively in LPS-stimulated BMDMs, as determined by quantitative PCR with reverse transcription (RT-qPCR) (Fig. 1e). Analyses of available quantitative proteomics data showed that ASS1 was upregulated, whereas levels of glutamic-oxaloacetic transaminase 2 (GOT2), ASL and FH were not significantly altered (Fig. 1f). FH levels were suppressed only at later time points of LPS treatment (Fig. 1g), which indicates that ASS1 induction is vital to the acute accumulation of fumarate.

Inhibition of the aspartate–argininosuccinate shunt with the GOT2 inhibitor aminoxyacetic acid (AOAA) reduced aspartate, aspara- gine, argininosuccinate and fumarate levels following LPS stimulation (Fig. 1h and Extended Data Fig. 1e). Knockdown of Asl also prevented fumarate accumulation (Extended Data Fig. 1f,g), which indicated the dependency of fumarate production on the aspartate–argininosuccinate shunt, which would increase cytosolic fumarate levels (Fig. 1i). Stable isotope-assisted tracing showed that glutamine-dependent anaplerosis is in part responsible for fumarate accumulation and drives the aspartate–argininosuccinate shunt. U-13C-glutamine tracing
demonstrated glutaminolysis as a carbon source for the TCA cycle, aspartate–argininosuccinate shunt metabolites, including fumarate, and glutathione (Extended Data Fig. 2). 14N-glutamine tracing also demonstrated that glutamine nitrogen is a source for glutathione synthesis and aspartate–argininosuccinate shunt metabolites (Extended Data Fig. 3). Notably, AOAA prevented the contribution of glutamine nitrogen to aspartate, asparagine, arginine and citrulline, thereby confirming its inhibition of GOT2. Metabolomics analysis of cytosolic fractions of resting and LPS-stimulated macrophages showed that metabolites such as itaconate and succinate accumulated in the cytosol following LPS stimulation (Extended Data Fig. 4a). Increased levels of argininosuccinate, fumarate and 2SC were also present in the cytosol (Extended Data Fig. 4b).

We proposed that lrg1 / BMDMs (which are unable to synthesize itaconate) would relieve the inhibition of succinate dehydrogenase 12,13 and exhibit greater accumulation of aspartate–argininosuccinate shunt metabolites. Metabolomics analysis of lrg1 / BMDMs revealed the expected decrease in itaconate and succinate levels, and increased aspartate–argininosuccinate shunt metabolites, including fumarate and nitric oxide (Extended Data Fig. 4c,d). This result provides further evidence linking mitochondrial TCA cycle activity to an aspartate–argininosuccinate shunt (Extended Data Fig. 4e).

**FH inhibition causes metabolic rewiring**

FH catalyses the hydration of fumarate to malate in mitochondria and the cytosol 11. Inhibition of this process increases cytosolic fumarate accumulation, perturbs urea cycle metabolism and leads to renal cyst development 17. FH levels remained stable during early LPS stimulation (Fig. 1g). Therefore, we used a well-established pharmacological inhibitor of FH (FHINA) 20 and a recently developed tamoxifen-inducible model of cre-ERT2 expressing Fh1 / mice to analyse the role of FH activity and fumarate accumulation in macrophages. However, because FH inhibition may lead to effects independent of fumarate accumulation through mitochondrial and redox stress 18, we also used low concentrations of cell-permeable dimethyl fumarate (DMF) to deliver a cysteine-reactive modulator of FH (FHINA) (Extended Data Fig. 5a). Tetramethylrhodamine methyl ester (TMRM) staining confirmed this result, as FHINA significantly increased staining, whereas DMF had no effect (Fig. 2h). Similarly, Fh1 / macrophages had increased MMP values, as previously reported in kidney epithelial cells 21 (Fig. 2h). We also observed a decreased aconitase/citrate ratio in FHINA-treated macrophages, which was indicative of impairment in the fumarate-sensitive and redox-sensitive TCA cycle enzyme aconitase 24 (Fig. 2i). Although the GSSG/GSH ratio was unchanged, FHINA led to a depletion of total glutathione (Fig. 2j), which is consistent with fumarate-mediated glutathione depletion 22,26. These data suggest that FH inhibition induces substantial redox stress responses.

**FH maintains appropriate cytokine responses**

To determine whether FH regulates macrophage activation and effector responses, we performed RNA sequencing (RNA-seq) and proteomics to assess changes in the transcriptome and proteome of FHINA-treated BMDMs. Gene set enrichment analysis (GSEA) identified an expected suppression in genes associated with metabolism. However, FHINA also decreased the expression of pathways that affect inflammation, including interleukin-1 (IL-1) and IL-10 signalling (Fig. 3a). Increased expression of components of the haem-regulated inhibitor stress response, amino acid metabolism and RNA ammocoylation was also observed (Fig. 3a), consistent with previous reports 28. Overrepresentation analysis of RNA-seq data revealed that tumour necrosis factor (TNF) signalling was the most highly upregulated pathway in our analysis (Fig. 3b).

Comparing FHINA with DMF on cytokine readouts allowed us to determine the role of protein succination following FH inhibition. FHINA and DMF decreased IL-10 release and expression, whereas TNF release and expression were increased (Fig. 3c and Extended Data Fig. 6a). This result validated our transcriptomics analysis. Both compounds also reduced IL-1β expression and IL-6 release (Extended Data Fig. 6b), consistent with previous reports 11,27, thereby demonstrating the widespread regulation of cytokine expression.

The less electrophilic fumarate ester monomethyl fumarate (MMF) exhibited the same effects on IL-10 and TNF expression (Fig. 3d), which provides support for a role for fumarate in their regulation. Shared transcriptomic changes induced by FHINA and DMF demonstrated strong downregulation of the ERK1 and ERK2 cascade and P38K signalling (Fig. 3e). A similar transcriptional fingerprint has been observed in FH-deficient leiomyomas 28. We also observed increased amino acid metabolism and transport and autophagy transcripts (Extended Data Fig. 6c). Following LPS stimulation, IL-10 is regulated by ERK1 and ERK2 and P38K-induced AP-1 activation 29, which suggests that downregulation of this signalling axis by FHINA and DMF may repress IL-10. However, we did not observe changes in the upstream kinases AKT, JNK, ERK and p38, which converge on AP-1 activation (Extended Data Fig. 6d). Although we did observe reduced Jun expression in our transcriptomics dataset.
(Extended Data Fig. 6e), this could indicate reduced autoregulation by AP-1 (ref. 39). In this dataset, Fos was not reduced (Extended Data Fig. 6f).

Notably, the thiol precursor N-acetyl cysteine (NAC) abrogated the suppression of Il10 by FHIN1 and DMF (Fig. 3f). The free thiols of NAC and its products would react with and sequester fumarate, thereby reducing the modification of protein thiols and suggesting that suppression of IL-10 results from a redox-dependent succination event. The electrophile sulforaphane has been shown to reduce AP-1 activation through the modification of Cys154 on c-Fos31. We therefore investigated whether FHIN1 or DMF may affect c-Fos activation, despite upstream regulators remaining unaffected. c-Fos transcription factor assays showed that FHIN1 and DMF strongly impaired c-Fos activation (Fig. 3g), which provides evidence of direct regulation of c-Fos, potentially through Salkylation.

IL-10 signalling has been shown to repress TNF expression32. We confirmed this effect using an IL-10 receptor (CD210) blocking antibody.
that targets IL-10-mediated STAT3 phosphorylation. This blockade leads to augmented LPS-induced TNF release (Fig. 3h and Extended Data Fig. 6g). We then examined whether recombiant IL-10 supplementation could rescue the increase in TNF release. IL-10 with FHIN1 failed to impair STAT3 phosphorylation or augment TNF production (Fig. 3j), which indicates that the induction of TNF driven by FHIN1 or DMF depends on the suppression of IL-10.

We sought to confirm the role of FH in regulating this axis. Inducible deletion of Fh1 in macrophages from heterozygous Fht+/− or homozygous Fht−/− mice (Extended Data Figs. 5c,d and 6h) resulted in decreased
IL-10 expression and release (Fig. 3c) and increased TNF release (Fig. 3i). Furthermore, FHIN1 also suppressed IL10 expression and increased TNF expression in LPS-stimulated human peripheral blood mononuclear cells (PBMCs) (Fig. 3m) and macrophages (Fig. 3n). This result indicates that the FH-regulated IL-10–TNF axis is also active in human cells. Establishing the role of LPS-driven fumarate accumulation in the release of these cytokines, AOAA, which reduces fumarate accumulation (Fig. 1h), modestly increased and reduced IL-10 and TNF release, respectively (Extended Data Fig. 6i). This result indicates that an increase in ASS1, which results in fumarate accumulation, mildly regulates IL-10 and TNF production. These effects were accentuated by pharmacological or genetic inhibition of FH, which led to increased fumarate accumulation (Extended Data Fig. 6j). Therefore, sustained expression and activity of FH may be viewed as protective against excessive fumarate accumulation and dysregulated production of IL-10 and TNF.

FH inhibition also resulted in the activation of a NRF2 and ATF4 stress response in macrophages (Extended Data Fig. 7a), which is in line with previous observations in epithelial cells. Proteomics analysis revealed that the inflammation-associated hormone GDF15 (refs. 33–35) was one of the most significantly increased proteins following FHIN1 and DMF treatment, whereas FHIN1 also increased the recently identified mitochondrial glutathione importer SLC25A39 (ref. 36). This result reinforces the fact that mitochondrial redox is perturbed (Extended Data Fig. 7b,c). In support of our proteomics data, FH inhibition drove GDF15 release from macrophages (Extended Data Fig. 7d). Both ATF4 and NRF2 have been reported to regulate GDF15 in different contexts34,35, and silencing of each revealed that FHIN1-driven GDF15 release partly depended on NRF2 but not ATF4 (Extended Data Fig. 7e,f). This work defines two previously unappreciated signalling axes linked to FH inhibition, uncovering its role in the regulation of IL-10, TNF and GDF15. Recent developments that have identified GDF15 as a mediator of immune tolerance, and the anti-inflammatory properties of colchicine and nonsteroidal anti-inflammatory drugs33,35, suggest that protective effects of DMF in models of inflammation could be mediated at least in part through GDF15. Moreover, increased TNF levels potentially explain adverse events reported with fumarate esters. Mechanistically, suppression of IL-10 may also explain why fumarate esters promote enhanced TNF production during trained immunity, in addition to reported epigenetic changes40.

**FH restrains mtRNA-driven IFNβ release**

RNA-seq analysis of type I interferon (IFN) response genes revealed divergent effects on IFN expression and signalling following FH inhibition, including an upregulation in Ifih1 (which encodes IFNβ) expression and several interferon-stimulated genes, such as Ifi1, Ifih1, Ratsd2 and Ifit2 (Fig. 4a). However, other interferon-stimulated genes, such as Lcn2, were suppressed by FHIN1 and DMF treatment (Fig. 4a and Extended Data Fig. 8a). Examination of specific type I IFN signalling components downstream of the IFNα/β receptor (IFNAR) revealed that both FHIN1 and DMF treatment limited IFNβ-induced STAT1 and JAK1 phosphorylation (Extended Data Fig. 8b), which indicated that there was modest suppression of JAK–STAT signalling. Activation of NRF2 by fumarate and derivatives (Extended Data Fig. 7) may be responsible41. Indeed, Ifih1 expression was increased after FHIN1 and DMF treatment following Nr2f2 silencing (Extended Data Fig. 8c,d), which suggests that NR2F2 restrains IFN transcription.

Notably, FHIN1, but not DMF or MEF, increased IFNβ release from LPS-stimulated macrophages (Fig. 4b,c). This effect was independent of N-acetyl-sensitised redox stress (Extended Data Fig. 8e) and was not due to augmented TLR4 signalling, as LPS-induced TRAF3 levels and IL-1β expression were not increased by FHIN1 (Extended Data Fig. 8f,g). FHIN1 and DMF modestly augmented LPS-induced p65 phosphorylation (Extended Data Fig. 8h), which may contribute to increased TNF release42. Given that FH inhibition causes mitochondrial stress (Fig. 2), which is associated with the release of immunostimulatory mitochondrial nucleic acids43–45, we proposed that the IFN response is driven by cytosolic nucleic acid sensors, such as cGAS, RIG-1 or MDA5. In support of this hypothesis, FH-deficient-erythroid leiomysomatosis and renal cell cancer tumours exhibit changes in mitochondrial DNA (mtDNA)46. We first used ethidium bromide to deplete mtDNA47 (Extended Data Fig. 8i) before treating cells with FHIN1 and LPS. FHIN1 no longer boosted LPS-induced IFNβ release in the presence of ethidium bromide (Fig. 4d), which indicated that the increased IFNβ release with FHIN1 may depend on mtDNA. We subsequently found that FHIN1 caused an increase in both mtDNA and mtRNA in cytosolic extracts (Fig. 4e and Extended Data Fig. 8j). Given the established role of mtDNA in driving IFN responses41,44, we examined whether the cGAS–STING or TLR9 DNA-sensing pathways are required for the increase in IFNβ. However, treatment with the STING inhibitor C-178 (ref. 48) or silencing of cGAS (which encodes cGAS) or Tmem173 (which encodes STING) had no effect on FHIN1-driven IFNβ induction (Extended Data Fig. 8k–n). Targeting TLR9 using the competitive inhibitor ODN 2088 (ref. 49) or using siRNA also had no effect on this response (Extended Data Fig. 8k–n). Suppression of Tmem173 expression by FHIN1 or DMF (Extended Data Fig. 8o) may explain why cGAS–STING signalling is redundant in our model, even in the presence of cytosolic mtDNA. ETC inhibition, as observed with FHIN1 treatment, has also been shown to inhibit STING activation48.

As cytosolic mtRNA was also increased by FHIN1 (Fig. 4e), we performed immunofluorescence staining with an antibody specific for double-stranded RNA (dsRNA). mtRNA has previously been shown to drive an IFN response in human cells50,51, and is known to be particularly immunostimulatory41. FHIN1 treatment led to an accumulation of dsRNA relative to cells treated with dimethylsulfoxide (DMSO) as control (Fig. 4f). We subsequently treated cells with both FHIN1 and IMT1, an inhibitor of mitochondrial RNA polymerase (POLRMT). The increase in mtRNA following FHIN1 was observed in the cytosolic fraction but not in the whole cell fraction and was inhibited in both conditions with IMT1 treatment (Extended Data Fig. 8p,q). Notably, IMT1 also partly abrogated the FHIN1-mediated increase in IFNβ release (Extended Data Fig. 8r), which implies that mtRNA has a role in driving this response. Mitochondrial single-stranded RNA (ssRNA), which results from a decline in mitochondrial integrity, has also been implicated in driving TLR7-dependent IFNβ signalling43,44. We subsequently silenced T7r7 or the dsRNA sensors Ddx58 (which encodes RIG-I) and Ifi1 (which encodes MDA5) (Extended Data Fig. 9a,b), all of which abrogated the increase in IFNβ release observed with FH inhibition (Fig. 4g,h). This result confirms that there is a non-redundant requirement of these sensors and mtRNA, rather than mtDNA, for the FHIN1-driven IFN response. Knockdown of the cell surface dsRNA sensor Tlr3 did not affect the augmentation in IFNβ release (Extended Data Fig. 9c). RIG-I and MDA5, although predominantly described as dsRNA sensors, can also bind ssRNA52, which indicates that the IFN response following FH inhibition is probably driven by a mixture of dsRNA and ssRNA species. It is notable that FHIN1 also reduced Ddx58 but not Ifi1 expression, which may warrant further investigation (Extended Data Fig. 9b). The signalling events downstream of RIG-I–MDA5 activation include mitochondrial antiviral signalling protein (MATS) oligomerization, followed by recruitment and phosphorylation of TANK-binding kinase 1 (TBK1). We observed MATS oligomerization and increased TBK1 phosphorylation following FHIN1 treatment (Fig. 4i and Extended Data Fig. 9d). Notably, Matk knockout did not impair the induction of IFNβ by FHIN1 (Extended Data Fig. 9e), which may indicate that compensatory TLR7 signalling is sufficient to drive type I IFN responses following FH inhibition with chronic MAVS deficiency.

We previously demonstrated that FH inhibition causes mitochondrial stress (Fig. 2). Changes in MMP have previously been correlated with increased type I IFN release46. Therefore we proposed that disturbances in MMP may be linked to mtRNA release and IFNβ induction following FH inhibition. To support this hypothesis, we induced changes in MMP by using the ATP synthase inhibitor oligomycin A, which increased MMP,
the K⁺ ionophore valinomycin A, which nonsignificantly reduced MMP, or the uncoupler CCCP, which significantly dissipated MMP (Extended Data Fig. 9b). All treatments boosted LPS-driven IFNβ release, similar to effects with FH1 (Extended Data Fig. 9g). MMP, which does not increase LPS-induced IFNβ expression (Fig. 4c), did not affect MMP (Extended Data Fig. 9a). Oligonucleotide treatment led to an accumulation of dsRNA to a similar extent to that observed in cells treated with FH1 or transfected with dsRNA (poly(I:C)), and increased mtRNA release into the cytosol (Extended Data Fig. 9j–l). Valinomycin treatment similarly drove dsRNA accumulation (Extended Data Fig. 9m,n), which indicated that compounds that alter MMP induce an accumulation of mtRNA. As we also observed an increase in cytosolic mtRNA levels following oligonucleotide treatment (Extended Data Fig. 9g), it is still possible that IFN responses following oligonucleotide, valinomycin and CCCP treatment are not exclusively driven by mtRNA. mtRNA release from chondrocytes has recently been implicated in activating the immune response and promoting osteoarthritis. As such, mitochondrial damage and nucleic acid release are emerging as key pathogenic processes that may underlie many immune-mediated diseases.

Tamoxifen-inducible Fh1⁻/⁻ BMDMs released more IFNβ after LPS stimulation than their Fh1⁺/⁺ counterparts (Fig. 4j). We also detected increased dsRNA accumulation in Fh1⁻/⁻ BMDMs (Fig 4k and Extended Data Fig. 9o), which, coupled with the fact that deletion of Fh1 also drives mitochondrial membrane hyperpolarization (Fig. 2h), demonstrate that both genetic and pharmacological targeting of FH drive similar mitochondrial retrograde type IFN stress responses.

We next considered whether this response could be applied to an endogenous model of LPS activation in the absence of pharmacological or genetic inactivation of FH. Given that LPS-induced FH suppression occurs predominantly during late-phase LPS stimulation (24–48 h)
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Article

Methods

Animal details

All mice were on a C57BL/6JolaHsd background unless stated below. Wild-type (WT) mice were bred in-house. The inducible Fh1+/– and Fh1–/– mice were generated on the C57BL/6 genetic background, and their hind legs were donated by C. Frezza (University of Cambridge, UK). Fh1–/– and Fh1+/– treated with vehicle (ethanol) were used as controls. Following treatment with 4-hydroxymoxofen (TAM), CRE-mediated chromatin excision results in the loss of either one (Fh1–/–) or both (Fh1+/–) copies of Fh1, thus generating either heterozygous or null animals. Hind legs from WT and Maus+/– mice were donated by C. Johannson (Imperial College London, UK). These strains, originally obtained from S. Akira (World Premier International Immunology Frontier Research Center, Osaka University, Osaka, Japan), were fna60+/+, but since fna60 expression was not a primary readout, the mice are designated as WT and Maus–/–. In vitro experiments were performed with BMDMs isolated from 6–18-week-old female and male mice. Although we did not use statistical methods to calculate sample sizes, we decided to use a minimum of three biological replicates per experiment to account for biological variability, considering the three Rs principle (replacement, reduction, and refinement) and the fact that most experiments were performed in primary macrophages from inbred mice. All in vitro treatment groups were randomly assigned. In vitro and in vivo experiments were not blinded owing to the lack of available experimenters with required expertise. In vivo models were performed using 6-week-old male mice, and littersmates were randomly assigned to experimental groups. Animals were maintained under specific pathogen-free conditions in line with Irish and European Union regulations. All animal procedures were approved by the Trinity College Dublin Animal Research Ethics Committee before experimentation and conformed with the Directive 2010/63/EU of the European Parliament.

Generation of mouse BMDMs

Mice (6–18 weeks old) were euthanized in a CO2 chamber, and death was confirmed by cervical dislocation. Bone marrow was subsequently collected from the tibia, femur and ilium and cells were differentiated in DMEM containing 10% FCS (10%), penicillin–streptomycin (1%), and penicillin–streptomycin (1/100) for 6 days, after which cells were counted and plated at 0.5 × 106 cells/ml unless otherwise stated. BMDMs were plated in 12-well cell culture plates and left overnight to adhere.

Isolation of human PBMCs

Human blood samples from healthy donors were collected and processed at the School of Biochemistry and Immunology at the Trinity Biomedical Sciences Institute (TCD). Blood samples were obtained anonymously, and written informed consent for the use of blood for research purposes was obtained from the donors. All the procedures involving experiments on human samples were approved by the School of Biochemistry and Immunology Research Ethics Committee (TCD). Experiments were conducted according to the TCD guide on good research practice, which follows the guidelines detailed in the National Institutes of Health Belmont Report (1978) and the Declaration of Helsinki. Whole blood (30 ml) was layered on 20 ml lymphoprep (Axis-Shield), followed by centrifugation for 20 min at 400g with the brake off, after which the upper plasma layer was removed and discarded. The layer of mononuclear cells at the plasma-density gradient medium interface was retained, and 20 ml PBS was added. Cells were centrifuged for 8 min at 300g and the resulting supernatant was removed and discarded. The remaining pellet of mononuclear cells was resuspended, counted and plated at 1 × 106 cells/ml in RPMI supplemented with FCS (10%) and penicillin–streptomycin (1%).

Generation of human macrophages

PBMCs were obtained, and CD14+ monocytes were isolated using a MagniSort Human CD14 Positive Selection kit (ThermoFisher) according to the manufacturer’s protocol. CD14 monocytes were then differentiated in T175 flasks in RPMI containing FCS (10%), penicillin–streptomycin (1%) and recombinant human M-CSF (1:1,000). After 6 days, the supernatant was discarded, cells were scraped and counted, and human monocyte-derived macrophages were plated in 12-well plates at 1 × 106 cells/ml in RPMI containing FCS (10%) and penicillin–streptomycin (1%).

Whole blood isolation from patients with SLE

All patients with SLE (as per the diagnostic criteria of the American College of Rheumatology) were recruited from the Cedars–Sinai Medical Center. Age- and sex-matched healthy donors who had no history of autoimmune diseases or treatment with immunosuppressive agents were included. All participants provided informed written consent, and the study received approval from the institutional ethics review board (IRB protocol number 19627). Blood was collected into PAXgene RNA tubes (2.5 ml blood plus 6.9 ml buffer) and stored at −80°C. Before isolation of RNA, the tubes were thawed at room temperature for 16 h. Total RNA was isolated using a PAXGene Blood RNA kit according to the manufacturer’s recommendations (PreAnalytiX, 08/2005, 762174).

Reagents

LPS from Escherichia coli, serotype EH100 (ALX-581-010-L001), was purchased from Enzo Life Sciences. High molecular weight poly(IC) (tirl-pic) and 2′-3′-cGAMP (tirl-nagca23) were purchased from InvivoGen. Recombinant mouse IFNβ (551802) and recombinant mouse IL-10 (417-ML-005/CF) were purchased from BioLegend. ATP disodium salt (A23833), DMSO (D8418), AOAA (C13420), valinomycin (V3639), TAM (H6278) and NAC (A7250) were purchased from Sigma Aldrich. Oligomycin A from Streptomyces diastatocromogenes (M02220) was purchased from Fluorochem. FHNIH (HY-1000004), DMF (HY-17363), MMF (HY-103252), IMT1 (HY-134339) and C-178 (HY-123963) were purchased from MedChemExpress. CPG ODN1826 (130-100-274) and ODN 2088 (130-105-815) was produced by Miltteny Biotec. CCR5 (M20036) was purchased from Thermofisher.

Compound treatments

All compounds used DMSO as a vehicle except for TAM (ethanol), NAC (PBS) and AOAA for tracing experiments (culture medium). LPS was used at a concentration of 100 ng/ml for indicated time points (2, 3, 4, 6, 8, 24 and 48 h). FHNIH (10 or 20 µM), MMF (50 or 100 µM), DMF (25 µM), AOAA (5 mM), oligomycin (10 µM), CCR5 (50 µM), NAC (1 mM) and IMT1 (10 µM) pre-treatments were performed for 3 h before the addition of LPS. Cells were treated with valinomycin (10 nM) 15 min before LPS stimulation. Anti-CD210 or IgG control (10 µg/ml) antibodies were added to cells 1 h before LPS stimulation. Recombinant mouse IL-10 protein (100 ng/ml) was added to cells at the same time as LPS. Cells were treated with IFNβ (220 ng/ml) for 3 h. Cells were treated with C-178 (1 µM) 1 h before LPS stimulation or transfection with 2′-3′-cGAMP (1.5 µg/ml) for 4 h to achieve cGAS–STING activation. Cells were treated with ODN 2088 (1 µM) 1 h before LPS stimulation or transfection with CPG ODN1826 (1.5 µg/ml) to achieve TLR9 activation. Three different time points of TAM (600 nM or 2 µM) or ethanol treatment were performed (specified in the individual figure legends). For 48-h treatments, ethanol and TAM were added on day 5 of 6 during the BMDM differentiation protocol. On day 6, they were plated with ethanol and TAM (left overnight) and treated the following day. For 72-h treatments, ethanol and TAM were added on day 4 of 6 during the BMDM differentiation protocol. On day 6, they were plated with ethanol and TAM (left overnight) and treated the following day. For 96-h treatments, ethanol and TAM were added on day 4 of 6 during the BMDM differentiation protocol. On day 6, they were plated with ethanol and TAM and treated 2 days later.

Antibodies

Working dilutions of antibodies were 1/1,000 unless otherwise stated. Anti-mouse lamin B1 (12S86), STAT1 (9172), p-STAT1 (9167), JAK1 (3344),
for each sample and gene of interest. Anti-dsRNA antibody (Merck) was diluted 1:60 in 0.1% Tween 20 in PBS. RT-qPCR was performed on the cDNA generated in the previous step, using primers designed in-house and ordered from Eurofins Genomics, as detailed in Supplementary Table 1. Isolated RNA samples were normalized and converted into cDNA using a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher). If necessary, samples were DNAse-treated after quantification using the lowest concentration across all samples with RNAse-free water. RNA extraction from cells was carried out using a Purelink RNA kit (Invitrogen). Details of anti-body validation are given in Supplementary Table 1.

RT–qPCR
RNA extraction from cells was carried out using a PureLink RNA kit (Invitrogen) according to the manufacturer’s instructions. BMDMs were treated as required, and following treatment were instantly lysed in 350 µl RNA lysis buffer. Isolated RNA was quantified using a NanoDrop 2000 spectrophotometer, and RNA concentration was normalized to the lowest concentration across all samples with RNAse-free water.

If necessary, samples were DNAse-treated after quantification using DNase I (Thermo Fisher) according to the manufacturer’s instructions. Isolated RNA samples were normalized and converted into cDNA using a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher) according to the manufacturer’s instructions. Next, 10 µl of RNA (at a maximum concentration of 100 ng µl⁻¹) was added to 10 µl of reverse transcription master mix to complete the reaction mixture. Real-time qPCR was performed on the cDNA generated in the previous step, using primers designed in-house and ordered from Eurofins Genomics, as detailed in Supplementary Table 2. The reaction was performed in a 96-well qPCR plate using a 7500 Fast Real-Time PCR machine (Thermo Fisher). Relative expression (2⁻ΔΔCt) was calculated from the Ct values for each sample and gene of interest.

RNA interference
Pre-designed silencer select siRNAs for Cgas (s103166), Tmem173 (s91058), Trl3 (s100579), Trl9 (s96268), Asl (s99640), Trl7 (s100720), Ddx58 (s106376), Ifih1 (s98797), Nr2f2 (s70522), Atf4 (s62689) and negative control (4398043) were ordered from Thermo Fisher. siRNA sequences are given in Supplementary Table 2. Cells were transfected with 50 nM siRNA using 5 µl Lipofectamine RNAiMAX according to the manufacturer’s instructions (Thermo Fisher). Cells were transfected in medium without serum and antibiotics, which was replaced with complete medium 8 h later. Cells were subsequently left for at least a further 12 h before treatment.

Immunofluorescence
Cells were plated on 20 mm cover slips in 12-well plates. Cells were treated as required and Mitotracker Red CMXRos (100 nM, Thermo Fisher) was added to medium 30 min before the end of cell treatments. After 30 min of incubation, cells were washed three times with warm PBS. Cells were subsequently fixed for 10 min with 4% paraformaldehyde in PBS at 37 °C. Cells were washed three times with PBS and permeabilized for 1 h in block solution (1% BSA, 22.52 mg ml⁻¹ glycine and 0.1% Tween 20 in PBS). Anti-dsRNA antibody (Merck) was diluted 1:60 in block solution and incubated with cells overnight at room temperature. Cells were washed three times with PBS for 5 min per wash. A mix containing AF488-conjugated goat anti-mouse IgG antibody (1:1,000) and DAPI (1:1,000, Thermo Fisher) was subsequently added to cells for 90 min at room temperature in the dark. Cells were subsequently washed three times with PBS for 5 min per wash. Cover slips were mounted onto microscope slides using 10–20 µl ProLong Gold antifade reagent (Thermo Fisher). Slides were imaged using a Leica SP8 scanning confocal microscope with a ×200 objective. Images were analysed using LAS X Life Science Microscope Software Platform (Leica). The same microscope instrument settings were used for all samples, and all images were analysed using the same settings. Quantification of dsRNA or Mitotracker Red CMXRos signal intensity was performed using the measure function in ImageJ 1.53t (NIH). The mean signal intensity was calculated for individual cells in single colour images and displayed relative to signal intensity of control cells.

Flow cytometry
Cells were plated in 12-well plates and treated as desired. CellROX Green (5 µM, Thermo Fisher) or TMRM (20 nM, Thermo Fisher) was added to cells 30 min before the end of cell treatments. Cells were washed once in PBS and scrunched into 200 µl FACS buffer (2 mM EDTA and 0.5% FCS in PBS). Acquisition of samples was performed using a BD Accuri C6 flow cytometer. The gating strategy used for all flow cytometry experiments consisted of debris exclusion by FSC-A versus SSC-A analysis and subsequent doublet exclusion by FSC-A versus FSC-H analysis. A sample gating strategy is provided in Supplementary Fig. 2. Overall, 10,000 cells were acquired per condition. The mean fluorescence intensity (MFI) was calculated for all cells in each condition using FlowJo v.10.

Liquid chromatography–mass spectrometry
Steady-state metabolomics. BMDMs (3 independent mice) were plated at 0.5 × 10⁶ cells per well in 12-well plates in technical triplicate per condition, treated as indicated, snap frozen and stored at −80 °C. For metabolomics of the cytosolic fraction, BMDMs were plated at 10 × 10⁶ cells per 10 cm dish, and rapid fractionation was performed as previously reported. Metabolite extraction solution (methanol:acetonitrile:water, 50:30:20 v/v/v) was added (0.5 ml per 1 × 10⁶ cells), and samples were incubated for 15 min on dry ice. The resulting suspension was transferred to ice-cold microcentrifuge tubes. Samples were agitated for 20 min at 4 °C in a thermomixer and then incubated at −20 °C for 1 h. Samples were centrifuged at maximum speed for 10 min at 4 °C. The supernatant was transferred into a new tube and centrifuged again at maximum speed for 10 min at 4 °C. The supernatant was transferred to autosampler vials and stored at −80 °C before analysis by liquid chromatography–mass spectrometry (LC–MS). HILIC chromatographic separation of metabolites was achieved using a Millipore Sequent ZIC-hILIC analytical column (5 µm, 2.1 × 150 mm) equipped with a 2.1 × 20 mm guard column (both 5 mm particle size) with a binary solvent system. Solvent A was 20 mM ammonium carbonate and 0.05% ammonium hydroxide; solvent B was acetonitrile. The column oven and autosampler tray were held at 40 °C and 4 °C, respectively. The chromatographic gradient was run at a flow rate of 0.200 ml min⁻¹ as follows: 0–2 min: 80% solvent B; 2–17 min: linear gradient from 80% solvent B to 20% solvent B; 17–17.1 min: linear gradient from 20% solvent B to 80% solvent B; 17.1–22.5 min: hold at 80% solvent B. Samples were randomized and analysed by LC–MS in a blinded manner, and the injection volume was 5 µl. Pooled samples were generated from an equal mixture of all individual samples and analysed interspersed at regular intervals within a sample sequence as a quality control. Metabolites were measured with a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer coupled to a Dionex Ultimate 3000 UHPLC or with Vanquish Horizon UHPLC coupled to an Orbitrap Exploris 240 mass spectrometer (both Thermo Fisher Scientific) through a heated electrospray ionization source. For the Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap Mass spectrometer (HRMS) coupled to a Dionex Ultimate 3000 UHPLC, the mass spectrometer was operated in full-scan, polarity-switching mode, with the spray voltage set to +4.5 kV/−3.5 kV, the heated capillary held at 280 °C and the heated electrospray ionization probe held...
at 320 °C. The sheath gas flow was set to 40 units, the auxiliary gas flow was set to 15 units and the sweep gas flow was set to 0 unit. HRMS data acquisition was performed in a range of m/z = 70–900, with the resolution set at 70,000, the AGC target at 1 × 10^6 and the maximum injection time (max IT) at 120 ms. Metabolite identities were confirmed using two parameters: (1) precursor ion m/z was matched within 5 ppm of theoretical mass predicted by the chemical formula; (2) the retention time of metabolites was within 5% of the retention time of a purified standard run with the same chromatographic method. Chromatogram review and peak area integration were performed using the Thermo Fisher software XCalibur Qual Browser, XCalibur Quan Browser software and Tracefinder S.0. The peak area for each detected metabolite was normalized against the total ion count of that sample to correct any variations introduced from sample handling through instrument analysis. Absolute quantification of 2SC was performed by interpolation of the corresponding standard curve obtained from serial dilutions of commercially available standards (Sigma Aldrich) running with the same batch of samples.

For the Orbitrap Exploris 240 mass spectrometer, MS1 scans, the mass range was set to m/z = 70–900, AGC target set to standard and maximum injection time (IT) set to auto. Data acquisition for experimental samples used full scan mode with polarity switching at an Orbitrap resolution of 120,000. Data acquisition for untargeted metabolite identification was performed using the AcquireX Deep Scan workflow, an iterative data-dependent acquisition strategy using multiple injections of the pooled sample. In brief, the sample was first injected in full scan-only mode in single polarity to create an automated inclusion list. MS2 acquisition was then carried out in triplicate, whereby ions on the inclusion list were prioritized for fragmentation in each run, after which both the exclusion and inclusion lists were updated in a manner such that fragmented ions from the inclusion list were moved to the exclusion list for the next run. Data-dependent acquisition full-scan ddMS2 method for AcquireX workflow used the following parameters: full scan resolution was set to 60,000, fragmentation resolution to 30,000 and fragmentation intensity threshold set to 5.0 × 10^6. Dynamic exclusion was enabled after 1 time and exclusion duration was 10 s. Mass tolerance was set to 5 ppm. The isolation window was set to 1.2 m/z. Normalized HCD collision energies were set to stepped mode with values at 30, 50 and 150. Fragmentation scan range was set to auto, AGC target at standard and max IT at auto. XCalibur AcquireX method modification was on. Mild trapping was enabled.

Metabolite identification was performed using the Compound Discoverer software (v3.2, Thermo Fisher Scientific). Metabolites were annotated at the MS2 level using both an in-house mzVault spectral database curated from 1,051 authentic compound standards and the online spectral library mzCloud. The precursor mass tolerance was set to 5 ppm and fragment mass tolerance set to 10 ppm. Only metabolites with mzVault or mzCloud best match score above 50% and 75%, respectively, and retention time tolerance within 0.5 min to that of a purified standard run with the same chromatographic method were exported to generate a list including compound names, molecular formula and retention time. The curated list was then used for further processing in the Tracefinder software (v5.0, Thermo Fisher Scientific), in which extracted ion chromatograms for all compounds were examined and manually integrated if necessary. False positive, noise or chromatographically unresolved compounds were removed. The peak area for each detected metabolite was then normalized against the total ion count of that sample to correct any variations introduced from sample handling through instrument analysis. The normalized areas were used as variables for further statistical data analysis. Statistical analysis was performed using MetaboAnalyst (v5.0)64.

Stable isotope-assisted tracing. BMDMs (3 independent mice) were plated at 0.5 × 10^5 cells per well in 12-well plates in technical triplicate per condition and treated as indicated in glutamine-free DMEM supplemented with U-13C-glutamine or U-15N-glutamine, respectively. For 13C and 15N-tracing analysis, the theoretical masses of 13C and 15N isotopes were calculated and added to a library of predicted isotopes in Tracefinder S.0. These masses were then searched with a 5 ppm tolerance and integrated only if the peak apex showed less than 1% deviation in retention time from the [U-13C or 15N] monoisotopic mass in the same chromatogram. The raw data obtained for each isotopologue were corrected for natural isotope abundances using the AccuCor algorithm (https://github.com/lparsons/accucor) before further statistical analysis.

Ethidium bromide treatment
BMDMs were plated in the presence or absence of ultrapure ethidium bromide (100 ng ml^-1) and incubated for a further 6 days before treatment. Depletion of mtDNA was determined by genomic DNA isolation followed by qPCR using primers specific for areas of mitochondrial DNA (D-loop) and areas of mtDNA that are not inserted into nuclear DNA (non-NUMT).

c-Fos activity assay
BMDMs from 3 mice were plated in 10 cm dishes at 0.5 × 10^5 cells per ml and left overnight. Cells were pre-treated with FHN1 or DMF (3 h) before LPS stimulation (4 h). After collection, nuclear extracts were isolated using a Nuclear Extraction kit (ab113474) purchased from Abcam. Nuclear extracts were quantified using a BCA assay and standardized. c-Fos relative activity was then quantified using the AP-1 transcription factor assay purchased from Abcam (Ab207196) according to the manufacturer's protocol.

Fumarate assay
Analysis of fumarate levels were assessed using a fumarate colorimetric assay kit (Sigma MAK060) that uses an enzyme assay, which results in a colorimetric (450 nm) product proportional to the fumarate present, as per the manufacturer's instructions.

Nitrite measurement
The Griess reagent system (Promega G2930) was used according to the manufacturer's instructions.

RNA-seq
BMDMs (three independent mice) were treated as indicated and RNA was extracted as detailed above. mRNA was extracted from total RNA using poly-T-oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers, followed by the second strand cDNA synthesis. The library was checked using Qubit and real-time PCR for quantification and a bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on a NovaSeq 6000 S4 (Illumina). Differential expression analysis of two conditions per group was performed using counted reads and the DESeq2R package65. Pathway enrichment analyses were performed as indicated in quantification and statistical analysis section below.

Proteomic analysis
Sample preparation. BMDMs (from five independent mice) were plated onto 10 cm dishes and treated as indicated. At the experimental end point, cells were washed with PBS on ice and centrifuged at 1,500 r.p.m. for 5 min at 4 °C and frozen at –80 °C. Cell pellets were lysed, reduced and alkylated in 50 µl of 6 M Gu-HCl, 200 mM Tris-HCl pH 8.5, 10 mM TCEP, 15 mM chloroacetamide by probe sonication and heating to 95 °C for 5 min. Protein concentration was measured using a Bradford assay and initially digested with LysC (Wako) with an enzyme/substrate ratio of 1:200 for 4 h at 37 °C. Subsequently, the samples were diluted tenfold with water and digested with porcine trypsin (Promega) at 37 °C overnight. Samples were acidified to 1% TFA, cleared by centrifugation.
Mass spectrometry analysis. The tryptic peptides were analysed using a Fusion Lumos mass spectrometer connected to an Ultimate Ultra3000 chromatography system (both Thermo Scientific) incorporating an autosampler. In brief, 2 μg of de-salted peptides were loaded onto a 50 cm emitter packed with 1.9 μm ReproSil-Pur 200 C18-AQ (Dr Maisch) using a RSLC-nano uHPLC system connected to a Fusion Lumos mass spectrometer (both Thermo). Peptides were separated using a 140 min linear gradient from 5% to 30% acetonitrile, 0.5% acetic acid. The mass spectrometer was operated in DIA mode, acquiring a MS 350–1,650 Da at 120 k resolution followed by MS/MS on 45 windows with 0.5 Da overlap (200–2,000 Da) at 30 k with a NCE setting of 27.

Data analysis. Raw files were analysed and quantified by searching against the UniProt Mus musculus database using DIA-NN 1.8 (https://github.com/vdemichev/DiaNN). Library-free search was selected, and the precursor ion spectra were generated from the FASTA file using the deep-learning option. Default settings were used throughout apart from using ‘Robust LC’ (high precision)\(^1\). In brief, carbamidomethylation was specified as the fixed modification whereas acetylation of protein amino termini was specified as the variable. Peptide length was set to a minimum of 7 amino acids, precursor false discovery rate (FDR) was set to 1%. Subsequently, missing values were replaced by a normal distribution (1.8 it shifted with a distribution of 0.3 n) to allow the following statistical analysis. Protein-wise linear models combined with empirical Bayes statistics were used for the differential expression analyses. We use the Bioco conductor package limma to carry out the analysis using the information provided in the experimental design table.

Digitonin fractionation

BMDMs were plated at 0.5 × 10^6 cells per well and treated as desired. After treatment, cells were washed once with room temperature PBS before being scraped on ice into ice-cold PBS and pelleted at 500g for 5 min at 4 °C. Supernatant was removed and discarded, and the pellet was resuspended in 400 μl extraction buffer (150 mM NaCl, 50 mM HEPES pH 7.4, and 25 μg ml⁻¹ digitonin). Samples were then placed in a rotating mixer at 4 °C for 10 min before centrifugation at 2,000g at 4 °C for 5 min. The resulting supernatant constituted the cytosolic fraction, from which RNA and DNA were isolated using an AllPrep DNA/RNA Mini kit (Qiagen). Alternatively, the cytosolic fraction was concentrated using Strataclean resin (Agilent) and analysed by western blotting. The pellet constituted a fraction containing membrane-bound organelles, which was lysed in RNA lysis buffer for RNA isolation or lysed in western blot lysis buffer for analysis by western blotting. To determine the presence of mtRNA and mtDNA in the cytosol, qPCR was performed using primers specific for mitochondrial DNA on cDNA, which had been reverse-transcribed from RNA isolated from the cytosolic fraction (mtRNA) and on DNA isolated from the cytosolic fraction (mtDNA). In both cases, values were normalized using a housekeeping control gene (Actb, which encodes β-actin) amplified in cDNA, which had been reverse-transcribed from RNA isolated from the membrane-bound fraction.

MAVS oligomerization

BMDMs were plated at 1 × 10^5 cells per well in technical triplicate and treated as desired. After treatment, cells were washed twice with 200 μl cold PBS before being lysed in crosslinking lysis buffer (50 mM HEPES, 0.5% Triton X-100 and 1× protease inhibitor cocktail). Samples were placed on ice for 15 min. Lysates were centrifuged for 15 min at 6,000g at 4 °C and the supernatant was removed and frozen down as the soluble fraction. Next, 20 μl of the soluble fraction was mixed with 5 μl of sample lysis buffer (0.125 M Tris pH 6.8, 10% glycerol, 0.02% SDS and 5% DTT) and run on a 10% gel. The insoluble pellet was resuspended in HEPES (50 mM) and washed 3 times by centrifuging at 6,000g at 4 °C and removing the supernatant each time. After the final wash, the pellet was resuspended in 500 μl crosslinking buffer (50 mM HEPES and 150 mM NaCl) and disuccinimidyl suberate (Thermo Fisher, made up in anhydrous DMSO) was added to the final concentration of 2 mM. Immediately following the addition of disuccinimidyl suberate, the sample was inverted several times and incubated for 45 min at 37 °C. The sample was then centrifuged for 15 min at 6,000g at 4 °C. After the supernatant was removed and the pellet was resuspended in 30 μl sample lysis buffer. The resuspended insoluble fraction was subsequently boiled for 5 mins at 95 °C before being run on a gel.

Seahorse XF glycolysis stress test

Cells were plated at 100,000 cells per well in 100 μl and were left overnight to adhere. The protocol was carried out according to the manufacturer’s instructions (Agilent). In brief, cells were treated as required, after which the medium was replaced with Seahorse medium containing glutamine (2 mM). Cells were then placed in a CO₂-free incubator for 1 h. The glycolysis stress test was subsequently performed using a Seahorse XF e96 analyzer (Agilent) with the following injections: glucose (10 mM); oligomycin (1 μM); and 2-DG (50 mM).

Analysis was performed using Seahorse Wave software (Agilent). Data shown are representative experiments containing at least three pooled biological replicates.

Seahorse XF mito stress test

Cells were plated at 100,000 cells per well in 100 μl and were left overnight to adhere. The protocol was carried out according to the manufacturer’s instructions (Agilent). In brief, cells were treated as required, after which the medium was replaced with Seahorse medium containing glutamine (2 mM), glucose (10 mM) and pyruvate (1 mM). Cells were then placed in a CO₂-free incubator for 1 h. The mito stress test was subsequently performed using a Seahorse XF e96 analyzer (Agilent) with the following injections: oligomycin (1 μM); FCCP (1 μM); and rotenone (500 nM).

Analysis was performed using Seahorse Wave software (Agilent). Data shown are representative experiments containing at least three pooled biological replicates.

LPS-induced inflammation model

Male mice (6 weeks old) were used, and littermates were randomly assigned to experimental groups. Compounds were resuspended in 10% DMSO followed by 90% cycloedrin in PBS (20% v/v). Mice were intraperitoneally injected with vehicle, FHIN1 or DMF (both 50 mg kg⁻¹) at a volume of 200 μl per injection. After 1 h, mice were intraperitoneally injected with PBS or LPS from E. coli (2.5 mg kg⁻¹, Sigma) at a volume of 100 μl per injection. After 2 h, mice were euthanized and blood was collected retro-orbitally. Blood was allowed to clot for 30 min at room temperature before it was centrifuged at 5,000g for 10 min at 4 °C. The serum was removed and the IFNβ concentration was determined by ELISA.

Western blotting

Supernatant was removed from cells following stimulation, and lysates were collected in 30–50 μl lysis buffer (0.125 M Tris pH 6.8, 10% glycerol, 0.02% SDS and 5% DTT) lysates were subsequently heated to 95 °C for 5 min to denature proteins. SDS–PAGE was used to resolve proteins by molecular weight. Samples were boiled at 95 °C for 5 min before loading into a 5% stacking gel. The percentage resolving gel depended on the molecular weight of the given protein. The Bio-Rad gel running system was used to resolve proteins, and the Bio-Rad wet transfer system was used for the electrophoretic transfer of proteins.
onto a PVDF membrane. Following transfer, the membrane was incubated in milk powder (5% in TBST) for 1 h and subsequently incubated in primary antibody rolling overnight at 4 °C. Primary antibodies targeting phospho-proteins were diluted in BSA (5% in TBST) as opposed to milk. The membrane was incubated for 1 h with secondary antibody (diluted in 5% milk powder) at room temperature. Before visualization, the membrane was immersed in WesternBright ECL Spray (Advanstar). Protein visualization was performed using a ChemiDoc MPTM imaging system (Bio-Rad), and both chemiluminescent and white light images were taken. Images were analysed using Image Lab 6.0.1 (Bio-Rad).

ELISA. DuoSet ELISA kits for IL-1β, TNF, IL-6, IL-10 and GDF15 were purchased from R&D Systems and were carried out according to the manufacturer’s instructions with appropriately diluted cell supernatants added to each plate in duplicate or triplicate. IFNβ was determined using a DuoSet ELISA kit from R&D Systems or Abcam (ab252363). Quantikine ELISA kit for IFNβ (R&D Systems) was used for determination of IFNβ concentrations in serum samples and from human cells, and these were also carried out according to the manufacturer’s instructions. Absorbance at 450 nm was quantified using a FLUOstar Optima plate reader. Corrected absorbance values were calculated by subtracting the background absorbance, and cytokine concentrations were subsequently obtained by extrapolation from a standard curve plotted using GraphPad Prism 9.2.0.

Quantification and statistical analyses
Details of all statistical analyses performed are provided in the figure legends. Data are expressed as the mean ± s.e.m. unless stated otherwise. Representative western blots are shown. For metabolomics data, MetaboAnalyst (v.5.0) was used to analyse, perform statistics and visualize the results. Autoscaling of features (metabolites) was used for heatmap generation. One-way analysis of variance (ANOVA) was performed for multiple comparisons using the Tukey statistical test was used, and a P-adjusted < 0.05 was set as the cut-off. For proteomics data, protein signal intensity was converted to a log2 scale, and biological replicates were grouped by experimental condition. Protein-wise linear models combined with empirical Bayes statistics were used for the differential expression analyses. The Bioconductor package limma was used to carry out the analysis using a R-based online tool. Data were visualized using a heatmap with autoscaled features (genes) and a volcano plot, which shows the log2 (fold change) on the x axis and the −log10 (adjusted P value) on the y axis. The proteomics cut-off values for analysis was log2 (fold change) of 0.5 and a FDR < 0.05, which were determined using t-statistics. RNA-seq cut-off values were set to log2 (fold change) of 1 and FDR < 0.05. Overrepresentation analysis of significant changes were assessed using Enrichr and the Bioconductor package clusterProfiler 4.0 in R (v.3.6.1). Further information on this visualization method is available. E-maps were generated using the enrichplot package in R (v.3.6.1). GSEA analysis of RNA-seq was performed using the Broad Institutes GSEA (v.4.1.0). Graphpad Prism v.9.2.0 was used to calculate statistics in bar plots using appropriate statistical tests depending on the data, including one-way ANOVA, two-tailed unpaired t-test and multiple t-tests. Adjusted P values were assessed using appropriate correction methods, such as Tukey, Kruskal–Wallis and Holm–Sidak tests. Sample sizes were determined on the basis of previous experiments using similar methodologies. All depicted data points are biological replicates taken from distinct samples unless stated otherwise. Each figure consists of a minimum of three independent experiments from multiple biological replicates unless stated otherwise. For in vivo studies, mice were randomly assigned to treatment groups. For metabolomics, proteomics and RNA-seq analyses, samples were processed in random order and experimenters were blinded to experimental conditions.

Data availability
Proteomics data from Fig. 1d were previously deposited to the ProteomeXchange Consortium through the PRIDE partner repository with the dataset identifier PXD029155. All other proteomics, RNA-seq data and metabolomics data have been deposited to Dryad (https://doi.org/10.3061/dryad.6wppzn28). All other data are available from the corresponding authors upon request. Source data are provided with this paper.

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Competing interests The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41586-023-05720-6. Correspondence and requests for materials should be addressed to Alexander Hoffman, Dylan G. Ryan or Luke A. J. O’Neill. Peer review information Nature thanks Navdeep Chandel and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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Extended Data Fig. 1 | LPS stimulation drives fumarate accumulation and protein succination. a–c, Fumarate-mediated protein succination with LPS (n = 3) and 2SC abundance in NS and LPS-stimulated BMDMs (n = 5; LPS 4 h). d, Heatmap of metabolites linked to aspartate-argininosuccinate shunt in NS and LPS-stimulated BMDMs (n = 5; LPS 24 h). e, Metabolite abundance of aspartate-argininosuccinate shunt metabolites in LPS-stimulated BMDMs pre-treated with DMSO or AOAA (n = 3; LPS 4 h; aspartate (P = 0.00000005)). f, Asl expression with silencing of Asl following LPS stimulation (n = 3; LPS 24 h). g, Fumarate levels with silencing of Asl following LPS stimulation (n = 3; LPS 24 h). c–g, Data are mean ± s.e.m. a, 1 representative blot of 3 shown. n = biological replicates. P values calculated using two-tailed Student’s t-test for paired comparisons or one-way ANOVA for multiple comparisons.
Extended Data Fig. 2 | LPS stimulation drives fumarate accumulation via glutamine anaplerosis and an aspartate-argininosuccinate shunt.

**a**, Schematic diagram indicating U-13C-glutamine tracing into distinct metabolic modules. **b**, U-13C-glutamine tracing into glutamate, α-KG and succinate in LPS-treated BMDMs (m+4 and m+5 labelling intensity and total isotopologue fraction distribution) (n = 3; LPS 4 h). **c**, U-13C-glutamine tracing into γ-glutamylcysteine, GSH and GSSG in LPS-treated BMDMs (m+5 labelling intensity and total isotopologue fraction distribution) (n = 3; LPS 4 h). **d**, U-13C-glutamine tracing into aspartate, argininosuccinate, fumarate and malate in LPS-treated BMDMs (m+4 labelling intensity and total isotopologue fraction distribution) (n = 3; LPS 4 h). Data are mean ± s.e.m. n = biological replicates. *P* values calculated using two-tailed Student’s *t*-test for paired comparisons.
Extended Data Fig. 3 | LPS stimulation drives fumarate accumulation via glutamine anaplerosis and an aspartate-argininosuccinate shunt.

a, Schematic diagram indicating $^{15}$N$_2$-glutamine tracing into distinct metabolic modules. b, $^{15}$N$_2$-glutamine tracing into glutamate and asparagine in LPS-treated BMDMs (m+1 and m+2 labelling intensity and total isotopologue fraction distribution) ($n$/uni2009 = 3; LPS 4 h).

c, $^{15}$N$_2$-glutamine tracing into GSH and GSSG in LPS-treated BMDMs (m+1 and m+2 labelling intensity and total isotopologue fraction distribution) ($n$/uni2009 = 3; LPS 4 h).

d, $^{15}$N$_2$-glutamine tracing into aspartate, arginine and citrulline in LPS-treated BMDMs (m+1 labelling intensity and total isotopologue fraction distribution) ($n$/uni2009 = 3; LPS 4 h; aspartate ($P$ = 0.000001)).

Data are mean ± s.e.m. $n$/uni2009 = biological replicates. $P$-values calculated using one-way ANOVA for multiple comparisons.
Extended Data Fig. 4 | Increase in aspartate-argininosuccinate shunt metabolites in cytosol and Irg1–/– macrophages. Heatmap (min-max) of metabolites linked to mitochondrial bioenergetics and redox signalling (a) and the aspartate-argininosuccinate shunt (b) in NS and BMDMs (n = 3; LPS 24 h). c, Metabolite abundance of TCA cycle and aspartate-argininosuccinate shunt metabolites in WT and Irg1–/– BMDMs (n = 3; LPS 24 h); itaconate (P = 0.00000000000002), succinate (P = 0.00000003), fumarate (P = 0.000018). d, Nitrite levels in WT and Irg1–/– BMDMs (n = 3; LPS 24 h). e, Schematic of metabolic changes occurring during mid-phase TCA cycle rewiring in WT and Irg1–/– BMDMs. Data are mean ± s.e.m. n = biological replicates. P values calculated using two-tailed Student’s t-test for paired comparisons or one-way ANOVA for multiple comparisons. Schematic in panel e was created using BioRender (https://biorender.com).
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | FH deletion increases bioenergetic stress, fumarate, and mitochondrial membrane potential. a, Bioenergetic ratios in BMDMs treated with DMSO or FHIN1 (n = 3). b, Fumarate and 2SC levels in BMDMs treated with DMSO or FHIN1 (n = 3). qPCR (n = 5) (c) and western blot (n = 2) (d) analysis of Fh1 expression in Fh1+/+ and Fh1−/− BMDMs (EtOH/TAM 72 h; LPS 4 h; Fh1+/+ NS vs Fh1+/+ LPS (P = 0.000000000002), Fh1+/+ NS vs Fh1+/+ LPS (P = 0.000000000001)). e, Bioenergetic ratios in Fh1+/+ and Fh1−/− BMDMs (n = 3; EtOH/TAM 48 h). f, Heatmap of top 50 significantly abundant metabolites in Fh1+/+ and Fh1−/− BMDMs (n = 3; LPS 4 h). g, Fumarate and 2SC levels in Fh1+/+ and Fh1−/− BMDMs (n = 3; EtOH/TAM 72 h). h, Glycolysis as measured by ECAR in BMDMs pre-treated with DMSO, FHIN1 or DMF (n = 8 (DMSO/FHIN1); n = 6 (DMF); LPS 4 h). n = technical replicates from 1 experiment performed with 3 pooled biological replicates. Data are mean ± s.d. i, Glyceraldehyde 3-phosphate (G3P) and 2,3-phosphoglycerate (2/3-PG) levels and ratio in BMDMs pre-treated with DMSO or FHIN1 (n = 3; LPS 4 h; G3P (P = 0.000004)). Immunofluorescence (j) and quantification (k) of Mitotracker red staining in BMDMs pre-treated with DMSO or FHIN1 (n = 8 (DMSO); n = 19 (FHIN1); LPS 4 h). n = technical replicates from representative experiment. Scale bar = 20 µm. Data are mean ± s.s.d. a–g, i Data are mean ± s.e.m. Representative blots or images of 2 (d) or 1 experiment(s) (j) shown. n = biological replicates unless stated otherwise. P values calculated using two-tailed Student’s t-test for paired comparisons or one-way ANOVA for multiple comparisons.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | FH inhibition remodels inflammatory gene expression. 

**a,** Il10 and Tnfa expression in BMDMs pre-treated with DMSO, FHIN1 or DMF ($n = 5$ (Il10); $n = 6$ (Tnfa); 1PS 4 h; FHIN1/Il10 $= 0.000001$, DMF/Il10 $= 0.0000004$). **b,** Il1b expression and IL-6 release in BMDMs pre-treated with DMSO, FHIN1 or DMF ($n = 6$; 4 h LPS; DMF/Il1b ($P = 0.000046$), DMF/IL-6 ($P = 0.00000002$)). 

**c,** Enrichment map plot of shared significantly increased genes in BMDMs pre-treated with DMF or FHIN1 compared to DMSO control ($n = 3$; LPS 4 h). **d,** Western blot of total and phospho-AKT, JNK, ERK and p38 levels in BMDMs pre-treated with DMSO, FHIN1 or DMF ($n = 2$). **e,** Jun expression in RNA seq from BMDMs pre-treated with DMF or FHIN1 compared to DMSO control ($n = 3$; LPS 4 h). **f,** Fos expression in RNA seq from BMDMs pre-treated with DMF or FHIN1 compared to DMSO control ($n = 3$; LPS 4 h). **g,** Western blot of total and phospho-STAT3 levels in BMDMs pre-treated with anti-CD210 antibody (1 h) ($n = 4$; LPS 4 h). **h,** FH protein and gene expression levels in Fh1+/− and Fh1−/− BMDMs ($n = 2$; EtOH/TAM 72 h). 

Data are mean ± s.e.m. 1 representative blot of 2 ($d, h$) or 4 ($g$) shown. 

$n =$ biological replicates. $P$ values calculated using two-tailed Student’s $t$-test for paired comparisons or one-way ANOVA for multiple comparisons. Schematic in panel j was created using BioRender (https://biorender.com).
Extended Data Fig. 7 | FH inhibition triggers the NRF2 and ATF4 stress response and promotes GDF15 release. a, Heatmap of significantly differentially expressed RNA seq data in BMDMs pre-treated with FHIN1 compared to DMSO control (n = 3; LPS 4 h). Volcano plots of proteomics in BMDMs pre-treated with DMSO, FHIN1 (b) or DMF (c) (n = 5; LPS 4 h). d, ELISA of GDF15 in BMDMs pre-treated with DMSO or FHIN1 (n = 3; LPS 4 h). e, Nrf2 expression or ATF4 protein levels after silencing of Nrf2 or Atf4, respectively, in BMDMs pre-treated with DMSO or FHIN1 (n = 6; LPS 4 h). f, Gdf15 expression after silencing of Nrf2 or Atf4 respectively in BMDMs pre-treated with DMSO or FHIN1 (n = 3; LPS 4 h; FHIN1/Nrf2 RNAi (P = 0.000048)). d-f, Data are mean ± s.e.m. e, 1 representative blot of 6 shown. n = biological replicates unless stated otherwise. P values calculated using one-way ANOVA for multiple comparisons.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | IFN-β release following FH inhibition is independent of cGAS-STING. 

**a**, Heatmap (min-max) of significantly differentially expressed RNA seq data in BMDMs pre-treated with DMSO or DMF (n = 3; LPS 4 h).

**b**, Phospho-STAT1, STAT1, phospho-JAK1 and JAK1 levels in BMDMs pre-treated with DMSO, FHIN1 or DMF (n = 3; LPS 4 h).

**c**, Ifnb1 expression after silencing of Nrf2 in BMDMs pre-treated with DMSO, FHIN1 or DMF (n = 3, LPS 4 h; FHIN1 (P = 0.0000008), DMF (P = 0.0000012)).

**d**, Nrf2 expression after silencing of Nrf2 in BMDMs pre-treated with DMSO, FHIN1 or DMF (n = 3, LPS 4 h; FHIN1 (P = 0.0000008), DMF (P = 0.0000012)).

**e**, Ifnb1 expression in BMDMs pre-treated with DMSO or FHIN1 in the presence of NAC (n = 3; LPS 4 h).

**f**, TRAF3 levels in BMDMs pre-treated with DMSO or FHIN1 (n = 3; LPS 4 h).

**g**, IL-1β levels in BMDMs pre-treated with DMSO, FHIN1 or DMF (n = 3).

**h**, p-p65 levels in BMDMs pre-treated with DMSO, FHIN1 or DMF (n = 3).

**i**, D-loop and Non-NUMT DNA fold expression in ethidium bromide (EtBr)-treated BMDMs (n = 5; D-loop (P = 0.0000000031), Non-NUMT (P = 0.0000000012)).

**j**, Lamin B1 and α-tubulin in cytosolic and membrane-bound organelle fractions following digitonin fractionation (n = 3).

**k**, IFN-β release from 2’,3’ cGAMP- or Cpg-transfected BMDMs pre-treated (1 h) with C-178 or ODN2088 (n = 3; 4 (CpG); 3 h).

**l**, Ifnb1 expression in BMDMs pre-treated with DMSO or FHIN1 in conjunction with C-178 or ODN2088 (1 h) respectively (n = 3; LPS 4 h).

**m**, Cgas, Tmem173 and Tlr9 expression with silencing of Cgas, Tmem173 and Tlr9 respectively in BMDMs pre-treated with DMSO or FHIN1 (n = 3; LPS 4 h).

**n**, IFN-β release with silencing of Cgas, Tmem173 and Tlr9 respectively from BMDMs pre-treated with DMSO or FHIN1 (n = 3; LPS 4 h).

**o**, Tmem173 expression in BMDMs pre-treated with DMSO, FHIN1 or DMF (n = 3; LPS 4 h).

**p**, ND4, ND5 and ND6 RNA levels in whole cell extracts of BMDMs pre-treated with DMSO or FHIN1 in the presence of IMT1 (n = 5; LPS 4 h; ND5 (P = 0.000052)).

**q**, ND4, ND5 and ND6 RNA levels in cytosolic extracts of BMDMs pre-treated with DMSO or FHIN1 in the presence or absence of IMT1 (n = 5; LPS 4 h).

**r**, IFN-β release in BMDMs pre-treated with DMSO or FHIN1 in the presence of IMT1 (n = 3; LPS 4 h).

Data are mean ± s.e.m. **b**, **f**, **h**, **j**, 1 representative blot of 3 shown. n = biological replicates. P values calculated using two-tailed Student’s t-test for paired comparisons or one-way ANOVA for multiple comparisons.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Mitochondrial membrane potential modifiers increase mtRNA and trigger IFN-β release. 

**a**, Tlr7 expression with silencing of Tlr7 in BMDMs pre-treated with DMSO or FHIN1 (n = 3; LPS 4 h). **b**, Ddx58 and Ifih1 expression with silencing of Ddx58 and Ifih1 respectively in BMDMs pre-treated with DMSO or FHIN1 (n = 5; LPS 4 h; DMSO/Ddx58 (P = 0.000000000002), FHIN1/Ddx58 (P = 0.0000000813792), DMSO/Ifih1 (P = 0.00000009), FHIN1/Ifih1 (P = 0.00000014)). **c**, Tlr3 expression and IFN-β release with silencing of Tlr3 in BMDMs pre-treated with DMSO or FHIN1 (n = 3; LPS 4 h; DMSO/Tlr3 (P = 0.000000007), FHIN1/Tlr3 (P = 0.000013487)). **d**, TBK1 and p-TBK1 in BMDMs pre-treated with DMSO or FHIN1 (n = 3; LPS 4 h). **e**, Ifnb1 expression in WT and Mavs–/– BMDMs pre-treated with DMSO or FHIN1 (n = 3; LPS 4 h; DMSO/Ifnb1 (P = 0.000000000002), FHIN1/Ifnb1 (P = 0.000000000002), DMSO/Ifih1 (P = 0.00000009), FHIN1/Ifih1 (P = 0.00000014)). **f**, MFI of TMRM staining in BMDMs pre-treated with DMSO, FHIN1, oligomycin or valinomycin (n = 3, LPS 4 h). **g**, IFN-β release from BMDMs pre-treated with DMSO, FHIN1, oligomycin or valinomycin (n = 4; LPS 4 h; oligomycin (P = 0.00000003)). **h**, MFI of TMRM staining and IFN-β release from BMDMs pre-treated with DMSO or CCCP (n = 4 (TMRM), n = 3 (IFN β)); LPS 4 h; CCCP/IFN-β (P = 0.000000008)). **i**, MFI of TMRM staining in BMDMs pre-treated with DMSO or MMF (n = 3, LPS 4 h). Immunofluorescence (j) and quantification (k) of dsRNA in BMDMs pre-treated with DMSO, FHIN1 or oligomycin or transfected with poly (I:C) (n = 8; LPS 4 h). n = technical replicates from representative experiment. Data are mean ± s.d. Scale bar = 20 µm. **l**, D-loop fold expression in DNA and RNA isolated from cytosolic fractions of digitonin-fractionated BMDMs pre-treated with DMSO or oligomycin (n = 4 for mtDNA, n = 5 for mtRNA). Immunofluorescence (m) and quantification (n) of dsRNA in BMDMs pre-treated with DMSO or valinomycin (n = 9 (DMSO); n = 6 (Valinomycin); LPS 4 h). n = technical replicates from representative experiment. Data are mean ± s.d. Scale bar = 20 µm. **o**, Quantification of dsRNA immunofluorescence in Fh1+/+ and Fh1–/– BMDMs (n = 7 (Fh1+/+ Control); n = 6 (Fh1–/– LPS); n = 12 (Fh1+/+ Control); n = 10 (Fh1–/– LPS); EtOH/TAM 72 h; LPS 4 h). n = technical replicates from representative experiment. Data are mean ± s.d. a–c,e–l Data are mean ± s.e.m. d,j,m,1 representative blot or image of 3 experiments shown. n = biological replicates unless stated otherwise. P values calculated using two-tailed Student’s t-test for paired comparisons, one-way ANOVA for multiple comparisons.
Extended Data Fig. 10 | Prolonged LPS stimulation increases mitochondrial membrane potential and dsRNA. a, MFI of TMRM staining in BMDMs (n = 3). Immunofluorescence (b) and quantification (c) of dsRNA in BMDMs (n = 8 (0/48 h); n = 9 (24 h)). n = technical replicates from representative experiment. Data are mean ± s.d. Scale bar = 20 µm. d, Ddx58 and Ifih1 expression in BMDMs (n = 4; LPS 4 h; Ddx58 (P = 0.0000000010), Ifih1 (P=0.00000012)). e, Fh1 expression in IFN-β-stimulated BMDMs (n = 3). a,d,e, Data are mean ± s.e.m. b, 1 representative image of 3 experiments shown. n = biological replicates unless stated otherwise. P values calculated using two-tailed Student’s t-test for paired comparisons, one-way ANOVA for multiple comparisons.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection

- XCalibur Qual Browser, XCalibur Quan Browser, Tracefinder 5.0, Leica LAS X Life Science Microscope Software

Data analysis

- Metaboanalyst 5.0, DESeq2 R package, Bioconductor Limma (3.16), DIA-NN 1.8, Agilent Seahorse Wave 2 (2.4.3), Enrichr, Bioconductor clusterProfiler 4.0, enrichplot package in R (version 3.6.1), Broad Institutes GSEA 4.1.0, GraphPad Prism 9.2.0, Leica LAS X Life Science Microscope Software Platform, FlowJo V10, Image Lab 8.0.3, ImageJ (1.53r)

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Proteomics data from Fig. 1d were previously deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD0291556. All other proteomics, RNA sequencing data and metabolomics data have been deposited to Dryad (doi:10.5061/dryad.6wwp2gn28). All original gel images are provided in the source data file. All other source data are available from the corresponding author(s) upon request.
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**Life sciences study design**

All studies must disclose these points even when the disclosure is negative.

**Sample size**

We have used at least 1 biological replicate for each individual experiment, which was performed on 3 independent occasions unless otherwise stated. Although we did not use statistical methods to calculate sample size, we decided to use a minimum of 3 biological replicates per experiment to account for biological variability, taking into account the 3 Rs principle and the fact that the majority of experiments were performed in primary murine macrophages from inbred mice. See statistical analyses section of methods for full details.

**Data exclusions**

No data were excluded from in vitro experiments. Data points were excluded from the in vivo experiment (Fig. 4J) on the basis that blood was harvested from the liver of these samples as it could not be harvested retroorbitaly (as was performed with all other samples) due to technical issues.

**Replication**

The in vitro experiments were highly reproducible. Each experiment was repeated on at least 3 independent occasions unless otherwise stated. The in vivo experiment was performed on two separate occasions, both of which were successful and included in the analysis, in order to ensure reproducibility.

**Randomization**

For in vivo studies, mice were randomly assigned to treatment groups. For metabolomics, RNA sequencing and proteomics experiments, samples were processed in random order and experimenters were blinded to experimental conditions. All in vitro treatment groups were randomly assigned.

**Blinding**

In vitro and in vivo experiments were not blinded due to lack of available experimenters with required expertise. For metabolomics, RNA sequencing and proteomics experiments, samples were processed in random order and experimenters were blinded to experimental conditions.

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**Reporting for specific materials, systems and methods**

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

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### Methods

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**Antibodies**

**Antibodies used**

- Anti-mouse Lamin B1 (Abcam 81) (12586); STAT1 [9172], p-STAT1 [9167], JAK1 [3344], p-JAK1 [3331], TBK1 [3504], p-TBK1 [5483]; STAT3 (30835), p-STAT3 (9163), Fli-1 (4672); ASK1 (7072); α-tubulin (2144), MAVS [4093], ATF4 [12815], p-AKT [13038], Akt [2920], p-JNK (9255), JNK (9252), p-ERK1/2, [9101], ERK1/2 [4695], p-p38 [4511], p-p38 [9212], TRAF3 [4729], p-p53 [3033] and GAPDH [2118];

**Antibodies were purchased from Cell Signaling. Anti-2SC antibody was kindly provided by Dr. Norma Frizzell (University of South Carolina, US). Anti-mouse β-actin antibody (AS5316) was purchased from SigmaAldrich. Horseradish peroxidase (HRP)-conjugated anti-mouse (115-035-003), anti-goat (705-035-003) and anti-rabbit (111-035-003) immunoglobulin G (IgG) antibodies were purchased from Jackson Immunoresearch. Anti-mouse CD210 (112710) and anti-mouse IgG (406601) antibodies were purchased from Biolegend. Anti-dsRNA antibody (clone j2) was purchased from Merck (MABR-1134). Alexa Fluor 488 goat-anti-mouse IgG2 antibody (A21121) was purchased from Invitrogen.**

**Validation**

Antibodies were used according to validation listed in manufacturer’s instructions. All details of antibody validation are given in Table S1.
Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals
In vitro experiments were performed with BMDMs isolated from 6-18-week-old female and male mice. In vivo models were performed with 6-week-old male mice and littermates were randomly assigned to experimental groups. All mice were on a C57Bl/6/OlaHsd background unless stated below. Wild-type (WT) mice were bred in-house. Mice were kept in 12 hour light/dark cycles, and the facility was maintained at 20–24°C and 45–65% humidity. The inducible Fh1+/- and Fh1-/- mice were generated on the C57Bl/6 genetic background and their hind legs were generously donated by Dr. Christian Frezza (University of Cambridge, UK). Vehicle (ethanol) treated Fh1+/- and Fh1-/- were used as controls. Upon treatment with 4-hydroxy tamoxifen, Cre-mediated chromatin excision results in the loss of either one (Fh1+/-) or both (Fh1-/-) copies of Fh1, thus generating either heterozygous or null animals. Hind legs from WT and Mavs-/- mice were generously donated by Dr. Cecilia Johansson (Imperial College London, UK). These strains, originally obtained from S. Akira (World Premier International Immunology Frontier Research Center, Osaka University, Osaka, Japan), were Ifnrg6/gp' but since Ifnrg6 expression was not a primary readout the mice are designated as WT and Mavs-/-.

Wild animals
The study did not involve wild animals.

Field-collected samples
The study did not involve field-collected animals.

Ethics oversight
Animals were maintained under specific pathogen-free conditions in line with Irish and European Union regulations. All animal procedures were ethically approved by the Trinity College Dublin Animal Research Ethics Committee prior to experiment and conformed with the Directive 2010/63/EU of the European Parliament.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics
For experiments performed in TCD which involved isolation of human PBMCs and macrophages for in vitro experiments: donors were defined as healthy and no further information was recorded.
For experiments performed in Cedars-Sinai: All SLE patients [as per ACR diagnostic criteria] were recruited from Cedars-Sinai Medical Center, CA, USA. Age- and sex-matched healthy donors who had no history of autoimmune diseases or treatment with immunosuppressive agents were included. There were no significant covariates between the two groups.

Recruitment
For experiments performed in TCD: Blood samples were obtained anonymously and written informed consent for the use of blood for research purposes was obtained from the donors.
For experiments performed in Cedars-Sinai: All participants provided informed written consent and the study received prior approval from the institutional ethics review board (IRB protocol 19627). No selection bias was noted.

Ethics oversight
School of Biochemistry and Immunology Research Ethics Committee (TCD), Cedars-Sinai Medical Center Institutional Ethics Review Board (IRB protocol 19627)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots
Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
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- All plots are contour plots with outliers or pseudocolor plots.
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Methodology

Sample preparation
Murine BMDMs were plated in 12-well plates and treated as desired. CellROX Green (5 μM, Thermo Fisher) or TMRM (20 nM, Thermo Fisher) was added to cells 30 mins prior to end of cell treatments. Cells were washed once in PBS and scraped into 200 μl FACS buffer (2 mM EDTA, 0.5% FCS in PBS). Acquisition of samples was performed on a BD Accuri C6 flow cytometer.

Debris was excluded by FSC vs SSC analysis and 10,000 cells was acquired per condition.

Instrument
BD Accuri C6

Software
FlowJo V10

Cell population abundance
Purity of samples was not determined. Debris was excluded by FSC vs SSC analysis.
Gating strategy

The gating strategy used for all flow cytometry experiments consisted of debris exclusion by FSC-A vs SSC-A analysis and subsequent doublet exclusion by FSC-A vs FSC-H analysis. A sample gating strategy is provided in Supplementary Fig. 2.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.
Dimethyl fumarate and 4-octyl itaconate are anticoagulants that suppress Tissue Factor in macrophages via inhibition of Type I Interferon

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Abstract
Excessive inflammation-associated coagulation is a feature of infectious diseases, occurring in such conditions as bacterial sepsis and COVID-19. It can lead to disseminated intravascular coagulation, one of the leading causes of mortality worldwide. Recently, type I interferon (IFN) signalling has been shown to be required for tissue factor (TF; gene name F3) release from macrophages, a critical initiator of coagulation, providing an important mechanistic link between innate immunity and coagulation. The mechanism of release involves type I IFN-induced caspase-11 which promotes macrophage pyroptosis. We have found that F3 is a type I IFN-stimulated gene. Furthermore, F3 induction by lipopolysaccharide (LPS) is inhibited by the anti-inflammatory agents dimethyl fumarate (DMF) and 4-octyl itaconate (4-OI). Mechanistically, inhibition of F3 by DMF and 4-OI involves suppression of Ifnb1 expression. Additionally, they block type I IFN- and caspase-11-mediated macrophage pyroptosis, and subsequent TF release. Thereby, DMF and 4-OI inhibit TF-dependent thrombin generation. In vivo, DMF and 4-OI suppress TF-dependent thrombin generation, pulmonary thromboinflammation, and lethality induced by LPS, E. coli, and S. aureus, with 4-OI additionally attenuating inflammation-associated coagulation in a model of SARS-CoV-2 infection. Our results identify the clinically approved drug DMF and the pre-clinical tool compound 4-OI as anticoagulants that inhibit TF-mediated coagulopathy via inhibition of the macrophage type I IFN-TF axis.

Key Words
Macrophage, type I interferon, tissue factor, dimethyl fumarate, 4-octyl itaconate, coagulation, thrombin, coagulopathy, LPS, SARS-CoV-2, caspase-11, inflammasome, pyroptosis

Introduction
Inflammation and coagulation are evolutionarily conserved host defence mechanisms that maintain haemostasis by rapidly forming blood clots in response to infection, thereby preventing dissemination of the invading pathogen. Excessive activation of
the coagulation cascade is intrinsically linked to increased activation of innate immune pathways and can lead to thrombosis, a pathological deviation from haemostasis. Thrombosis is an integral part of inflammation, and this interplay between innate immunity and coagulation can lead to disseminated intravascular coagulation (DIC), an infection-induced life-threatening illness characterized by organ dysfunction often accompanied by coagulopathy, and the leading cause of death in intensive care units.

Tissue factor (TF; also known as coagulation factor 3 (gene name F3)) is the primary initiator of trauma-induced coagulation. TF is essential for survival via rapid formation of blood clots. Its deletion is fatal in mice. Under normal conditions, TF is expressed at low, basal levels in complex with coagulation factor VII on the membrane of circulating immune cells, such as macrophages, and cells in the blood vessel wall. This TF:FVIIa complex serves as the key regulator of physiological haemostasis, triggering local coagulation cascade activation which ultimately leads to thrombin generation and blood clot formation. However, under inflammatory conditions such as exposure to lipopolysaccharide (LPS) from gram-negative bacteria, which account for approximately 60% of all sepsis cases, F3 is rapidly induced and its procoagulant activity is increased up to 100-fold. Recently, LPS-mediated type I interferon (IFN) and non-canonical inflammasome signalling, which involves caspase-11 or its human homologue caspase-4, have been shown to be critical for TF release from immune cells via pyroptosis, a proinflammatory, lytic form of cell death. Extracellular LPS induces caspase-11 via type I IFN production. Cytosolic LPS then binds and activates caspase-11, which in turn cleaves gasdermin D (GSDMD), leading to pyroptosis. Procoagulant TF is thereby released on extracellular vesicles through pyroptotic pores in the macrophage cell membrane which can lead to aberrant thrombin generation and pathological thrombosis. In addition to its primary function in blood clotting, excessive thrombin generation can trigger detrimental feed-forward inflammation via activation of protease-activated receptors (PARs), which can synergize with Toll-like receptors (TLRs) to induce proinflammatory cytokines and IFN-β. Thrombin can also cleave and activate the proinflammatory cytokine IL-1α. Collectively, this process is termed thromboinflammation.
The immunomodulatory agents dimethyl fumarate (DMF), which is clinically approved for the treatment of multiple sclerosis and psoriasis, and the related compound 4-octyl itaconate (4-OI), are potently anti-inflammatory. Both are based on endogenous metabolites. DMF is a derivative of the tricarboxylic (TCA) cycle intermediate fumarate, and 4-OI is a derivative of itaconate, which is synthesized in macrophages from the TCA cycle intermediate aconitate via the enzyme ACOD1. 4-OI and itaconate have been shown to have a range of anti-inflammatory effects, including activation of the transcription factor NRF2, and inhibition of NLRP3, glycolysis, TET DNA dioxygenases, and the kinase JAK1. Some of these effects such as NRF2 activation and inhibition of glycolysis, NLRP3, and JAK1 are shared with DMF. Both DMF and 4-OI have also been shown to inhibit IFN-β production.

We have therefore examined whether DMF and 4-OI might block inflammation-associated coagulation via inhibition of type I IFN signalling and the release of TF. We found that F3 is a type I IFN-stimulated gene (ISG), and inhibition of F3 induction by DMF and 4-OI is likely to involve suppression of Ifnb1. DMF and 4-OI also block type I IFN- and caspase-11-driven macrophage pyroptosis, thereby inhibiting release of procoagulant TF, leading to suppression of inflammation-associated coagulation in vivo in response to LPS, E. coli, S. aureus, and SARS-CoV-2. DMF and 4-OI are therefore identified as anticoagulants that inhibit the macrophage type I IFN-TF axis with potential to limit infection-associated coagulopathy.

**Results**

DMF and 4-OI inhibit LPS-mediated F3 induction and TF-dependent thrombin generation in macrophages

Recent evidence has identified macrophages as important contributors to the total pool of TF available for the initiation of blood clotting. Using publicly available data, we first confirmed that macrophages are a major expressor of F3 mRNA (Supplementary Fig. 1), supporting the use of macrophages as an appropriate cell type for this study. Differential gene expression of LPS-stimulated mouse bone marrow-derived macrophages (BMDMs) revealed that DMF suppressed induction of F3 in addition to F7 (Fig. 1a), suggesting that DMF may inhibit TF:FVII(a) complex formation. DMF also inhibited induction of F10 and F2r (PAR1) which indicated that
DMF may be a potent inhibitor of the extrinsic pathway of coagulation as well as downstream thrombin signalling. Furthermore, expression of Casp4/11 and Gsdmd, which are required for non-canonical inflammasome-mediated pyroptosis, were also reduced (Fig. 1a). We therefore confirmed by PCR that DMF and 4-OI downregulated LPS-induced mRNA expression of F3 in both BMDMs and human peripheral blood mononuclear cells (PBMCs) (Fig. 1b, c), in addition to suppressing TF protein levels in BMDMs (Fig. 1d, e). We next assessed the effect of DMF and 4-OI on TF-dependent thrombin generation, a functional readout of TF procoagulant activity. Thrombin generation was assessed using FXII-deficient plasma to examine the effect of DMF and 4-OI on TF-dependent, and therefore FXII-independent, thrombin generation39. After stimulating BMDMs with LPS, DMF and 4-OI both significantly increased TF-dependent thrombin generation lagtime in normal platelet-poor plasma (Fig. 1f-h), indicating functional inhibition of thrombin generation via suppression of F3.

**F3 is a type I IFN-stimulated gene with JAK-STAT-dependency**

To assess if DMF and 4-OI regulate type I IFN in the context of coagulation, we first examined their effect on LPS-induced type I IFN production. Consistent with previous studies29,37, DMF and 4-OI blocked LPS-mediated transcriptional induction of Ifnb1 (Fig. 2a) and subsequent IFN-β release from BMDMs (Fig. 2b). We next hypothesized that F3 is induced directly via type I IFN signalling, and found that LPS-mediated induction of F3 is significantly decreased in BMDMs from Ifnar−/− mice (Fig. 2c), which do not express the type I IFN receptor. Furthermore, IFN-β itself induced F3 after 4 h, which was transient (Fig. 2d), indicating that F3 is an ISG. IFN-β-induced F3 was inhibited by DMF and 4-OI (Fig. 2e).

Both DMF and 4-OI exert their anti-inflammatory effects in part via NRF2 activation29,35, and NRF2 activation has recently been shown to inhibit type I IFN signalling40. We confirmed that DMF and 4-OI drive the NRF2-dependent genes Gclm and Hmox1 (Supplementary Fig. 2a, b). However, knockdown of NRF2 in BMDMs did not alter the suppression of LPS-induced F3 by DMF and 4-OI (Supplementary Fig. 2c). We confirmed the knockdown in Supplementary Fig. 2d, whereby Gclm
expression was decreased in the NRF2-deficient cells. This suggests that the
mechanism of inhibition of F3 by DMF and 4-OI is NRF2-independent.

Type I IFNs act upon their receptor, IFNAR, to induce hundreds of ISGs via the JAK-
STAT signalling pathway\textsuperscript{41}. We next explored this component of the type I IFN
pathway further and found that LPS-induced F3 was blocked by baricitinib (Fig. 2f), a
specific JAK1/2 inhibitor which limits type I IFN signalling. It has recently been shown
that JAK1 is modified and inhibited by both DMF and 4-OI\textsuperscript{34,36}. Knockdown of JAK1
significantly reduced LPS-mediated F3 induction (Fig. 2g; knockdown confirmed in
Supplementary Fig. 3a), further indicating a JAK1-dependency for F3 induction. We
next analyzed transcription factor binding sites in the region of the F3 gene promoter
using the publicly available Interferome database\textsuperscript{42} which predicted STAT-binding
sites in the promoter of the F3 gene (Fig. 2h; coordinates in Supplementary Table 1).
We verified these findings using the ChIP-Atlas enrichment analysis tool\textsuperscript{43} which also
predicted STAT- and IRF-binding sites in the downstream region of the human F3
promoter (Fig. 2i), further suggesting a type I IFN- and STAT-dependency for F3. The
inhibition by DMF and 4-OI of F3 induction in response to LPS would therefore appear
to involve suppression of type I IFN production and signalling via JAK-STATs.

DMF and 4-OI inhibit caspase-11-mediated pyroptosis, suppressing TF release
from pyroptotic macrophages and subsequent thrombin generation

We next examined whether DMF and 4-OI might block release of TF by inhibiting type
I IFN- and caspase-11-mediated pyroptosis. As caspase-11 is an ISG\textsuperscript{44} (as confirmed
in Supplementary Fig. 3b), DMF and 4-OI dose-dependently blocked LPS-induced
Casp11 expression in BMDMs (Fig. 2j; Supplementary Fig. 3c, d). DMF and 4-OI also
inhibited induction of the ISGs Isg15 and Usp18 (Supplementary Fig. 3e, f), indicating
a broad reduction in type I IFN signalling. Detection of cytosolic LPS activates
caspase-11, which in turn cleaves GSDMD to form pyroptotic pores\textsuperscript{20,21}. We therefore
next assessed caspase-11-mediated pyroptosis. BMDMs were primed with LPS and
subsequently transfected with LPS (which delivers LPS to the cytosol to activate
caspase-11\textsuperscript{45}). Pre-treatment, but not post-treatment, of BMDMs (Supplementary Fig.
3g) with DMF and 4-OI dose-dependently blocked pyroptosis, as measured by LDH
release (Figure 2k) and PI staining (Supplementary Fig. 3h), consistent with induction
of Ifnb1 and subsequent Casp11 as being the process being targeted here. Caspase-
11- and pyroptosis-mediated TF release (assessed by release into the supernatant) was also suppressed by DMF and 4-OI (Fig. 2l-n), resulting in decreased TF procoagulant activity and TF-dependent thrombin generation after activation of the non-canonical inflammasome, shown in Fig 2o for DMF, Fig 2p for 4-OI, and lagtime in Fig 2q.

**Therapeutic treatment with DMF and 4-OI suppresses LPS-induced thrombin generation* in vivo, improving survival in mice**

Following our findings that DMF and 4-OI block the induction and release of TF in response to LPS, thereby blocking thrombin generation *in vitro*, we next investigated the effects of DMF and 4-OI *in vivo* in a systemic model of LPS-induced inflammation and coagulopathy. Intraperitoneal LPS injection significantly induced thrombin generation in mice (Fig. 3a-f). Therapeutic administration (2 h after LPS injection) revealed that DMF (Fig. 3a) and 4-OI (Fig. 3b) significantly inhibited thrombin generation *in vivo*, as did baricitinib (Fig. 3c) and the specific anti-TF antibody, 1H1, which blocks TF:FVIIa assembly46 (Fig. 3d). These data are quantified in Figure 3e and 3f. Collectively, this demonstrates the multiple points of TF control as the compounds used target three separate stages of TF activity: F3 induction and suppression of pyroptosis via inhibition of type I IFN (DMF and 4-OI), downstream JAK1 activity (baricitinib), and extrinsic-mediated coagulation via inhibition of TF:FVIIa (1H1).

We next assessed the effects of DMF and 4-OI in a model of LPS-induced lethality. We challenged mice intraperitoneally with a lethal dose of LPS for 24 h, before treating therapeutically with DMF or 4-OI. We also included a mouse group that received heparin as a control anticoagulant and caspase-11 inhibitor47. All DMF- and heparin-treated mice survived a lethal dose of LPS, with 4-OI reducing mortality by 80% compared with PBS-treated mice (Fig. 3g). Clinical scores of mice (assessing weight loss, activity level, eye closure, appearance of fur and posture) highlighted the protective roles of DMF and 4-OI when administered 24 h after LPS compared with PBS-treated mice (Fig. 3h). This is highlighted by the divergence in clinical scores of DMF- and 4-OI-treated mice compared with PBS-treated mice when the treatments were administered 24 h after LPS injection (Fig. 3h). Prophylactic administration of DMF and 4-OI also improved survival, with all DMF- and 4-OI-treated mice surviving
a lethal dose of LPS, as did all Casp11−/− mice (Supplementary Fig. 4a), thus supporting the critical role played by caspase-11 in LPS-induced sepsis and coagulopathy. Clinical scoring of mice further showed the protective effects of DMF and 4-OI (Supplementary Fig. 4b). Taken together, these results indicate that DMF and 4-OI can protect against LPS-induced lethality, with inhibition of type I IFN- and TF-mediated thrombin generation likely to be a critical aspect of their protective effects.

**DMF and 4-OI inhibit TF release and TF-mediated thrombin generation following infection with *E. coli* and *S. aureus***

Having shown an inhibitory effect against LPS, we next turned to infectious agents. *Escherichia coli* (*E. coli*) infection represents a clinically relevant model of TLR4, gram-negative bacterial-mediated coagulation. Consistent with our LPS data, DMF significantly inhibited, with 4-OI non-significantly reducing, *E. coli*-induced thrombin generation *in vivo* (Fig. 3i-k).

We next assessed whether the anticoagulant effects of DMF and 4-OI could extend beyond gram-negative bacterial infection. We thus tested if DMF and 4-OI could inhibit coagulopathy induced by two different strains of the gram-positive bacterium *Staphylococcus aureus* (*S. aureus*), which is driven by F3 induction and TF procoagulant activity48. We first demonstrated inhibition of F3 induction by DMF and 4-OI *in vitro* when BMDMs were infected with *S. aureus* (Fig. 3l). *In vivo*, DMF and 4-OI both reduced thrombin generation following intraperitoneal injection with *S. aureus* (Fig. 3m, n). Consistent with this reduction in TF-dependent thrombin generation, DMF and 4-OI markedly reduced TF deposition in the lungs of intraperitoneally-infected mice (Fig. 3o). DMF and 4-OI also reduced thrombin generation following intravenous injection of *S. aureus* (Supplementary Fig. 5a-c). Histological analysis showed decreased TF expression in the lungs of DMF- and 4-OI-treated mice in this model compared with PBS-treated mice (Supplementary Fig. 5d), demonstrating inhibition of *S. aureus*-driven coagulopathy via different modes of infection. As *S. aureus*-mediated coagulopathy occurs in a caspase-11-independent manner49, this finding highlights the importance of the inhibition by DMF and 4-OI on F3 induction and subsequent TF procoagulant activity in blocking aberrant thrombin generation in bacterial-driven sepsis.
4-OI suppresses lung inflammation with associated coagulopathy in mice after SARS-CoV-2 infection

Having established that DMF and 4-OI potently inhibit type I IFN- and TF-mediated thrombin generation in vivo following bacterial infection, we next assessed the broader anticoagulant effects of these compounds in a model of viral-induced coagulopathy. TF is a key contributor to viral-associated coagulation, so we next tested the effects of DMF and 4-OI on the prothrombotic effect of the viral dsRNA mimetic, poly(I:C). Both DMF and 4-OI inhibited F3 induction in BMDMs in vitro after stimulation with poly(I:C) (Fig. 4a). Consistent with our LPS data, poly(I:C) stimulation also induced Casp11, which was potently inhibited by DMF and 4-OI (Fig. 4b).

Coagulopathy is a hallmark of COVID-19-associated pathology, and dysregulated immunothrombosis is a critical driver of this aberrant state of coagulation. Inflammasome activation (in particular via caspase-11 activation) and TF expression and procoagulant activity have been shown to exacerbate SARS-CoV-2-associated immunothrombosis and coagulopathy. Recent evidence indicates a prothrombotic shift in the phenotype of monocytes following COVID-19 infection. Furthermore, dysregulated type I IFN signalling is a critical mediator of COVID-19-associated pathology. We therefore tested 4-OI in a mouse model of SARS-CoV-2-infection and assessed broader anticoagulant effects. SARS-CoV-2 infection in mice induced extensive lung inflammation and an increase in the presence of inflammatory cells in the parenchyma (Fig. 4c; lower left-hand quadrant). 4-OI treatment reduced lung inflammation and infiltration of inflammatory cells (Fig. 4c; lower right-hand quadrant). Diffuse pulmonary damage and coagulopathy following SARS-CoV-2 infection is characterized by elevated lung von Willebrand Factor (vWF) expression and extensive intraalveolar fibrin deposition. Following SARS-CoV-2 infection, we found a significant elevation of vWF and fibrinogen/fibrin surrounding the parenchyma, blood vessels, and airways in the lungs (Fig. 4d-g; lower left-hand quadrant). Mice that received 4-OI had significantly lower levels of both vWF and fibrinogen/fibrin (Fig. 4d-g; lower right-hand quadrant), which was indistinguishable from mock-infected mice. Increased collagen deposition indicates dysregulated tissue repair in the lungs, and SARS-CoV-2-infected mice also displayed elevated collagen deposition (Fig. 4, i; lower left-hand quadrant), a marker of lung damage induced by
inflammation-associated coagulation\textsuperscript{66}. There was a significant reduction in collagen deposition in the lungs of 4-OI-treated mice (Fig. 4h, i; lower right-hand quadrant).

4-OI treatment also reduced total numbers of neutrophils (Fig. 4j), lymphocytes (Fig. 4k), and leukocytes (Fig. 4l) in the bronchoalveolar lavage fluid (BALF) of SARS-CoV-2-infected mice. This is notable as neutrophils are a significant contributor to SARS-CoV-2-associated inflammation-driven coagulopathy\textsuperscript{67,68}. 4-OI also significantly reduced infectious viral load in the BALF (Fig. 4m) and lung homogenates (Fig. 4n) of infected mice, indicating an anti-viral effect of 4-OI, consistent with previous reports\textsuperscript{69,70}. Whilst SARS-CoV-2-infected mice exhibited significant weight loss, the weight loss of 4-OI-treated mice following SARS-CoV-2 infection was not significantly different compared with mock-infected mice (Supplementary Fig. 6a). Clinical scoring of mice (assessing weight loss, activity level, eye closure, appearance of fur and posture) highlighted the protective role of 4-OI in this model (Fig. 4o). At 6-days post infection, \textit{Ifnb1} expression was significantly attenuated by 4-OI (Fig. 4p), providing further indication that suppressing type I IFN signalling is critical in reducing TF-mediated coagulopathy in COVID-19. Notably, 4-OI significantly increased \textit{Nfe2l2} expression after SARS-CoV-2-infection (Fig. 4q). Although NRF2 knockdown did not directly alter LPS-induced \textit{F3} expression (previously shown in Supplementary Fig. 3c), NRF2 activation by 4-OI may contribute to decreased type I IFN signalling\textsuperscript{40,69} by an as-yet unknown mechanism and therefore decrease coagulopathy in COVID-19. Furthermore, 4-OI limited SARS-CoV-2-induced expression of the proinflammatory genes \textit{Ifng} and \textit{Il1b} (Supplementary Fig. 6a,b), indicating a broad reduction in inflammation upon 4-OI treatment. Finally, \textit{F3} expression was elevated in PBMCs from SARS-CoV-2-infected patients compared with healthy controls (Fig. 4r), in addition to \textit{CASP4} (Fig. 4s), confirming the procoagulant genotype associated with COVID-19.

\textbf{Discussion}

TF mediates haemostasis upon vessel injury, but infection and other proinflammatory stimuli can trigger rapid \textit{F3} induction in macrophages to provoke aberrant coagulation, with inflammation-associated coagulation an underlying driver of sepsis-associated coagulopathies. In particular, it has recently been shown that type I IFN signalling is a critical mediator of coagulation\textsuperscript{17}. Type I IFN induction facilitates increased
expression of caspase-11, which is activated and cleaved upon detection of cytosolic LPS, leading to the formation of pyroptotic pores in macrophages\(^{18-21}\). Procoagulant TF is released from macrophages on extracellular vesicles through these pyroptotic pores, initiating rapid, pathological blood clotting\(^{22-24}\).

Whilst current anticoagulant therapies are effective, they are associated with an increased bleeding risk for patients\(^{27,71}\). This bleeding is significantly enhanced in septic patients and can manifest as life-threatening bleeds such as haemorrhagic stroke\(^{72}\). Thus, developing anticoagulants without an associated bleeding risk is a key goal for the treatment of coagulopathies\(^{73}\). Notably, no specific TF inhibitor has been clinically approved for anticoagulation in humans due to the essential role of TF in maintaining haemostasis and the associated bleeding risk of solely targeting the TF:FVIIa complex. Furthermore, it was recently shown that the anticoagulant heparin does not improve the likelihood of survival-to-hospital-discharge when administered to critically ill COVID-19 patients\(^{74}\), highlighting the urgent need to counteract the innate immune signalling component of coagulopathies and develop broad-spectrum treatments for coagulopathies associated with excessive inflammation.

This recognition of the role of innate immune signalling as a key contributor to pathological thrombosis has led to a recent surge in assessing clinically approved anti-inflammatory therapies as potential anticoagulants\(^{75-78}\). The clinically approved drug DMF and the pre-clinical tool compound 4-OI are potently anti-inflammatory agents and share multiple targets, including NRF2 activation\(^{29,35}\), and inhibition of glycolysis\(^{28,32}\) and NLRP3 inflammasome activation\(^{30,31}\). Furthermore, they also inhibit type I IFN induction\(^{29,37}\) which contributes to aberrant coagulation. Therefore, we hypothesized that DMF and 4-OI might regulate coagulation via modulation of the type I IFN-TF axis in macrophages.

In our study, we have identified DMF and 4-OI as inhibitors of inflammation-associated coagulation and report for the first time their anticoagulant effects via suppression of the type I IFN-TF axis in macrophages (Supplementary Fig. 7), which has been implicated in aberrant coagulation\(^{17}\). Mechanistically, we have found that F3 is an ISG with predicted STAT-binding sites in the F3 gene promoter, and that DMF and 4-OI likely inhibit F3 induction at least in part via suppression of \(Ifnb1\). Additionally, they
block caspase-11-mediated release of TF via suppression of macrophage pyroptosis, again most likely as a consequence of inhibiting type I IFN production with a subsequent decrease in caspase-11 expression. DMF and 4-OI protected against aberrant TF-mediated thrombin generation *in vivo* in models of bacterial- (both gram-negative and gram-positive) and viral-infection and via multiple routes of administration. In particular, our results showing reduced deposition of TF in the lungs with DMF and 4-OI treatment might be particularly relevant in acute respiratory distress syndrome, a leading cause of death in bacterial sepsis due to wholesale destruction of the lung endothelial barrier induced by excessive inflammation and coagulation. 4-OI also reduced deposition of fibrinogen/fibrin in the lungs of mice following SARS-CoV-2 infection. This is likely due to inhibition of excessive TF-dependent thrombin generation, which cleaves fibrinogen into fibrin. The presence of persistent fibrin amyloid microclots has been linked with the long-term debilitating effects of long COVID, and furthermore, elevated type I IFN production has been detected at least 8 months after the onset of COVID-19 infection. This suggests potential utility for 4-OI and inhibitors of the type I IFN-TF axis in long COVID. Furthermore, vWF deposition, which was blocked by 4-OI, has recently been shown to activate proinflammatory signalling in macrophages. This suggests that, in addition to its primary anti-inflammatory and anticoagulant properties, 4-OI might also inhibit downstream amplification of thromboinflammation.

We also found that 4-OI reduces viral titres following SARS-CoV-2 infection. Emerging evidence points to itaconate derivatives including 4-OI as being anti-viral. For example, 4-OI attenuates pathology associated with HSV-1 and- 2, vaccinia, and zika viruses as well as influenza A. It has recently been shown that thrombin can directly cleave the SARS-CoV-2 spike protein, augmenting viral entry into the lungs. This suggests a broader anti-viral effect of therapeutic thrombin inhibition in the treatment of COVID-19, as this will likely suppress pathological type I IFN production (as a result of reduced viral uptake) as well as thromboinflammation. This also might be a critical downstream effect of the inhibition of TF-mediated thrombin generation by 4-OI following SARS-CoV-2 infection and may explain the reduction in viral titres following 4-OI treatment.
Collectively, our work supports recent clinical trials testing anti-inflammatory compounds as potential inhibitors of inflammation-associated coagulation\textsuperscript{75-78}. There is an extensive crosstalk between inflammation and coagulation and therefore the broad reduction in inflammation exerted by DMF and 4-OI may contribute to their anticoagulant properties. However, our study identifies a new, targetable pathway (the type I IFN-TF axis) in thromboinflammation and our work supports redeploying the clinically approved DMF in clinical trials for inflammation-associated coagulopathies, in particular for autoimmune and thromboinflammatory diseases including systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS), which are characterized by excessive type I IFN production and are associated with significantly greater risk of developing thrombotic cardiovascular disease\textsuperscript{84,85}. This is likely due in part to significantly elevated expression of $F3$ mRNA in PBMCs from patients with SLE\textsuperscript{86} and APS\textsuperscript{87,88}, which correlates with risk of vascular disease. Our work also supports the future development of 4-OI-based compounds as a new class of anticoagulants that inhibit inflammation-associated coagulation. For example, DMF and 4-OI-based compounds may have utility in sepsis-associated DIC, which is exacerbated by increased thrombin levels in patients\textsuperscript{72}.

In summary, these anticoagulant effects that we describe add to the well-characterized anti-inflammatory properties of DMF and 4-OI, further supporting their use in infectious diseases where both inflammation and coagulation are key pathologic features.
Methods

Animal details
All mice were on a C57BL/6J OlaHsd background unless stated below. Wild-type (WT) mice were bred in-house. Caspase-11−/− mice on the C57BL/6J background were backcrossed onto the C57BL/6J background for another 8 generations. Heterozygous breeding pairs were used to generate wild-type and Caspase-11−/− littermates, which were used for all experiments described. Ifnar−/− mice were also generated on the C57BL/6J background and age- and sex-matched WT mice were used in all experiments described. Experiments were performed with 6-to-12-week-old male and female mice bred under specific pathogen-free conditions, under license and approval of the local animal research ethics committee (Health Products Regulatory Authority) and European Union regulations. In vivo models were performed with 6-12-week-old C57BL/6J OlaHsd mice and littermates were randomly assigned to experimental groups. All animal procedures were ethically approved by the Trinity College Dublin Animal Research Ethics Committee prior to experimentation, and conformed with the Directive 2010/63/EU of the European Parliament.

Generation of murine BMDMs
6-12-week-old mice were euthanized in a CO₂ chamber and death was confirmed by cervical dislocation. Bone marrow was subsequently harvested from the tibia, femur, and ilium. For monocyte/macrophage isolation, cells were differentiated in DMEM containing L929 supernatant (10%), fetal calf serum (FCS) (10%), and penicillin/streptomycin (1%) for 6 days, after which cells were counted and plated at 0.5 x 10⁶ cells/mL unless otherwise stated.

Isolation of human PBMCs
Human blood samples from healthy donors and SARS-CoV-2-infected patients were collected and processed at the School of Biochemistry and Immunology in TBSI (TCD) or the St James's, Tallaght University Hospital, Trinity Alliance for Research (STTAR) Bioresource, St. James's Hospital, Tallaght University Hospital, and Trinity Translational Medicine Institute (TCD) (for PBMCs isolated from SARS-CoV-2-infected patients). Blood samples were obtained anonymously and written informed consent for the use of blood for research purposes was obtained from the donors. All
the procedures involving experiments on human samples were approved by the School of Biochemistry and Immunology Research Ethics Committee (TCD). The collection of human samples for STTAR was approved by the National Research Ethics Committee. Experiments were conducted according to the TCD guide on good research practice, which follows the guidelines detailed in the National Institutes of Health Belmont Report (1978) and the Declaration of Helsinki. 30 mL whole blood was layered on 20 mL Lymphoprep (StemCell Technologies, Inc.), followed by centrifugation for 20 min at 400 x g with the brake off, after which the upper plasma layer was removed and discarded. The layer of mononuclear cells at the plasma-density gradient medium interface was retained, and 20 mL PBS was added. Cells were centrifuged for 8 min at 300 x g and the resulting supernatant was removed and discarded. The remaining pellet of mononuclear cells was resuspended, counted and plated at 1 x 10⁶ cells/mL in RPMI supplemented with FCS (10%) and penicillin-streptomycin (1%).

Reagents

Dimethyl fumarate (DMF) (242926) and 4-octyl itaconate (4-OI) (SML2338) were dissolved in dimethyl sulfoxide (DMSO) (D8418), all purchased from Sigma. Baricitinib (7222) was purchased from R&D and also dissolved in DMSO. Recombinant mouse IFN-ß was purchased from BioLegend (581304). High molecular weight poly(I:C) (tlrl-pic) was purchased from Invivogen. LPS from E. coli (ALX-581-010-L002) was purchased from Enzo Life Sciences for in vitro experiments. LPS from E. coli (L4524) and heparin (SRE-0027) were purchased from Sigma for in vivo experiments. 1H1 anti-TF antibody was a kind gift from Dr Helen Bettencourt (Genentech, Inc. South San Francisco, CA). Cells were transfected (to activate caspase-11) using FuGENE HD (Promega).

Non-canonical inflammasome assay

Cells were treated with DMF (5-10 μM) and 4-OI (125-250 μM) for 1 h before being primed with LPS (100 ng/mL) for 3 h, after which the medium was replaced and 2 mg LPS was transfected using FuGENE HD (Promega) overnight (16 h) in serum-free media in order to activate the inflammasome by cleaving and activating caspase-11.
Total RNA was isolated using the PureLink RNA Mini Kit (Invitrogen) and quantified using a Nanodrop 2000 UV-visible spectrophotometer. cDNA was prepared using 20–100 ng/μL total RNA by a reverse transcription-polymerase chain reaction (RT-PCR) using a high capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer’s instructions. Real-time quantitative PCR (qPCR) was performed with an ABI 7500 Fast real-time PCR system (Applied Biosystem) on cDNA using SYBR Green (Invitrogen). Data were normalized to murine Rps18 or Hprt and human RPS13 as endogenous controls, and mRNA expression fold change relative to controls was calculated using the $2^{-ΔΔCt}$ method. All fold changes are expressed normalized to the untreated control. The following primers were used:

### Mouse Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casp4_F</td>
<td>GGTGGTGAAAGAGGAGCTTAC</td>
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<tr>
<td>Casp4_R</td>
<td>CCAGGAATGTGCTGTCTGAT</td>
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<tr>
<td>F3_F</td>
<td>ACCCAAACCCACAACTATAC</td>
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<tr>
<td>F3_R</td>
<td>GGTCACATCCTTCAGATCTC</td>
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<tr>
<td>Gclm_F</td>
<td>TGGAGTTCCCAAATCAGCCC</td>
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<tr>
<td>Gclm_R</td>
<td>TGCATGGGACATGGTCATT</td>
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<td>Hmox1_F</td>
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<td>Ifnb1_F</td>
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<tr>
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<td>Isg15_F</td>
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<tr>
<td>Usp18_F</td>
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<tr>
<td>Usp18_R</td>
<td>TCCGTGATCTGGCCTAGTT</td>
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### SARS-CoV-2 Primers

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<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
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<td>Hprt_F</td>
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<td>Human Primers</td>
<td>Primer</td>
</tr>
<tr>
<td>---------------</td>
<td>--------</td>
</tr>
<tr>
<td>Hprt_R</td>
<td>CAACTTGCGCTCATCTTGGCTTT</td>
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<tr>
<td>Ifnb1_F</td>
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<tr>
<td>Ifng_R</td>
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<td>GGACATGGAGCAAGTTTGGC</td>
</tr>
<tr>
<td>Nfe2l2_R</td>
<td>CCAGCGAGGAGATCGATGAG</td>
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</tbody>
</table>

Table 1 – Mouse and human primer sequences for quantitative Real-Time PCR

RNA silencing (siRNA)

Pre-designed mouse-specific silencer select siRNAs for Jak1 (s68537; Ref Seq NM_146145; sense sequence: CACUGAUUGUCCACAAUATT; antisense sequence: AUAAUGUGGACAAUCAGUGGG), Nrf2 (s70522; Ref Seq NM_010902; sense sequence: CAGGAGAGGUAAGAAUAATT; antisense sequence: UUUAUUCUUACCCUCUCUGCG), and negative control (4390843) were ordered from Thermo Fisher. Cells were transfected with 50 nM siRNA using 5 μL lipofectamine RNAiMAX according to manufacturer’s instructions (Thermo Fisher). Cells were transfected in medium without serum and antibiotics which was replaced with complete medium 8 h later. Cells were subsequently left for a further 12 h prior to treatment.

RNA sequencing
BMDMs (3 independent mice) were treated as indicated and RNA was extracted as previously detailed. mRNA was extracted from total RNA using poly-T-oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers, followed by the second strand cDNA synthesis. The library was checked with Qubit and RT-PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on the NovaSeq 6000 S4 (Illumina).

**ELISA**

A DuoSet ELISA kit for IFN-β (R&D) in the supernatant of BMDMs was purchased and carried out according to the manufacturer's instructions with cell supernatants added to each plate in duplicate or triplicate. Absorbance was read at 450 nm and quantified using a FLUOStar Optima plate reader. Corrected absorbance values were calculated by subtracting the background absorbance and cytokine concentrations were subsequently obtained by extrapolation from a standard curve plotted on GraphPad Prism 9.0.

**Western blotting**

Supernatant was removed from cells following stimulation and lysates were harvested in 30-50 μL lysis buffer (0.125 M Tris pH 6.8, 10% glycerol, 0.02% SDS, 5% DTT). Lysates were subsequently heated to 95°C for 5 min to denature proteins. To concentrate supernatants for western blot, 5 μL Strataclean Resin (Agilent) was added to 500 μL of supernatant and vortexed for 1 min. Supernatants were then centrifuged at 210 x g for 2 min at 4°C. Supernatants were removed and discarded, and the remaining pellet was resuspended in 30 μL lysis buffer. SDS-PAGE was used to resolve proteins by molecular weight. Samples were boiled at 95°C for 5 min prior to loading into a 5% stacking gel. The percentage resolving gel (8-12%) depended on the molecular weight of the given protein. The Bio-Rad gel running system was used to resolve proteins and the Bio-Rad wet transfer system was used for the electrophoretic transfer of proteins onto PVDF membrane. Following transfer, the membrane was incubated in blocking solution (5% milk powder or 5% BSA in TBST) for 1 h and subsequently incubated in primary antibody (5% milk powder or 5% BSA in TBST) rolling overnight at 4°C. The membrane was incubated for 1 h with
secondary antibody (diluted in 5% milk powder or 5% BSA in TBST) at room temperature. Prior to visualization, the membrane was immersed in WesternBright ECL Spray (Advansta). Proteins were visualized on a ChemiDoc MPTM Imaging System (Bio-Rad), and both chemiluminescent and white light images were taken. Quantification of western blot images was performed using Image Lab Software (Bio-Rad). Adjusted band volume was calculated for each band and for each experimental condition this was presented as target protein/housekeeping protein. Uncropped and unprocessed blots are available as source data in the Source Data file.

The following antibodies were used for western blotting: mouse-reactive anti-rat CASPASE-11 (14340), anti-rabbit GAPDH (2118), anti-rabbit JAK1 (3344), anti-rabbit α-TUBULIN (2144), and anti-rabbit TF (44861) were purchased from Cell Signaling. Working dilutions of primary antibodies for western blotting were 1:1000. Horseradish peroxidase (HRP)-conjugated anti-rat (112-035-003) and anti-rabbit (111-035-003) IgG antibodies (both 1:2500) were purchased from Jackson Immunoresearch. All antibodies have been validated for mouse reactivity and for western blotting.

**LDH assay**

The CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) was used to quantify lactate dehydrogenase (LDH) release from cells as a measure of pyroptotic cell death in BMDMs following non-canonical inflammasome stimulation. Freshly harvested supernatants were used in this assay. 25 μL of each supernatant was added to 25 μL Cytotox 96 Reagent and incubated in the dark at room temperature for 30 min. 12.5 μL acetic acid was added to stop the reaction, and the absorbance at 492 nm was measured using a FLUOstar optima. 100 μL total lysis solution was added to untreated cells 30 min before harvesting and served as a maximum LDH release control. Medium alone was used to correct for background absorbance.

**Live-cell imaging**

Pyroptosis was visualized by adding propidium iodide (PI) (R37169, Invitrogen) (1 drop/1 x 10^6 cells/mL) at time of transfection and imaged using the IncuCyte S3 Live-Cell Analysis System (Essen BioScience). PI is not permeant to live cells; pyroptotic
dead cells stained red. Images and movies were saved in tiff format, exported, and analyzed in ImageJ (NIH).

**LPS (endotoxin)-induced model of inflammation with associated coagulopathy**

6-week-old female mice were used, and littermates were randomly assigned to experimental groups. Compounds were resuspended in 10% DMSO followed by 90% cyclodextrin/PBS (40% w/v). For therapeutic assessment, mice were challenged with intraperitoneally (i.p.) injected LPS (1 mg/kg) for 2 h before i.p. injection of DMF (50 mg/kg), 4-OI (50 mg/kg), baricitinib (50 mg/kg), 1H1 anti-TF antibody (15 mg/kg), or vehicle (PBS in 40% cyclodextrin) for 4 h. Mice were euthanized in a CO₂ chamber and lungs were harvested and whole blood samples were collected in 3.2% sodium citrate-coated tubes to prevent coagulation. For platelet-poor plasma (PPP) isolation, whole blood was spun at 2000 rpm at 4°C for 15 min, and plasma (the top, liquid layer of the buffy coat) was used fresh. For the survival trial, mice were challenged i.p. with LPS (15 mg/kg) for 24 h before treatment i.p. with DMF (50 mg/kg), 4-OI (50 mg/kg), or vehicle (PBS in 40% cyclodextrin) for 48 h. Heparin (200 IU/kg) was injected subcutaneously 24 h after LPS injection. Mice were assessed every 6 h for 72 h post LPS-challenge, and graded clinically from 0 to 3 (assessing weight loss, activity level, eye closure, and appearance of fur and posture), with 0 representing healthy and 3 reaching humane endpoint. Mice reaching humane endpoint were euthanized in a CO₂ chamber.

**Staphylococcus aureus-systemic infection model with coagulopathy**

For *in vitro* infection with *S. aureus*, BMDMs were treated with DMF (5-10 μM) or 4-OI (125-250 μM) for 1 h before being primed with 5x10⁶ CFU LAC for 3 h prior to harvesting cell lysates. For the community-acquired MRSA strain *S. aureus* USA300-LAC *in vivo*, mice were co-treated via intravenous injection with 5x10⁷ CFU LAC ± DMF (50 mg/kg), 4-OI (50 mg/kg), or PBS in 40% cyclodextrin for 6 h before harvesting as per our LPS (endotoxin)-induced septic shock model. For *S. aureus* PS80-strain, mice were co-treated i.p. with 5x10⁸ CFU PS80 ± DMF (50 mg/kg), 4-OI (50 mg/kg), or PBS in 40% cyclodextrin for 6 h before a second dose of inhibitor and harvesting after 18 h.
**E. coli-induced systemic infection model with coagulopathy**

Mice were co-treated i.p. with $1 \times 10^7$ CFU CFT073 *E. coli* ± DMF (50 mg/kg), 4-OI (50 mg/kg), or PBS in 40% cyclodextrin for 6 h before a second dose of inhibitor and harvesting after 18 h.

**SARS-CoV-2 model of inflammation with associated coagulopathy**

Heterozygous male K18-hACE c57BL/6J mice (strain: 2B6.Cg-Tg(K18-ACE2)2Prlmn/J) were obtained from The Jackson Laboratory. Animals were housed in groups and fed standard chow diets. Mice were administered $10^3$ PFU SARS-CoV-2 (Wuhan isolate; VIC01/2020) via intranasal administration and intranasally treated with 4-OI (10 mg/kg) from 1 day post-infection, with daily treatments continuing until the termination of the experiment at day 6 post-infection. Numbers of total neutrophils, lymphocytes, and leukocytes were assessed in the bronchoalveolar lavage fluid (BALF) and lung homogenates of mice. A plaque assay was utilized to assess viral titres from SARS-CoV-2-infected mice. Collagen deposition was quantified using Sirius Red-stained lung histological sections, with the area surrounding the primary airways (red) enumerated for each mouse. PBMCs were isolated from whole blood for assessment of mRNA expression. Clinical scoring of mice assessed weight loss, activity level, eye closure, and appearance of fur and posture. Mice were graded clinically with increasing severity in 4 categories, from 0 to 3.

**Cell-based thrombin generation assay**

Cells were seeded onto a 96-well plate and left overnight to adhere. Following the described cell stimulations, supernatants were removed and cells washed once with PBS. MP-reagent (Thrombinoscope) was used as a source of phospholipids in 80 μL FXII-deficient plasma (Haemtech). Thrombin generation of cells in situ in the plate was measured by comparing experimental wells against thrombin activity of a calibrator, as recently described$^{39}$. As BMDM TGA was carried out with cells in situ, changes in thrombin generation will most likely be as a result of surface-bound TF rather than TF released from the surface. The reaction was initiated with 20 μL FluCa-kit (0.42 mM fluorometric substrate, 16.67 nM CaCl$_2$). Fluorescence was quantified using Thrombinoscope software on the Fluoroskan for 60 min. Data was analysed on GraphPad Prism 9.0.
**Plasma thrombin generation assay**

Plasma was diluted 1:3 for all experiments described. MP-reagent which contains phospholipids was added to wells to allow coagulation complex formation. Mouse thrombin converts the fluorogenic substrate at a rate which is 20% lower than human thrombin, therefore to correct for this discrepancy, calibrator activity was set to 20% higher in the Thrombinscope settings. The temperature of the Fluoroskan was set to 33°C. 20 µL plasma and 20 µL of MP-reagent were added to wells and the reaction was initiated with 20 µL FluCa-kit (0.42 mM fluorometric substrate, 8.2 nM CaCl₂). Fluorescence was quantified using Thrombinscope software on the Fluoroskan for 60 min. Data was analysed on GraphPad Prism 9.0.

**Histology**

The superior, middle, and inferior lobes of right lungs were rapidly removed from mice, fixed with 4% paraformaldehyde in PBS, and embedded in paraffin using an automatic tissue processor (Leica Microsystems). 3 μM-thick lung sections were deparaffinized in xylene and rehydrated through graded ethanol washes. The antigen retrieval was performed in Tris-EDTA buffer (10 mM Tris pH 9.0, 1 mM EDTA) for 30 min at 95°C followed by incubation with proteinase K for 5 min at room temperature. The lung tissue sections were blocked with 10% BSA in TBS for 1 h at room temperature and then incubated overnight at 4°C with either a rabbit anti-fibrinogen/fibrin (cat. no.: A0080; Dako, Gostrup, Denmark), a rabbit anti-von Willebrand factor (VWF; cat. no.: A0082; Dako), or a rabbit anti-Tissue Factor antibody (Novus Bio; NBP2-15139). Afterwards, the lung tissue sections were extensively washed with TBS and incubated with a secondary antibody labelled with Alexa Fluor™ 555 (Thermo-Fisher Scientific; Waltham, MA) for 1 h at room temperature. Finally, the slides were embedded in Vectashield Mounting Medium with DAPI (Vector Laboratories Inc., Burlingame, CA). Images were taken with a Leica DM 6000 microscope (Leica, Wetzlar, Germany) using a 20x objective and processed using Leica Application Suite Advanced Fluorescence (LAS AF) software. Alternatively, antigen detection was performed using a Zytochem-Plus AP Polymer Kit in accordance with the manufacturer's instructions (Zymed Systems, Berlin, Germany) and the slides were scanned with a Miramax slide scanner (Zeiss, Oberkochen Germany).
Quantification, statistical analysis, and reproducibility

Details of all statistical analyses performed can be found in the figure legends. Data were expressed as mean ± standard error of the mean (SEM) unless stated otherwise. *P*-values were calculated using two-tailed Student’s t-test for pairwise comparison of variables and one-way ANOVA for multiple comparison of variables. A Sidak’s or Tukey’s multiple comparisons test was used as a post-test when performing an ANOVA. A Mantel-Cox test was used for log-rank analysis of the survival Kaplan-Meier curve. A confidence interval of 95% was used for all statistical tests. For statistical significance, exact *P*-values are included with each panel, and for *P*-values < 0.0001, exact *P*-values are included in the figure legends. Sample sizes were determined on the basis of previous experiments using similar methodologies. All depicted data points are biological replicates taken from distinct samples. Each figure consists of a minimum of 3 independent experiments from multiple biological replicates unless stated otherwise in the figure legends. n = the number of animals or the number of independent experiments with primary BMDMs. For *in vivo* studies, age- and sex-matched mice were randomly assigned to treatment groups, unless otherwise stated.

Data availability

RNA sequencing data ([https://doi.org/10.5061/dryad.6wwpzgn28](https://doi.org/10.5061/dryad.6wwpzgn28)) is available via the Dryad Data Platform. Data generated from the Immunological Genome Project (ImmGen) bulk-population RNA-seq database\(^38\), Interferome v2.0\(^42\), and the ChIP-Atlas enrichment analysis tool\(^43\) are publicly available. Source data are provided with this paper in the Supplementary Information.
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Authorship contributions

assisted with and advised on transcription factor promoter analyses. M.W. performed
and advised on histological analysis. R.J.S.P. assisted with experimental design and
provided critical input. Z.Z. designed experiments and oversaw a portion of the
research project. L.A.J.O’N. obtained funding and oversaw the research project.
Correspondence to Luke A.J. O’Neill, laoneill@tcd.ie.

Disclosure of conflicts of interest
The authors declare no competing interests.
**Figure 1** – DMF and 4-OI inhibit LPS-mediated F3 induction and TF-dependent thrombin generation in macrophages

(a) Heatmap from RNA sequencing of selected inflammation-associated coagulation genes in LPS-stimulated mouse macrophages (BMDMs) pre-treated (3 h) with DMF compared to DMSO control prior to LPS stimulation (4 h). (b) BMDMs (n = 3) and (c) PBMCs (n = 4) were pre-treated with DMSO, DMF, or 4-OI (1 h) prior to LPS stimulation (3 h) and harvesting cell lysates. Quantification of F3 mRNA by qRT-PCR in (b) BMDMs (DMSO – 250 µM 4-OI, P = 0.000178) and (c) PBMCs. (d) Western blot and (e) densitometry analysis of TF in BMDM cell lysates pre-treated with DMSO, DMF, or 4-OI (1 h) prior to LPS stimulation (3 h), with GAPDH as loading control. (f-h) BMDMs were pre-treated with DMSO, (f) DMF, or (g) 4-OI (1 h) before LPS priming (3 h). Thrombin generation was measured in BMDMs in situ in plate wells using FXII-deficient plasma. (h) Lagtime represents time-to-clot formation. NS – DMSO, P = 0.000015; DMSO – 5 µM DMF, P = 0.00001; DMSO – 10 µM DMF, P = 0.0000006.

Data from (b-c) are mean ± SEM from 3-4 independent experiments. Blot from (i) is 3 mice from 3 independent experiments. Data from (e-h) are mean ± SD from 3 independent experiments. P values calculated one-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.
Figure 2 – F3 is a type I IFN-stimulated gene with JAK-STAT-dependency, and DMF and 4-OI suppress TF release from macrophages via inhibition of type I IFN- and caspase-11-mediated pyroptosis

(a) BMDMs were pre-treated with DMSO, DMF, or 4-OI (3 h) before LPS priming (16 h) and harvesting cell lysates (n = 6). Ifnb1 mRNA was quantified by qRT-PCR. DMSO – 250 µM 4-OI, P = 0.00004. (b) BMDMs were pre-treated with DMSO, DMF, or 4-OI (3 h) prior to LPS priming (6 h). IFN-β in the supernatant was measured by enzyme-linked immunosorbent assay (ELISA) (n = 3). (c) BMDMs from WT and Ifnar−/− mice (n = 4) were stimulated with LPS (3 h). F3 mRNA was quantified by qRT-PCR. LPS 3 h, WT – KO, P = 0.0000000488. (d) BMDMs were stimulated with recombinant mouse IFN-β for a timecourse as indicated (n = 3). F3 mRNA was quantified by qRT-PCR. (e) BMDMs pre-treated with DMSO, DMF, or 4-OI (1 h) before stimulation with recombinant mouse IFN-β (4 h) and harvesting cell lysates (n = 9). F3 mRNA was quantified by qRT-PCR. DMSO – 4-OI, P = 0.0000018. (f) BMDMs (n = 4) were pre-treated with DMSO, DMF, 4-OI, or baricitinib (1 h) before LPS stimulation (3 h). F3 mRNA was quantified by qRT-PCR. DMSO – DMF, P = 0.0000144; DMSO – 4-OI, P = 0.0000133; DMSO – baricitinib, P = 0.00000134. (g) BMDMs (n = 6) were transfected with Ctrl or Jak1 siRNA and stimulated with LPS (3 h). F3 mRNA was quantified by qRT-PCR. (h) Predicted transcription factor sites in the promoter of the F3 gene via the Interferome database42. Data presented shows the predicted location of transcription factors from the region spanning -1500 bp to +500 bp from the start site of the F3 gene promoter. (i) The location of STAT1- and IRF1-transcription factor sites in the promoter of the human F3 gene, analyzed from enrichment analysis of 6 independent experiments from publicly available data using the ChIP-Atlas43. (j) Representative western blot of CASPASE-11 in BMDM cell lysates pre-treated with DMSO, DMF, or 4-OI (1 h) prior to LPS stimulation (3 h), with α-TUBULIN as loading control. Blot is representative of 3 independent experiments. (k) BMDMs (n = 3) were pre-treated with DMSO, DMF, or 4-OI (1 h) before priming with LPS (3 h) and LPS transfection (16 h). Pyroptosis is represented as percentage cell death measured by LDH release in BMDM supernatants. DMSO – 10 µM DMF, P = 0.0000247; DMSO – 250 µM 4-OI, P = 0.000084. (l) Representative western blot of TF in BMDMs pre-treated with DMSO, DMF, or 4-OI (1 h) prior to LPS priming (3 h) and LPS transfection.
Blots are representative of 3 independent experiments. Densitometry analysis of TF in BMDM (m) supernatants and (n) cell lysates, with GAPDH as loading control. (o-q) BMDMs were pre-treated with DMSO, (o) DMF, or (p) 4-OI (1 h) before priming with LPS (3 h) and LPS transfection (16 h). Thrombin generation was measured in BMDMs in situ in plate wells using FXII-deficient plasma. (q) Lagtime represents time-to-clot formation. NS – DMSO, $P = 0.000017$; DMSO – 5 µM DMF, $P = 0.000044$; DMSO – 10 µM DMF, $P = 0.0000019$; DMSO – 250 µM 4-OI, $P = 0.00007985$. Data from (a-g and k) are mean ± SEM from 3-6 independent experiments. Data from (m-q) are mean ± SD from 3 independent experiments. $P$ values calculated using two-tailed Student’s t-test for paired comparisons or one-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.
LPS intraperitoneal injection murine model

- a, b: Graphs showing changes in a distinct parameter over time for different groups (PBS, LPS + PBS, LPS + DMF, LPS + 4-Oi).
- e, f: Graphs showing peak changes in another parameter for different groups.

LPS intraperitoneal injection lethality murine model

- g: Graph showing probability of survival over time post LPS challenge for different groups (WT, WT (DMF), WT (4-Oi), WT (Heparin), Casp11^−/−).

LPS intraperitoneal injection lethality murine model

- h: Graph showing clinical score over time post LPS challenge for different groups (WT, WT (DMF), WT (4-Oi), WT (Heparin), Casp11^−/−).

E. coli intraperitoneal injection murine model

- i: Graph showing changes in a parameter for different groups (E. coli, E. coli + DMF, E. coli + 4-Oi).

S. aureus intraperitoneal injection murine model

- l: Graph showing peak changes in another parameter for different groups (S. aureus, S. aureus + PBS, S. aureus + DMF, S. aureus + 4-Oi).

- m: Graph showing changes in a distinct parameter over time for different groups (NS, DMF (μM), 4-Oi (μM)).

- o: Images showing fluorescence staining for different conditions (PBS, DMF, 4-Oi).
Figure 3 – Therapeutic treatment with DMF and 4-OI suppresses thrombin generation in vivo induced by LPS, E. coli, and S. aureus, improving survival in mice

(a-f) Mice were intraperitoneally injected with 1 mg/kg LPS (2 h) followed by treatment with PBS, (a) 50 mg/kg DMF, (b) 50 mg/kg 4-OI, (c) 50 mg/kg baricitinib, or (d) 15 mg/kg 1H1 anti-TF antibody (for a further 4 h) via intraperitoneal injection. Citrated plasma was harvested and thrombin generation was assessed (n = 4 per group). Thrombin generation in (a-d) is compared relative to PBS-treated mice +/- LPS challenge. (e) Peak thrombin in mouse citrated plasma treated as in (a-d). PBS – LPS+PBS, P = 0.00000637; LPS+PBS – LPS+DMF, P = 0.0000008; LPS+PBS – LPS+4-OI, P = 0.0000167; LPS+PBS – LPS+1H1, P = 0.000000055. (f) Total thrombin generation (ETP = endogenous thrombin potential) in mouse citrated plasma treated as in (a-d). LPS+PBS – LPS+1H1, P = 0.00000135. (g) Kaplan-Meier survival curve of mice injected intraperitoneally with 15 mg/kg LPS (24 h) followed by treatment with PBS, 50 mg/kg DMF, 50 mg/kg 4-OI, or 200 IU/kg heparin (for a further 48 h) (n = 10 per group). LPS+PBS – treatment groups, P = 0.0000003. (h) Mice from (g) were scored clinically (assessing weight loss, activity level, eye closure, and appearance of fur and posture) every 6 h for the duration of the 72 h experiment. LPS+PBS – LPS+DMF, P = 0.0000001997. Data points indicate individual mice in (g-h). For (g-h) Mantel-Cox survival analysis was performed. (i) Mice were intraperitoneally injected with 1x10^7 CFU E. coli (CFT073) and co-treated with PBS, 50 mg/kg DMF, or 50 mg/kg 4-OI (6 h), followed by supplemental treatment via intraperitoneal injection with PBS, 50 mg/kg DMF, or 50 mg/kg 4-OI (for a further 18 h). Citrated plasma was harvested and thrombin generation was assessed (n = 5 per group). (j) Peak and (k) total thrombin generation in mouse citrated plasma treated as in (i) (n = 5 per group). (l) BMDMs were pre-treated with DMSO, DMF, or 4-OI (1 h) before infection with 5x10^6 CFU S. aureus (USA300-LAC) (3 h) and harvesting cell lysates. F3 mRNA was quantified by qRT-PCR. (m) Mice were intraperitoneally injected with 5x10^6 CFU S. aureus (PS80) and co-treated with PBS, 50 mg/kg DMF, or 50 mg/kg 4-OI (6 h), followed by supplemental treatment via intraperitoneal injection with PBS, 50 mg/kg DMF, or 50 mg/kg 4-OI (for a further 18 h). Citrated plasma was harvested and thrombin generation was assessed (n = 5 per group). (n) Peak thrombin generation in mouse citrated plasma treated as in (m). (o) TF-positive
regions (red) in the lungs of mice treated as in (m). Representative lung tissue sections are shown. Magnification 20×. Scale bar = 10 μm. Data from (a-k and m-n) are mean ± SD. Data from (l) are mean ± SEM from 3 independent *in vitro* experiments. *P* values calculated using two-tailed Student’s t-test for paired comparisons or one-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.
**Figure 4 – 4-OI suppresses lung inflammation with associated coagulopathy after SARS-CoV-2 infection**

(a-b) BMDMs were pre-treated with DMSO, DMF, or 4-OI (1 h) before poly(I:C) stimulation (3 h) and harvesting cell lysates. (a) F3 and (b) Casp4 (caspase-11) mRNA was quantified by qRT-PCR. (c) Representative images of H&E staining examining histological changes in lung structure and inflammatory cell infiltration in the primary airways, parenchyma and vasculature from male K18-hACE2 mice infected with 10³ PFU SARS-CoV-2 (Wuhan isolate; VIC01/2020) or mock infection and intranasally treated with PBS or 4-OI (10 mg/kg) from 1 day post-infection, with daily treatments continuing until the termination of the experiment at day 6 post-infection (n = 5-6 per group). (d) Representative images and (e) quantification of vWF staining in lung sections from mice treated as in (c) (n = 5-6 per group). Mock+PBS – SARS-CoV-2+PBS, P = 0.000000000002; SARS-CoV-2+PBS – SARS-CoV-2+4-OI, P = 0.000000000002. (f) Representative images and (g) quantification of fibrinogen staining in lung sections from mice treated as in (c) (n = 5-6 per group). Mock+PBS – SARS-CoV-2+PBS, P = 0.000016; SARS-CoV-2+PBS – SARS-CoV-2+4-OI, P = 0.000002887. (h) Representative images and (i) quantification of collagen deposition in lung sections from mice treated as in (c). Total numbers of (j) neutrophils, (k) lymphocytes, and (l) leukocytes (Mock+PBS – SARS-CoV-2+PBS, P = 0.000078) in the bronchoalveolar lavage fluid (BALF) of mice treated as in (c). Viral titres in the (m) BALF and in (n) lung homogenates from SARS-CoV-2 infected mice treated as in (c), detected using plaque assays. n = 5-6 per group for (i-n). (o) Clinical scoring of mice (assessed for weight loss, activity level, eye closure, and appearance of fur and posture) treated as in (c). Clinical scoring of mice assigned SARS-CoV-2-infected mice to both categories 2 and 3 (83% to category 2, 17% mice to category 3), while SARS-CoV-2-infected mice that received therapeutic administration of 4-OI were dispersed across no clinical score, category 1, and category 2 groups (33% mice to no clinical score, 50% mice to category 1, and 17% mice to category 2). Quantification of (p) Ifnb1 (Mock+PBS – SARS-CoV-2+PBS, P = 0.000003) and (q) Nfe2l2 mRNA by qRT-PCR in PBMCs of mice treated as in (c) (n = 5-6 per group). Human PBMCs from healthy controls (HC) (n = 5-6) and SARS-CoV-2-infected patients (n = 9-11) were assessed for quantification of (q) F3 and (r) CASP4 mRNA by qRT-PCR. Data from (a-b and r-s) are mean ± SEM from 3 independent in vitro experiments. Data
from (e, g, i-n, and p-q) are mean ± SD. Scale bar for (c, d, f, and h) = 50 μm. P values calculated using two-tailed Student’s t-test for paired comparisons or one-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.
Supplementary Information for:

Dimethyl fumarate and 4-octyl itaconate are anticoagulants that suppress Tissue Factor in macrophages via inhibition of Type I Interferon

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Supplementary Figure 1 – Macrophages are a major expressor of F3 mRNA

F3 mRNA expression analysis by cell type from the Immunological Genome Project (ImmGen) bulk-population RNA-seq database. Source data are provided as a Source Data file.
Supplementary Figure 2 – Inhibition of LPS-induced F3 expression by DMF and 4-OI is independent of NRF2 activation

(a-b) BMDMs were pre-treated with DMSO, DMF, or 4-OI (1 h) before LPS priming (3 h) and harvesting cell lysates. (a) Gclm and (b) Hmox1 mRNA were quantified by qRT-PCR. (c-d) BMDMs were transfected with Ctrl or Nrf2 siRNA, before treatment with DMSO, DMF, or 4-OI (1 h) prior to LPS stimulation (3 h). (c) F3 and (d) Gclm mRNA were quantified by qRT-PCR. (d) Ctrl siRNA DMF – Nrf2 siRNA DMF, P = 0.00000976; Ctrl siRNA 4-OI – Nrf2 siRNA 4-OI, P = 0.0000007. Data from (a-d) are mean ± SEM from 3 independent experiments. P values calculated using (a-b) one-way ANOVA and (c-d) two-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.
Supplementary Figure 3 – DMF and 4-OI inhibit type I IFN- and caspase-11-mediated pyroptosis in macrophages

(a) Representative western blot of JAK1 in BMDM cell lysates following transfection with Ctrl or Jak1 siRNA prior to stimulation with LPS (3 h). GAPDH is used as loading control. Blot is representative of 3 independent experiments. (b) BMDMs were stimulated with recombinant mouse IFN-β for a timecourse as indicated. Casp4 (caspase-11) mRNA was quantified by qRT-PCR. NS – 4h, P = 0.00002. (c) BMDMs were pre-treated DMSO, DMF, or 4-OI (1 h) before LPS priming (3 h). Casp4 (caspase-11) mRNA was quantified by qRT-PCR. DMSO – 5 µM DMF, P = 0.0000868; DMSO – 10 µM DMF, P = 0.0000004. (d) Densitometry analysis of CASPASE-11 in BMDM cell lysates pre-treated with DMSO, DMF, or 4-OI (1 h) prior to LPS stimulation (3 h), with α-TUBULIN as loading control. (e-f) BMDMs were pre-treated with DMSO, DMF, or 4-OI (1 h) before LPS priming (3 h). (e) Isg15 and (f) Usp18 mRNA (DMSO – 250 µM 4-OI, P = 0.00004) were quantified by qRT-PCR. (g) BMDMs were either pre-treated or post-treated as indicated with DMSO or 4-OI (1 h) before or after priming with LPS (3 h) followed by LPS transfection (16 h). Pyroptosis is represented as percentage cell death measured by LDH release in BMDM supernatants. (h) BMDMs were pre-treated with DMSO, DMF, or 4-OI (1 h) before priming with LPS (3 h) and LPS transfection (16 h). Representative images of pyroptotic cell death after propidium iodide staining was added to cell media at time of LPS transfection. Red hue indicates pyroptotic cell death. Scale bar = 400 µm. Images are representative of 3 independent experiments. Data from (b-c and e-g) are mean ± SEM from 3 independent experiments. Data from (d) are mean ± SD from 3 independent experiments. P values calculated using two-tailed Student’s t-test for paired comparisons or one-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.
Supplementary Figure 4 – Prophylactic administration of DMF and 4-OI improves survival in vivo

(a) Kaplan-Meier survival curve of mice pre-treated with PBS, 50 mg/kg DMF, or 50 mg/kg 4-OI (1 h), or 200 IU/kg heparin (30 min), followed by intraperitoneal injection with 15 mg/kg LPS (for a further 72 h) (n = 10 per group). PBS+LPS – treatment groups, P = 0.0000000001. (b) Mice from (a) were scored clinically (assessing weight loss, activity level, eye closure, and appearance of fur and posture) every hour for 72 hours. PBS+LPS – DMF+LPS, P = 0.00000000019. Data points indicate individual mice in (a-b). For (a-b) Mantel-Cox survival analysis was performed. Data from (a-b) are mean ± SD. Source data are provided as a Source Data file.
**S. aureus intravenous injection murine model**

(a) Mice were intravenously injected with $5 \times 10^7$ CFU *S. aureus* (USA300-LAC) and co-treated with PBS, 50 mg/kg DMF, or 50 mg/kg 4-OI (6 h). Citrated plasma was harvested and thrombin generation was assessed ($n = 10$ per group). (b) Peak thrombin generation in mouse citrated plasma treated as in (a). PBS – DMF, $P = 0.0000067$; PBS – 4-OI, $P = 0.00003$. (c) Total thrombin generation in mouse citrated plasma treated as in (a). (d) TF-positive regions (red) in the lungs of mice treated as in (a). Representative lung tissue sections are shown. Magnification 20×. Scale bar = 10 µm. Data from (a-c) are mean ± SD. $P$ values calculated using one-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.
Supplementary Figure 6 – 4-OI limits weight loss and proinflammatory gene induction in vivo after SARS-CoV-2 infection

(a) Daily body weight measurements from male K18-hACE2 mice infected with $10^3$ PFU SARS-CoV-2 (Wuhan isolate; VIC01/2020) or mock infection and intranasally treated with PBS or 4-OI (10 mg/kg) from 1 day post-infection, with daily treatments continuing until the termination of the experiment at day 6 post-infection (n = 5-6 per group). Quantification of (b) Ifng and (c) Il1b mRNA by qRT-PCR in PBMCs of mice treated as in (a) (n = 5-6 per group). Data from (a-c) are mean ± SD. P values calculated using one-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.
Supplementary Figure 7 – DMF and 4-OI are anticoagulants that suppress inflammation-associated coagulation via inhibition of the macrophage type I IFN-TF axis

Schematic summarizing the anticoagulant effects of DMF and 4-OI. After bacterial or viral infection, F3 is induced in macrophages via type I IFN signalling, as F3 is a type I IFN-stimulated gene. This triggers excessive TF procoagulant activity and TF-dependent thrombin generation leading to coagulopathy. DMF and 4-OI block F3 induction via inhibition of Ifnb1. DMF and 4-OI therefore suppress aberrant thrombin generation, downstream thromboinflammation, and improve survival in mice. Thus, the clinically approved drug DMF and anti-inflammatory tool compound 4-OI are anticoagulants that suppress inflammation-associated coagulation via inhibition of the macrophage type I IFN-TF axis.
Table 1 – Predicted location of transcription factor sites in the region of the F3 promoter

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Supplementary Table 1 – Predicted location of transcription factor sites in the region of the F3 promoter

The coordinates of the predicted location of transcription factor sites in the region spanning -1500 bp to +500 bp from the start site of the F3 promoter, generated using the publicly available Interferome database\textsuperscript{42}.
Source Data for Supplementary Figure 3a

JAK1  GAPDH
Metabolic function of PKM2 regulates ROS production and microbial killing by neutrophils

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Summary

Neutrophils rely mainly on glycolytic metabolism for their biological functions, including reactive oxygen species (ROS) production. Pyruvate kinase M2 (PKM2) is a glycolytic enzyme involved in metabolic reprogramming and gene transcription of many immune cell types, but its role in neutrophils remains poorly understood. Here we report that PKM2 regulates ROS production and microbial killing by neutrophils. Zymosan-activated neutrophils showed increased expression of cytoplasmic PKM2, which is required for aerobic glycolysis. Inhibition or genetic deficiency of PKM2 in neutrophils reduced ROS production and microbial killing. Mechanistically, pharmacological inhibition or genetic deficiency of PKM2 resulted in phosphoenolpyruvate (PEP) accumulation and decreased dihydroxyacetone phosphate (DHAP) production, that is required for de novo synthesis of diacylglycerol (DAG) from glycolysis. DAG synthesis from glycolysis supplements DAG production by phospholipase C (PLC), and both contribute to protein kinase C (PKC) and subsequent NADPH oxidase activation. In vivo, PKM2-deficiency in myeloid cells impaired the control of bacterial infection. Our results fill the gap in the current knowledge of the importance of lower glycolysis for ROS production in neutrophils, highlighting the role of PKM2 in regulating the DHAP-DAG-PKC-NADPH oxidase pathway to promote ROS production in neutrophils.
Introduction

Neutrophils are the most abundant leukocytes in human blood and are usually the first immune cell type recruited into the site of infection\(^1\). Once activated, neutrophils produce a large amount of superoxide, the precursor of hydrogen peroxide and other reactive oxygen species (ROS), through the activation of the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase complex. Defects in ROS production impair pathogen elimination by neutrophils leading to recurring infections, as seen in Chronic Granulomatous Disease patients\(^2\). The NADPH oxidase is an enzymatic complex composed of two membrane-bound (gp91-\textit{phox}, p22-\textit{phox}) and three cytosolic (p47-
\textit{phox}, p40-\textit{phox}, p67-\textit{phox}) components. The activation of NADPH oxidase requires the phosphorylation of these subunits, mainly performed by protein kinase C (PKC), which is activated by diacylglycerol (DAG) in a calcium-dependent manner. DAG is produced from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) by phospholipase C (PLC), which is activated by a wide variety of pro-inflammatory stimuli that signal mainly through G protein-coupled receptors (GPCRs)\(^3\). In addition, it is broadly known that neutrophil metabolism reprogramming is essential for ROS production. Neutrophil metabolism relies mainly on glycolysis rather than oxidative phosphorylation (OXPHOS) for energy production since they harbour fewer mitochondria than other immune cells\(^4\). Interestingly, the inhibition of glycolysis using 2-deoxyglucose (2-DG) severely impairs phagocytosis, ROS production, and bacterial killing by neutrophils\(^7,8\). In this context, it is well-known that glycolysis fuels the pentose phosphate pathway (PPP) that utilises the glycolytic intermediate glucose-6-phosphate (G6P) to generate NADPH, essential for NADPH oxidase-dependent ROS production\(^9\). The importance of PPP for neutrophil activation is observed in patients with glucose-6-phosphate dehydrogenase (G6PD) syndrome with recurrent infections associated with less ROS production by neutrophils\(^10\). Furthermore, Rossi and colleagues\(^11\) showed that the \textit{de novo} synthesis of DAG from glucose represents a substantial source of DAG in
activated neutrophils. In this pathway, fructose-1,6-bisphosphate (F1,6BP) is converted into glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) by fructose-bisphosphate aldolase and the balance between GAP and DHAP is regulated by triosephosphate isomerase (TPI). DHAP is further converted to glyceral-3-phosphate (G3P) by glycerol-3-phosphate dehydrogenase. G3P is metabolised to phosphatidic acid and, subsequently, to DAG by phosphatidate phosphohydrolase. However, whether DAG produced from glycolysis is important for ROS production is unknown.

The importance of the last steps of glycolysis for ROS production is less understood. The pyruvate kinase (PK) is a rate-limiting glycolytic enzyme that catalyses the last step of glycolysis, converting phosphoenolpyruvate (PEP) to pyruvate. There are four PK isoforms: PKL, PKR, PKM1, and PKM2. PKL and PKR are expressed in the liver and erythrocytes, respectively, whereas PKM1 and PKM2 are expressed in many cell types, including neutrophils. Particularly, PKM2 can be activated allosterically by endogenous regulators such as F1,6BP, some amino acids, and small synthetic molecules such as TEPP-46 that affect its PEP-binding affinities. In the absence of these activators, PKM2 assumes a dimeric or monomeric form, resulting in the accumulation of glycolytic intermediates. While dimeric PKM2 can translocate to the nucleus to function as a coactivator of transcriptional factors, regulating genes important for cell proliferation and glycolysis, allosteric activated tetrameric PKM2 has a high glycolytic capacity. Although several reports have recently shown the importance of PKM2 in regulating the metabolic reprogramming and the immune function of macrophages and lymphocytes, the role of PKM2 in the cellular metabolism and transcriptional activity in neutrophils remains poorly characterised.

Here we show that ROS production in neutrophils is dependent on glycolytic flux. In this context, PKM2 regulates the DHAP-DAG-PKC-NADPH oxidase axis to promote ROS production and killing activity in neutrophils. Our results show a new role of PKM2
in neutrophils and highlight the importance of PKM2 in host defence and as a potential therapeutic target in neutrophil-mediated diseases.

Results

PKM2 expression increases in the cytoplasm of activated neutrophils

We initially compared the expression of Pkm2 between murine neutrophils and other myeloid cells by analysing data from the Immunological Genome Project. We found that Pkm2 expression is higher in neutrophils (NØ) compared to macrophages (MØ), basophils (BØ), or eosinophils (EØ) (Fig. 1a). Additionally, we found that thioglycollate-elicited peritoneal neutrophils expressed higher amounts of Pkm2 than bone marrow neutrophils, indicating that Pkm2 expression increases in activated neutrophils (Fig. 1a). To investigate the PKM2 protein expression pattern, wild-type and PKM2-deficient neutrophils were isolated from the bone marrow of Pkm2fl/fl and Pkm2ΔlzM mice, respectively (Extended Data Fig. 1), and activated with serum-opsonised zymosan (Zy/op) for 6h. We found that PKM2 is constitutively expressed in control (Ctrl) neutrophils and increased when neutrophils were activated with Zy/op (Fig. 1b). Of note, PKM2-deficient neutrophils showed very low levels of PKM2 protein expression (Fig. 1b).

PKM2 exists in different conformations as a monomer, dimer, and tetramer. The stabilised tetrameric form has high glycolytic activity, converting PEP to pyruvate, and is located in the cytoplasm. The dimeric form has low glycolytic activity but can translocate into the nucleus, acting as a nuclear transcriptional coactivator regulating gene expression. We found an increase in the PKM2-tetrameric form but not in the PKM2-dimeric form in Zy/op-activated neutrophils (Fig. 1c). Accordingly, using confocal microscopy, we found that PKM2 is primarily located in the cytoplasm of neutrophils (Fig. 1d). We also evaluated PKM2 expression in human neutrophils. Neutrophils were isolated from the blood of 3 healthy subjects and activated with Zy/op. Consistent with
the data observed in mouse neutrophils, human neutrophils also showed a constitutive
basal expression of PKM2, increasing when neutrophils were activated with Zy/op for
6h (Fig. 1e). Moreover, the confocal analysis showed that PKM2 was also mainly
expressed in the cytoplasm of human neutrophils (Fig. 1f). Together these results show
that PKM2 expression is upregulated in Zy/op-activated neutrophils and is localised
mainly in the cytoplasm.

PKM2 is required for ROS production and killing capacity by neutrophils

We then conducted a series of experiments to evaluate the role of PKM2 in neutrophils’
functions. We first performed flow cytometry analysis to evaluate phagocytosis in wild-
type and PKM2-deficient neutrophils activated with Zy/op-FITC. We found that PKM2
did not affect the phagocytic activity of neutrophils since the percentage of neutrophils
bearing Zy/op-FITC and the amount of phagocytosed Zy/op-FITC was similar between
wild-type and PKM2-deficient neutrophils (Fig. 2a). Also, there was no difference in the
production of neutrophil extracellular traps (NETs) by PKM2-deficient neutrophils
(Extended Data Fig. 2a). We next evaluated real-time ROS production. PKM2-deficient
neutrophils produced a lower amount of total ROS and superoxide than wild-type
neutrophils when activated with Zy/op (Fig. 2b and Extended Data Fig. 2b).

ROS is an important tool for the microbicidal activity of neutrophils\(^ {19}\). Thus, we next
determined the killing activity of neutrophils exposed to \( S. \) aureus and found that it was
impaired in PKM2-deficient neutrophils (Fig. 2d). To extend the evaluation of the role of
PKM2 to human neutrophils, we isolated cells from the blood of 4 healthy subjects and
pre-treated them with oxalate, a non-selective pyruvate kinase activity inhibitor\(^ {20}\), for 1h
before the activation with Zy/op. We also pre-treated human neutrophils with TEPP-46,
which activates PKM2 in the tetrameric form, maintaining PKM2 in the glycolytic
pathway and impairing its migration into the nucleus\(^ {21}\). The inhibition of PK with oxalate
decreased ROS production (Fig. 2e). In contrast, activation of PKM2 with TEPP-46 increased ROS production, although this effect varies for each subject (Fig. 2f). Accordingly, killing activity by human neutrophils was impaired by oxalate and enhanced by TEPP-46 (Fig. 2g).

In addition to neutrophils, macrophages are also important phagocytic cells that produce ROS and contribute to bacterial killing. To verify whether our findings in neutrophils can also be extended to macrophages, we activated PKM2-deficient bone marrow-derived macrophages (BMDMs) with Zy/op (Extended Data Fig. 3a,b) or *S. aureus* (Extended Data Fig. 3c-e). Similar to neutrophils, PKM2 deficiency did not affect phagocytosis in BMDMs (Extended Data Fig. 3a,c). However, ROS production was actually slightly increased in BMDMs (Extended Data Fig. 3b,d). Despite this, there was no difference in the killing capacity of PKM2-deficient BMDMs (Extended Data Fig. 3e). Thus, PKM2 regulates ROS production and, consequently, killing activity in neutrophils, but not in macrophages.

**Aerobic glycolysis is essential for ROS production in neutrophils**

The NADPH oxidase complex catalyses superoxide production, the primary source of ROS in neutrophils, a process that requires a large amount of NADPH. Among the different pathways contributing to NADPH production, the activation of PPP is crucial to trigger oxidative burst in neutrophils. Since ROS production was impaired in murine and human neutrophils in the absence of glucose (Extended Data Fig. 4a), we next evaluated the relationship between glycolytic activity and ROS production. To this end, we performed a kinetic experiment using two simultaneous assays, ROS production by chemiluminescence and glycolysis stress test using the Seahorse XF96 Analyzer (Agilent Technologies), plotting the results in the same graph for comparison (Fig. 3a). Murine neutrophils in glucose-free media were stimulated with Zy/op just before starting the assays. For both assays, glucose was added at 18 min (5.56 mM) after initiation. At 36 min, it was added rotenone and antimycin A (Rot/AA), complex I and
complex III inhibitors, respectively, to shut down mitochondrial respiration; and, at 54 min, it was added 2-DG to inhibit glycolysis. Fig. 3b shows ECAR (extracellular acidification rate, blue line) and ROS production (black line). We found that Zy/op could not increase ECAR or ROS production without glucose. Interestingly, the addition of glucose increased ECAR, while Rot/AA had no effect, and 2-DG reduced ECAR to basal levels (blue line) (Fig. 3b). In parallel, we observed that ROS production increased when glucose was added with a slight decrease upon adding Rot/AA, but it was completely abolished when 2-DG was added. Together, these results show that glycolytic metabolism is crucial for ROS production by activated neutrophils. Since activated neutrophils increase oxygen consumption to produce ROS\(^2\), we next assessed the correlation between OCR (oxygen consumption rate), glucose metabolism, and ROS production (Fig. 3c). As shown in the red line, Zy/op was unable to increase OCR in the absence of glucose; however, OCR increased when glucose was added, slightly decreased when Rot/AA was added and returned to basal levels with the addition of 2-DG. Together with ROS results (black line), this highlights that while a portion of oxygen consumption and ROS production is due to mitochondrial respiration, both oxygen consumption and ROS production are dependent on glycolysis.

We then conducted a set of experiments targeting different steps of the glycolytic pathway to elucidate the importance of each one for regulating ROS production (Fig. 3d). First, wild-type and PKM2-deficient neutrophils were treated with the PPP inhibitor 6-AN (6-aminonicotinamide). We observed that while PKM2 deficiency partially reduced ROS production, 6-AN treatment resulted in a massive impairment in ROS production regardless of PKM2 presence (Fig. 3e), highlighting the well-known importance of PPP for this function\(^8\). To further explore whether PKM2-deficiency could impair PPP activation, thus leading to a decreased ROS production, we evaluated the NADPH and NADP concentrations in Zy/op-activated neutrophils, but no differences were found (Fig. 3f). We then examined the expression of some NADPH oxidase
subunits. There were no differences in the gene expression of gp91-phox or protein expression of gp91-phox and p47-phox between wild-type and PKM2-deficient neutrophils (Fig. 3g,h). It has been shown that activated neutrophils have increased glutathione turnover, which may be consumed when ROS is produced\(^\text{23}\). Glutathione is an antioxidant molecule present at high concentrations in neutrophils, and the ratio between its reduced (GSH) and oxidised (GSSG) forms can be used to measure intracellular oxidative balance. However, PKM2 deficiency did not alter the concentration of total glutathione or GSH/GSSG ratio in neutrophils (Extended Data Fig. 4b). Second, we treated murine and human neutrophils with 2-DG to inhibit hexokinase (HK), 3PO to inhibit PFKFB3 and oxalate to inhibit PK, at doses that showed comparable inhibition of lactate production. As shown in Fig. 3i, the inhibition of glycolysis at HK, PFKFB3, or PK decreased ROS production. Altogether these results show that different glycolytic steps are important for ROS production; however, PKM2 regulates ROS production by a PPP-independent mechanism. Besides being dependent on glycolysis, ROS production also depends on PLC activation, which results in calcium release, DAG production, and, consequently, PKC activation\(^\text{24}\). We performed two experiments to understand better the role of the PLC-dependent and the glucose-dependent pathways in ROS production. In the first one, wild-type and PKM2-deficient neutrophils were pre-treated with a PLC inhibitor (iPLC) for 1h before being stimulated with Zy/op. PLC inhibition decreased ROS production in both cells, but it had an additive effect in PKM2-deficient neutrophils (Fig. 3j), suggesting that PKM2 regulates ROS production independent of PLC. In the second one, murine or human neutrophils were activated with Zy/op without glucose (Fig. 3k). After 30 min, glucose was added to the media, and an increase in ROS production was observed. After 30 min, we added vehicle (black line), 3PO (blue line), or iPLC (red line) and found that both inhibitors decreased ROS production, showing that both pathways are important for this function. Finally, after 30 min, we added vehicle (black line), iPLC (blue line) or 3PO (red line) and found that, in both cases, ROS production
decreased to basal levels, showing that PLC-dependent and glucose-dependent pathways complement each other for ROS production. Together, these results suggest that PKM2-mediated glycolysis and PLC pathway are independently required for ROS production in activated neutrophils.

The metabolic activity of PKM2 regulates ROS production in neutrophils

Because PKM2, acting as a transcriptional coactivator, has been reported to have an important role in the expression of glycolytic genes and induction of glycolytic flux, we next investigated whether PKM2 deficiency affects the expression of glucose transporter 1 (GLUT1) or glucose uptake (evaluated by flow cytometer analysis using 2-NBDG) in activated neutrophils. As expected, we found an upregulation of GLUT1 expression (Fig. 4a) and glucose uptake (Fig. 4b) in Zy/op-activated neutrophils compared to control neutrophils. However, PKM2 deficiency does not affect these parameters (Fig. 4a,b). We then evaluated total pyruvate kinase activity (including PKM1 and PKM2) and found a significant reduction in PKM2-deficient neutrophils activated with Zy/op (Fig. 4c). In accordance, Zy/op-activated control neutrophils showed increased pyruvate (Fig. 4d) and lactate (Fig. 4e) production when compared to PKM2-deficient neutrophils. In line, oxalate-treated human neutrophils showed a decreased lactate production (Fig. 4e). Despite mitochondrial ROS production being increased in PKM2-deficient neutrophils (Extended Data Fig. 5a), no mitochondrial dysfunction was observed in these cells, as evaluated by MitoTracker Green and Red (Extended Data Fig. 5b). Taken together, these results show that PKM2 is required for the canonical metabolic function (pyruvate kinase activity) rather than transcriptional control activity in activated neutrophils.

To further explore the metabolic changes regulated by PKM2, we measured the redistribution of glucose metabolism flux during neutrophil activation by a tracing study with ^13C-glucose. Zy/op-activated wild-type neutrophils showed an increase in glycolysis metabolites, including G6P, F1,6BP, pyruvate, and lactate, consistent with
the activation of the glycolytic pathway. Importantly, DHAP and G3P, the precursors for DAG de novo synthesis from glucose, were also increased (Fig. 4f). We then evaluated the glucose metabolism flux in PKM2-deficient neutrophils and found that, as in wild-type neutrophils, F1,6BP production was also increased in PKM2-deficient neutrophils activated with Zy/op. However, pyruvate and lactate, in addition to DHAP and G3P, were all decreased (Figure 4f and Extended Data Fig. 6a,b). Importantly, PEP was hugely accumulated in PKM2-deficient neutrophils. To better understand the direction of the glycolytic flux in neutrophils (i.e. whether it favours lactate or DAG production), we evaluated the GAP and DHAP concentrations in Zy/op-activated murine neutrophils. Both metabolites were boosted, confirming that both pathways were activated during neutrophil activation (Extended Data Fig. 6c,d). In addition, we found that GAP accumulates, and the production of DHAP is impaired when PKM2 is inhibited by oxalate (Extended Data Fig. 6c,d). These data indicate that PKM2 activity regulates the DHAP pathway leading to de novo synthesis of DAG from glucose.

Phosphoenolpyruvate accumulation reduces ROS production in neutrophils

To elucidate whether the DHAP pathway that leads to DAG production from glucose is important for ROS production, we pre-treated murine neutrophils with 5-pentadecylresorcinol, an inhibitor of glycerol-3-phosphate dehydrogenase (GDP), or propranolol, an inhibitor of phosphatidate phosphohydrolase (Fig. 5a). We found that both inhibitors impaired ROS production in Zy/op-activated neutrophils (Fig. 5b). These results, together with the glucose tracing analysis, indicate that PKM2 deficiency or inhibition could decrease ROS production by downregulating DHAP and G3P production and, consequently, DAG synthesis from glucose, thus decreasing NADPH oxidase complex activation. In fact, PKM2-deficient neutrophils activated with Zy/op had reduced phosphorylation of p47-phox, indicating an impaired activation of the NADPH oxidase complex (Fig. 5c). In addition, neutrophil activation with PMA – a PKC direct activator – bypassed the PKM2 deficiency role in ROS production (Fig. 5d),
showing that the impairment in ROS production in PKM2-deficient neutrophils is due to an upstream event for PKC activation.

To further explore the importance of DAG on ROS production, we examined whether 1-oleoyl-2-acetyl-sn-glycerol (OAG), a synthetic DAG analogue, would rescue ROS production in PKM2-deficient neutrophils or oxalate-treated human neutrophils. We found that OAG restored ROS production in Zy/op-activated neutrophils in both cases (Fig. 5e). Together, these data indicate that PKM2 regulates ROS production in activated neutrophils by modulating DAG de novo synthesis from glucose. In this way, two independent pathways - through PLC activation and de novo synthesis from glucose - are responsible for maintaining DAG levels in neutrophils, which is important for PKC and NADPH oxidase activation.

Grüning and collaborators26 showed that a decrease in pyruvate kinase activity leads to PEP accumulation in yeast, inhibiting the enzyme TPI by binding to its catalytic pocket. Accordingly, we also found that PEP accumulates in PKM2-deficient neutrophils (Fig. 4f). TPI interconverts GAP and DHAP27, and PEP competes with GAP for binding to TPI26. We then hypothesised that PEP accumulates and inhibits TPI activity when PKM2 is absent or inhibited in neutrophils. This would decrease DHAP accumulation and, consequently, DAG production, impairing ROS production. To further explore this hypothesis, we treated wild-type neutrophils with PEP for 1h prior to the activation with Zy/op and found that ROS production was impaired (Fig. 5f). In addition, DHAP (Fig. 5g) and G3P (Fig. 5h) concentrations and TPI activity (Fig. 5i) were also reduced. Furthermore, PEP and oxalate treatment decreased phosphorylation of p47-phox, which indicates a decreased NADPH oxidase complex activation (Fig. 5j). We then evaluated TPI activity in PKM2-deficient neutrophils and oxalate-treated human neutrophils. We found that the absence or inhibition of PKM2 decreased TPI activity (Fig. 5k). To further assess TPI importance for ROS production, we utilised CRISPR/Cas9-based editing to disrupt Pkm2 and Tpi (Extended Data Fig. 7a-c) in NB4 cells, a promyelocytic leukaemia cell line that differentiates to maturate neutrophils.
upon exposure to all-trans retinoic acid (ATRA). We found that ROS production was impaired in the absence of PKM2 and TPI (Fig. 5l). Together, these results show that PKM2 deficiency reduces ROS production in activated neutrophils by increasing PEP accumulation, which inhibits TPI activity and DHAP metabolism, impairing NADPH oxidase activation and ROS production (Figure 5m). In this way, PKM2 regulates the DHAP-DAG-PKC-NADPH oxidase axis to promote ROS production in neutrophils.

**PKM2 is important for *S. aureus* killing in vivo**

*S. aureus* is a Gram-positive bacterium that is a common pathogen responsible for a skin infection. A hallmark of *S. aureus* infections is neutrophil recruitment and abscess formation, which is required to eliminate the pathogen. To determine the importance of PKM2 in vivo, we used two different mouse models: peritonitis and cutaneous infection with *S. aureus* in *Pkm2* and *Pkm2* mice and evaluated the infection progression. For the first model, we inoculated mice with *S. aureus* via intraperitoneal (i.p.) injection, and the animals were assessed for 18h (Fig. 6a). We found that *Pkm2* mice were more susceptible to *S. aureus* infection than *Pkm2* mice, showing higher bacterial loads in the blood (Fig. 6b) and in the peritoneal exudate (Fig. 6c). Despite that, neutrophils (Fig. 6d) and mononuclear cells (Fig. 6e) counts were unchanged. In addition, IL-6 was increased in the plasma (Fig. 6f) of *Pkm2* mice, but no differences were found in CXCL2 (Fig. 6g) and CCL2 (Fig. 6h) levels in the exudate.

It has been shown that mice inoculated intracutaneously with high doses of *S. aureus* display clinical and histopathological signs of local infection and inflammation and massive neutrophil recruitment within 48h. In this way, for the second model, we performed two 3 mm punches through the dermis in the back of shaved mice and inoculated the wound with *S. aureus* (Fig. 6i). The wound lesion sizes of anaesthetised
mice were evaluated for 11 days and determined by measuring the total lesion size (square centimeters). We found that sham \( Pkm2^{fl/fl} \) and \( Pkm2^{\DeltaLyz2} \) mice had the same wound healing timing, showing no difference in the cicatrisation processes in mice that do not express PKM2 in myeloid cells (Fig. 6f). However when inoculated with \( S. aureus \), \( Pkm2^{\DeltaLyz2} \) mice had larger wounds than \( Pkm2^{fl/fl} \) mice in the first four days of infection (Fig. 6j,k). Moreover, compared to \( Pkm2^{fl/fl} \), the wound from \( Pkm2^{\DeltaLyz2} \) had more colony-forming units (CFU) of \( S. aureus \) at 48h of inoculation despite having the same number of neutrophils at later time points (Fig. 6l,m). In addition, there was no difference in the concentrations of inflammatory cytokines KC (Fig. 6n), TNF-\( \alpha \) (Fig. 6o), and IL-6 (Fig. 6p) in the wound tissue homogenates. Altogether these results show that PKM2 is important for controlling \( S. aureus \) infection \textit{in vivo}.

**Discussion**

In the past few years, the role of metabolic reprogramming as a key immuno-regulatory phenomenon has been investigated by its influence on cell differentiation, function, and fate\(^{34} \). In this context, the enzyme PKM2 has been implicated as a critical regulator of aerobic glycolysis and recently has generated significant interest due to its upregulation in activated immune cells\(^{15} \). Of note, it was shown that in macrophages and lymphocytes, PKM2 has a major transcriptional role, migrating to the nucleus and regulating gene expression\(^{16-18} \). Conversely, we show here that PKM2 is mainly expressed in the cytoplasm of neutrophils. Its expression in the tetrameric form is upregulated during neutrophil activation, indicating that PKM2’s primary role is on the glycolytic pathway in activated neutrophils.

Neutrophils are rapidly recruited to tissues to phagocyte and eliminate invading pathogens via antimicrobial actions, especially ROS production\(^{2,35} \). It is worth noting that neutrophils recognise both Zymosan and \( S. aureus \) by TLR2, which bind to ligands.
with very diverse structures such as lipoproteins/lipopeptides, peptidoglycan,
glycopolymers, and proteins. Here, we showed that while PKM2 was not important for
the phagocytosis process, it regulates ROS production and, consequently, the killing
capacity in neutrophils. It is already known that ROS production is dependent on
glycolysis, but until now, this was mainly associated with the PPP activation and the
balance of NADPH/NADP in neutrophils. Surprisingly, we found that, in the
absence of PKM2, ROS production was reduced without impairments in NADPH/NADP
balance, while PK activity and lactate production was decreased.

We found a difference between murine and human neutrophil responses during our
experiments utilising glucose-free media. The absence of glucose almost totally
impaired ROS production in murine neutrophils and reduced it by 50% in human
neutrophils. In this regard, it is interesting to note that neutrophils contain glycogen
within granules, which is important to their function and survival. Furthermore, human
neutrophils contain more glycogen than murine neutrophils, which could explain the
more drastic effect of glucose starvation in ROS production of murine neutrophils.
Moreover, we found that ROS production is associated with increased glycolytic rate
and the inhibition of glycolysis in different steps of this pathway impaired ROS
production. Previously, the canonical pathway for ROS production was not thought to
involve the direct activation of the glycolytic pathway, and it has been shown that
glycolysis provides the NADPH used during NADPH oxidase activation for ROS
production. However, our results highlight that PEP, a glycolytic metabolite at PKM2
enzymatic reaction, is involved in regulating ROS production in activated neutrophils.

DAG is an important PKC activator and can be produced from glucose in neutrophils in
a pathway dependent on DHAP metabolism. However, if DAG produced from
glucose/DHAP metabolism contributes to ROS production is unknown. We found that
the inhibition of DAG production from glycolysis by blocking the glycerol-3-phosphate
dehydrogenase, which converts DHAP into G3P, and the phosphatidate
phosphohydrolase, which converts phosphatidic acid into DAG, impaired ROS production. In addition, as demonstrated by others, we found here that neutrophil activation increased DHAP production. However, DHAP and G3P concentrations were decreased in PKM2-deficient and oxalate-treated neutrophils, indicating that PKM2 somehow regulates the DHAP downstream pathway. As aforementioned, this pathway is important for de novo synthesis of DAG. In fact, the treatment of neutrophils with OAG restored ROS production in PKM2-deficient and oxalate-treated neutrophils, suggesting that ROS production is impaired in these cells because DAG production from glucose is decreased.

It has been shown that a decrease of pyruvate kinase activity leads to PEP accumulation, which can inhibit the activity of triose phosphate isomerase (TPI) by binding to its catalytic pocket. TPI has high catalytic efficiency and enhances the movement of a single proton to constantly interconvert DHAP and GAP, where the equilibrium favours the synthesis of DHAP. Here we found a decrease in TPI activity in PKM2-deficient and oxalate-treated neutrophils. We hypothesised that PKM2 modulates TPI activity by regulating PEP concentration, which would impact on DHAP concentration and, ultimately, ROS production, explaining, at least in part, our results. Indeed, when neutrophils were treated with PEP, there was a decrease in ROS production, DHAP, and G3P concentrations. Conversely, it has been shown that the low TPI activity increases DHAP concentration in erythrocytes and yeast and mediates an increase in PPP metabolites concentrations and oxidant resistance in yeast. However, PKM2-deficient neutrophils had normal glutathione concentrations, suggesting that, in contrast to what is seen in other cell types, the low TPI activity did not alter the PPP activation in neutrophils. Thus, further studies are needed to understand the role of TPI in neutrophil function.

Finally, we showed that PKM2 is important for early infection resolution in vivo in two different S. aureus infection models, peritonitis and skin infection, where a massive
neutrophil infiltration is observed in the first hours of infection\textsuperscript{43}. The importance of
glycolysis for \textit{S. aureus} killing by neutrophils was shown by Boxer and collaborators\textsuperscript{8},
where neutrophils treated with 2-DG had impaired killing capacity. Furthermore, Burge
and collaborators\textsuperscript{44} showed that a patient with recurrent \textit{S. aureus} infections had a
defect in PK activity. In both models we evaluated, there was a higher number of \textit{S.}
aureus CFU despite having the same number of neutrophils in the peritoneum and the
wound. It is known that defects in neutrophil functions, including ROS production,
chemotaxis, and granules disorders impair \textit{S. aureus} elimination and infection
resolution\textsuperscript{43}. Together with our \textit{in vitro} findings, these results indicate that PKM2
regulation of ROS production in neutrophils is important for bacterial elimination \textit{in vivo}.
However, because we used LysM\textsuperscript{cre} PKM2\textsuperscript{fl/fl} mice, we cannot exclude that PKM2 plays
additional roles in macrophages that could account for controlling \textit{S. aureus} infection \textit{in vivo}.
Our results reveal a new role of PKM2 that regulates the DHAP-DAG-PKC-NADPH
oxidase axis and, at least in part, ROS production in neutrophils by impairing PEP
accumulation. These findings fill the gaps in the current knowledge of the importance of
the glycolytic pathway for ROS production and elucidate for the first time the
mechanisms underlying PKM2’s metabolic role in neutrophils.

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Author contributions


Declaration of interests

The authors declare no competing interests.
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METHODS

Animals

C57BL/6 wild-type mice were purchased from Charles River. Lyz2$^{Cre}$ (004781) and PKM2$^{floxflox}$ (024048) mice were obtained from Jackson Laboratories. Myeloid cell-(Pkm2$^{ΔLyz2}$) specific-Pkm2-deficient mice were generated by crossing the Pkm2$^{floxflox}$ mice with Lyz2$^{Cre}$ mice on a C57BL/6 background. Pkm2$^{fl/fl}$ mice without Lyz2$^{Cre}$ gene were used as controls for all experiments. All experiments were carried out with 6–7-week-old female and male littermate mice according to the guidelines of the Animal Welfare Committee of the Ribeirão Preto Medical School, University of São Paulo (protocol number: 143/2017). Animals were bred and maintained under specific pathogen-free conditions at the animal facility of the Ribeirão Preto Medical School, University of São Paulo. Mice were randomly assigned to experimental groups.

Human blood samples

Thirty healthy donors of both sexes with ages between 18-40 years were included in this study. The exclusion criteria were smoking, obesity, diabetes, pregnancy and anti-inflammatory use. The study was approved by the Human Subjects Institutional Committee of the Ribeirão Preto Medical School, Brazil (License number: 31033519.4.0000.5440).

Neutrophil isolation and culture

Murine neutrophils were obtained from mouse bone marrow. Briefly, mouse femurs and tibias were removed and flushed with phosphate buffer solution (PBS, Corning) and then isolated by immunomagnetic negative selection with the Neutrophil Isolation Kit, following manufacturer’s instructions (Miltenyi Biotech, 130-097-658).

Human neutrophils were obtained from peripheral blood by Percoll (GE Healthcare, 17-0891-01) density gradient. Briefly, whole blood was collected into Vacutainer® Citrate Tubes with 3.2% buffered sodium citrate solution as anticoagulant (BD Bioscience).
Blood was laid on top of a 4-layer Percoll gradient (72%, 63%, 54% and 45% in PBS) and then centrifuged at 650 g, 30 min, at 20 °C. Neutrophils were recovered at the interface of the 63%-72% fractions. The remaining erythrocytes were lysated (1X RBC Lysis Buffer, Thermo, 00-4333-57). Both murine and human neutrophils were resuspended in Hank’s balanced saline solution (HBSS, Corning, 21-020-CM) containing 2 % of bovine serum albumin (BSA) and calcium. Cell suspensions contained > 95 % of viable neutrophils, as established by flow cytometry.

**Bone marrow-derived macrophages (BMDM) isolation and culture**

BMDMs were obtained using L929 cell-conditioned medium, as previously described\(^\text{46}\). Briefly, bone marrow cells were obtained by flushing both mice femurs and tibias with RPMI-1640 culture media (Corning, 15-040-CV). After centrifugation, cells were resuspended in RPMI-1640 containing 20 % L929, 10 % foetal bovine serum (FBS, GE Life Sciences, SV30160.03), L-glutamine (2 mM, Sigma, G7513), penicillin (100 U/mL, Sigma, P4333), and fungizone (2.5 μg/mL, Gibco, 15-290-018) and seeded for 7 days. After the end of this period, BMDMs were harvested for subsequent experiments.

**CRISPR/Cas9 Gene Editing in NB4 cell line**

NB4 cell line (ATCC, ACC-207) was cultured in RPMI-1640 culture medium supplemented with 10% FBS, penicillin (100 U/ml), and L-glutamine (2 mM) at 37°C and 5% CO\(_2\). For the generation of NB4 deficient for PKM2 and TPI, we used the CRISPR/Cas9 plasmid PXL (plasmid # 75349, Addgene). For the selection of the gene region to be targeted by the Cas9 endonuclease, we utilised Benchiling (Biology Software), selecting the exon 10 of the coding sequence of the PKM gene or exon 3 of the coding sequence of TPI, all responsible for the production of their respective protein. Succinctly, based on those sequences, guide RNAs were selected using CRISPOR\(^{47}\). The oligonucleotides synthesised carrying the targeting sequence of
PKM2 were 5’-caccATTTGAGGAACTCCGCCGCC-3’ and 5’-aaacGGCGGCGGAGTTCCTCAAAT -3’. For TPI 5’-acaccGCAAAGACTCGGGAGCCACGTG-3’ and 5’-aaaacACGTGGCTCCGCAGTCTTTGCG-3’ (Exxtend). The oligos were then cloned into the PXL vector (Addgene, 75349). Briefly, E. coli DH5-Į chemo-competent bacteria were transformed with the plasmid, and on the next day, the plasmids were purified by the method of MiniPrep, following the manufacturer’s instructions (Qiagen-Hilden, 27104). NB4 cells (2 x 10⁶ cells) were resuspended in 100 µl of Cell Line Nucleofector Solution V buffer. To this solution was added 2 µg of the cloned PXL plasmid. This mixture was transferred to cuvettes, and electroporation was performed using the X-001 (Nucleofector I Device) protocol. After transfection, 500µl of RPMI-1640 supplemented with 10% FBS was immediately added, and the cells were plated. After 48 h, NB4 cells were differentiated into granulocytes with 1 µM ATRA (Sigma-Aldrich) for 5 days. Granulocytic differentiation was evaluated by cell morphology.

Mouse model of skin wound infection

The mouse model of skin wound infection was done as described by Guo and collaborators with modifications. Briefly, mice were anesthetised by inhalation administration of isoflurane (1-3 %), and their posterior backs were shaved. On the following day, two full-thickness wounds were made through the dermis using a 3 mm biopsy punch. The wounds were subsequently inoculated with Staphylococcus aureus (ATCC, Strain 6538, 1 x 10⁸ CFU per 7.5 µl of PBS in each wound) using a micropipette. Wounds of sham mice were not inoculated. All mice were given analgesics (12.5 mg.kg⁻¹, tramadol, Agener União, subcutaneous) beginning 30 min before making the wounds and then every 12 h up to day 3. Measurements of total lesion size (square centimetres) were made daily. Wound healing was evaluated for 11 days. For wound analysis, biopsy skin specimens were obtained from the lesions of infected mice. Biopsy of healthy skin from the same
mouse was used as a control. Tissues were weighed before being homogenised in 500 mL PBS. Recovered bacteria were counted by plating serial dilutions onto Mueller-Hinton agar (Difco, 225250) plates. Samples were centrifuged, and supernatants were used for cytokines measurements. The pellet was used for MPO activity assay by measuring the change in OD at 450 nm using tetramethylbenzidine. This assay was used as a quantitative measurement of neutrophil migration and accumulation in the wound.

**Mouse model of peritonitis infection**

Mice were anesthetised by inhalation administration of isoflurane (1-3 %) and inoculated with *S. aureus* (1x10^8 CFU) intraperitoneally (i.p.). After 18h, mice were euthanised, and exudate and blood were obtained. Recovered bacteria were counted by plating serial dilutions onto Mueller-Hinton agar plates. Samples were centrifuged and supernatants were used for cytokines measurements.

**Zymosan opsonisation**

For Zymosan opsonisation, 10 mg of zymosan (Sigma, Z4250) was washed in PBS and then incubated with murine or human serum diluted twice in PBS at 37 °C for 30 min. Zymosan opsonised (Zy/op) was washed twice, resuspended in PBS and stored at -20 °C. For phagocytosis experiments, Zymosan was first labelled with Fluorescein isothiocyanate isomer I (FITC, Sigma, F4274) and then incubated with serum (Zy-FITC/op).

**Bacteria**

*Staphylococcus aureus* was obtained from the American Type Culture Collection (ATCC, USA, Strain 6538). Bacteria were cultivated from frozen stocks for 24 h at 37°C in Brain Heart Infusion Agar (Oxoid, CM1135). Bacteria suspension was prepared in sterile NaCl 0.9 % and the calculations were estimated by measuring the OD of
solutions at 600 nm. CFU were verified by plating serial dilutions onto Mueller-Hinton agar plates.

**Western Blotting**

For standard western blot protocols, neutrophils (3 x 10^6) were activated with Zy/op (100 µg/mL) at 37 °C and 5 % CO₂. After 6 hours, cells were harvested in boiling Laemmli Sample Buffer (Biorad, 161-0737), followed by heating at 95 °C for 5 min. For crosslink assay, neutrophils were incubated with 500 µM disuccinimidyl suberate (Sigma-Aldrich, S1885) for 30 min, and then cell lysates were prepared as described above. Protein samples were resolved on SDS-PAGE gels and transferred onto a nitrocellulose membrane (GE Healthcare). Membranes were blocked with 5% (wt/vol) nonfat milk (Cell Signalling) in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature and then incubated overnight at 4°C with 1:1000 dilutions of primary antibodies against PKM2, β-actin (Cell Signalling), gp91-phox (BD Transductions), p47-phox (Millipore) and phospho-p47-phox (Thermo). Membranes were repeatedly washed with TBST and incubated for 2 h with the appropriate HRP-conjugated secondary antibody (1:5000 dilution; Sigma-Aldrich). Immunoreactivity was detected using the ECL prime reagent (GE Healthcare, RPN2236), and then the chemiluminescence signal was recorded on the ChemiDoc XRS Imager (Bio-Rad Laboratories). Data were analysed with Image Lab software (Bio-Rad Laboratories). Total β-actin levels were used as a loading control.

**Confocal microscopy**

Neutrophils (8 x 10^4) were incubated on poly-L-lysine–coated coverslips for 30 min and activated with Zy/op (100 µg/mL) for 6h. After this time cells were fixed (2% PFA), permeabilised and stained with anti-PKM2 (1:200, Cell signalling, 4053P) overnight. After incubation with a secondary antibody (1:800, AlexaFluor 488, Abcam, ab150065), coverslips were washed in PBS and mounted onto microscope slides using a DAPI-
containing mounting medium (Vector Laboratories, H-1200-10). Images were acquired by Axio Observer combined with LSM 800 microscope (Carl Zeiss) and DMI6000B microscope (Leica Microsystems) and analysed with Fiji/ImageJ.

*Phagocytosis*

Neutrophils (0.3 x 10^6) were activated with Zy-FITC/op (100 µg/mL) for 20 min at 37 ºC and then washed with PBS. Fluorescence of the samples was measured in a flow cytometer (FACSVersetm, BD Biosciences) before and after the addition of the quenching solution (0.4 % Trypan blue in PBS citrate, pH 4.4), as described by Nuutila and collaborators50, with modifications. Ten thousand cells were analysed.

*ROS production*

ROS production was measured by luminol-dependent chemiluminescence (CL) assay as described by Kanashiro and collaborators51. Briefly, neutrophils (0.2 x 10^6) were pre-treated (when indicated) for 1h with oxalate (3 mM, Sigma, 71800), TEPP-46 (30 µM, Millipore, 5054870001), 2-Deoxy-D-glucose (2-DG, 3 mM, Sigma, D8375), 3PO (10 µM, Calbiochem, 525330), phosphoenolpyruvate (PEP, 1 mM, Sigma, P0564), 5-pentadecylresorcinol (30 µM, Sigma, 91822) or propranolol (30 µM, Sigma, P0884). Neutrophils were then activated with Zy/op (100 µg/mL) or S. aureus (MOI=3) in the presence of luminol (Sigma, A8511) 10^-4 M or lucigenin (Sigma, M8010). The reaction was monitored in a luminometer (FlexStation 3 Multi-Mode Microplate Reader, Molecular Devices, San Jose, CA) for 1h, at 37 ºC, and the results were expressed as the area under the time-course CL curve (AUC).

*Killing capacity*

Neutrophils (1 x 10^6) were exposed to S. aureus (3 x 10^6) opsonised with serum (MOI=3) for 2h, at 37 ºC and 5 % CO2. For human samples, neutrophils were pre-treated with oxalate (3 mM) or TEPP-46 (30 µM) for 1h before being exposed to S.
aureus. Cells were centrifuged and the supernatant discarded. Neutrophils were lysed by adding distilled H$_2$O. The lysates were serially diluted in PBS and plated on Mueller-Hinton agar plates and incubated overnight at 37 °C. The results were expressed by CFU per mL.

**Neutrophil Extracellular Trap assay**

This procedure was performed as previously described\(^45\). Briefly, plasma or culture supernatant was added to a 96-well clear-bottom black plate coated with anti-MPO antibody (5 µg/ml, Thermo Fisher Scientific, PA5-16672) and incubated overnight at 4 ℃. The amount of DNA bound to MPO was quantified using the Quant-iT™ PicoGreen® kit (Invitrogen, P11496). The fluorescence intensity (excitation at 488 nm and emission at 525 nm) was quantified by a FlexStation 3 Microplate Reader (Molecular Devices, CA, USA).

**Biochemical parameters**

Neutrophils (0.2 x 10$^6$) were activated with Zy/op (100 µg/mL) for 6h, at 37 °C and 5 % CO$_2$. Lactate concentration in cultured supernatant was measured with the Enzymatic Lactate Kit (Labtest, 138).

For Dihydroxyacetone Phosphate, Glycerol-3-Phosphate, Triose Phosphate Isomerase, NADP/NDPH, glutathione (GSH/GSSG/Total), Pyruvate Kinase activity and Pyruvate analysis, we used commercial kits. Briefly, murine or human neutrophils (1 x 10$^6$) were activated with Zy/op (100 µg/mL) for 1h, at 37 °C and 5 % CO$_2$. For human samples, neutrophils were pre-treated for 1h with oxalate (3 mM) before being activated with Zy/op. Samples were centrifuged and prepared according to manufacturer's instructions. The following kits were used: PicoProbe™ Dihydroxyacetone Phosphate (DHAP) Fluorometric Assay Kit, Glycerol-3-Phosphate (G3P) Colorimetric Assay Kit, Triose Phosphate Isomerase (TPI) Activity Colorimetric Assay Kit, NAD/NADH Quantitation Colorimetric Kit, Glutathione (GSH/GSSG/Total)
Fluorometric Assay Kit and Pyruvate Kinase Activity Colorimetric/Fluorometric Assay kit (Biovision, K673, K641, K670, K337 and K709, respectively); and Pyruvate Assay kit (Cayman, 700470).

**Glucose tracing analysis**

Neutrophils were activated with Zy/op at 37 °C for 1h in the presence of D-(+)-Glucose-\textsuperscript{13}C\textsubscript{6} (Cayman, 26707) under slow rotation. Samples were centrifuged and pellets were extracted with 200 µL of methanol/water (8:2 v:v), and centrifuged at 21,000 xg, 4 °C, for 10 min (Boeco M-240R, Germany). The supernatant (100 µL) was evaporated up to dryness (Analitica Christ RVC2-18, Sao Paulo, Brazil) and the residue was reconstituted in 80 µL of water. The analysis was performed using an Acquity UPLC-TQD MS/MS System (Waters Corp., Massachusetts, EUA) and metabolites were separated on a Kinetex F5 column 50 mm x 2.1 mm, 1.7 µm (Phenomenex, California, EUA) with a pre-column of the same material, maintained at 40° C. The mobile phase was composed by solvent A (10 mM tributylamine and 15 mM acetic acid in water) and B (methanol and water, 8:2 v:v). The elution program was as follows: 1% B (initial), 1-10% B (3 min), 10-90% B (4 min), 1% B (4.1 min), 1% B (6.5 min). The flow rate was 0.4 mL.min\textsuperscript{-1}, the injection volume was 5 µL in full loop mode, and the autosampler temperature was set to 15 °C. The mass spectrometry (MS) was operated in the negative ion and selected ion recording (SIR) mode using an electrospray voltage of 2.5 kV, source temperature of 150 °C and desolvation temperature of 500 °C. Nitrogen was used as desolvation gas (1,000 L h\textsuperscript{-1}) and as a cone gas (50 L h\textsuperscript{-1}). Identification of isotopically labelled metabolites was based on its unit resolution m/z and retention time corresponding to unlabelled metabolites determined with chemical standards (Sigma). The analyses were performed using MassLynx V 4.1 Software (Waters Corp.) and the abundance of labelled glycolytic intermediates was determined by the peak height.

**Flow cytometry analysis**
Neutrophils (0.3 x 10⁶) were activated with Zy/op (100 µg/mL) for 1h, at 37 °C and 5 % CO₂. Neutrophils were washed in PBS and incubated with MitoSOX (5 µM, Thermo, M36008) or Mitotracker Red (50 nM, Thermo, M7512) and Green (100 nM, Thermo, M7514) for 30 min. Samples were washed twice in PBS and immediately analysed by flow cytometry.

For glucose uptake assay, neutrophils (0.5 x 10⁶) in glucose-free RPMI medium supplemented with the fluorescent glucose analogue 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG, Invitrogen, 30 µM, N13195) were activated with Zy/op (100 µg/mL) for 30 min, at 37 °C and 5 % CO₂. Neutrophils were washed twice in PBS and immediately analysed by flow cytometry.

To evaluate the expression of surface antigens, neutrophils were incubated with specific antibodies to GLUT1 (Abcam), Ly6G (BD Bioscience) CD15 or CD11b or the appropriate isotype controls for 1h. Viable cells were assessed by incubating cells with Fixable Viability Dye (Thermo).

The fluorescence of samples was measured in a flow cytometer (FACSVersé™, BD Biosciences) and analysed using FlowJo software (Tree Star). Ten thousand cells were analysed.

**Analysis of ECAR and OCR with the Seahorse XF Platform**

To evaluate the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR), neutrophils (0.1 x 10⁶) in Seahorse XF RPMI Medium without glucose (Agilent) were seeded in a 96-well XF Cell Culture Microplate (Agilent) pre-coated with Poly-lysine. Cells were left to adhere for one hour at 37 °C without CO₂. Neutrophils were activated with Zy/op (100 µg/mL), and ECAR and OCR were then measured on a Seahorse XF Analyser (Agilent) in basal conditions, and upon the sequential addition of glucose (10 mM), Rotenone/Antimycin (5 µM), and 2-deoxyglucose (2-DG; 50 mM).

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Prism 8 software (GraphPad) was used for data analysis. We observed normal distribution. Comparisons for two groups were calculated using unpaired two-tailed Student’s t tests and multiple comparisons by one-way ANOVA with Tukey’s post hoc test. Comparisons for the time course of skin infection were performed using two-way ANOVA with Bonferroni’s post hoc test. Data are represented as mean ± SEM or bacterial load as a median. Statistical significance: *p < 0.05.

Data Availability Statements

All data generated or analysed during this study are included in this published article (and its supplementary information files). Data sharing not applicable to this article as no datasets were generated or analysed during the current study.
**Figures Legends**

**Fig. 1** | PKM2 expression increases in activated neutrophils. 

- **a**, Pkm2 expression analysis in myeloid cells from the Immunological Genome Project (ImmGen) bulk-population RNA-seq database (Heng et al., 2008).
- **b**, Immunoblot analysis of PKM2 expression in BM-isolated neutrophils from wild-type (Pkm2\(^{fl/fl}\)) and PKM2-deficient (Pkm2\(^{fl/l}\)Lyz2) mice. Cells were activated with Zyl/op (100 µg/mL) or medium only (Ctrl) for 6h. β-actin was used as the loading control.
- **c**, Immunoblot analysis of PKM2 conformational states in murine neutrophils after activation with Zyl/op for 6h using DSS cross-linking assay.
- **d**, Confocal immunofluorescence analysis of Zyl/op-activated murine neutrophils stained for PKM2 (green) and DAPI (blue). The scale bar indicates 5 µm.
- **e**, Immunoblot analysis of PKM2 expression in blood-isolated neutrophils from three healthy volunteers. Cells were activated with Zyl/op for 6h. β-actin was used as the loading control.
- **f**, Confocal immunofluorescence analysis of Zyl/op-activated human neutrophils stained for PKM2 (green) and DAPI (blue). The scale bar indicates 5 µm.

**Fig. 2** | PKM2 deficiency impairs ROS production and microbial killing by neutrophils. 

- **a**, Phagocytosis of Zyl/op-FITC (100 µg/mL) by wild-type (Pkm2\(^{fl/fl}\)) and PKM2-deficient (Pkm2\(^{fl/l}\)Lyz2) neutrophils determined by flow cytometry. Representative dot plots, histograms and graphs showing the frequency and the median fluorescence intensity (MFI) of phagocytic cells. n = 3. 
- **b**, Kinetic measurement of ROS production by wild-type and PKM2-deficient neutrophils activated with Zyl/op determined by luminol-dependent chemiluminescence assay. Representative time-response graph and area under the curve (AUC) graph bar. n = 3. 
- **c**, ROS production by wild-type and PKM2-deficient neutrophils exposed to S. aureus (2 x 10\(^6\)) opsonized with serum. n = 3. 
- **d**, Quantification of viable S. aureus recovered from lysates of wild-type and PKM2-deficient neutrophils after 2h. n = 3. 
- **e**, ROS production by human neutrophils pre-treated with oxalate (3 mM) for 1h and then activated with Zyl/op. Representative time-response graph and area under the curve (AUC) graph bar. n = 3. 
- **f**, ROS production by human neutrophils pre-treated with TEPP-46 (30 µM) for 1h and then activated with Zyl/op. Representative time-response graph and area under the curve (AUC) graph bar. 
- **g**, Number of viable S. aureus recovered after 2h from lysates of human neutrophils pre-treated with oxalate or TEPP-46 for 1h. n = 5. Error bars are mean ± SEM. p values were determined by two-tailed unpaired Student’s t-test (a, d) or one-way ANOVA followed by Tukey’s post hoc test (b, c, e-g). *p < 0.05.

**Fig. 3** | Aerobic glycolysis is essential for ROS production in activated neutrophils. 

- **a**, Schematic representation of the 2 simultaneous assays pooled together in the graphs of Fig. 3b,c. 
- **b**, Kinetic profile of ECAR and ROS production in wild-type neutrophils activated with Zyl/op (100 µg/mL). ECAR and ROS production were measured in real-time by Seahorse and by luminol-dependent chemiluminescence, respectively, under basal conditions and in response to glucose, Rotenone/Antimycin, and 2-DG at the indicated time points. n = 5. 
- **c**, Kinetic profile of OCR and ROS production in wild-type neutrophils activated with Zyl/op. OCR and ROS production were measured in real-time by Seahorse and by luminol-dependent chemiluminescence, respectively, under basal conditions and in response to glucose, Rotenone/Antimycin, and 2-DG at the indicated time points. n = 5. 
- **d**, Illustration showing the glycolytic pathway and the steps where the drugs inhibit glycolysis. 
- **e**, ROS production by wild-type (Pkm2\(^{fl/fl}\)) and PKM2-deficient (Pkm2\(^{fl/l}\)Lyz2) pre-treated with 6-AN (3 mM) for 1h and then activated with Zyl/op for 1h. n = 3. 
- **f**, NADPH and total NADP production by wild-type and PKM2-deficient neutrophils activated with Zyl/op for 30 min determined by colorimetric assay. n = 4. 
- **g**, gp91-phox mRNA expression in wild-type and PKM2-deficient neutrophils activated with Zyl/op for 3h. Data were normalised to Gapdh; fold-change is relative to unstimulated control.
neutrophils. n = 5. h, Immunoblot analysis of gp91-phox and p47-phox expression in neutrophils from wild-type and PKM2-deficient mice. Cells were activated or not with Zy/op for 6h. β-actin was used as the loading control. i, ROS production by wild-type and human neutrophils pre-treated with 2-DG (3 mM), 3PO (10 µM) or oxalate (3 mM) for 1h and then activated with Zy/op for 1h. 5 = 3 and j, ROS production by wild-type and PKM2-deficient pre-treated with a PLC inhibitor (iPLC, 1 µM) for 1h and then activated with Zy/op for 1h. n = 5. k, Representative real-time ROS production in wild-type and human neutrophils activated with Zy/op. ROS production was measured by chemiluminesence under basal conditions and in response to glucose, 3PO (10 µM), and iPLC at the indicated time points. n = 3. Error bars are mean ± SEM. p values were determined by two-tailed unpaired Student’s t-test (f) or one-way ANOVA followed by Tukey’s post hoc test (b, c, e, g, i-k). *, p < 0.05. $, statistically different from untreated wild-type neutrophils activated with Zy/op. &; statistically different from untreated PKM2-deficient neutrophils activated with Zy/op.

Fig. 4 | Metabolic activity of PKM2 regulates ROS production in neutrophils. a, GLUT1 expression in wild-type (Pkm2<sup>+/+</sup>) and PKM2-deficient (Pkm2<sup>Δ/Δ</sup>) activated with Zy/op (100 µg/mL) for 1h determined by flow cytometry. Representative histogram and graph bar showing the GLUT1 median fluorescence intensity (MFI). n = 6. b, Glucose uptake in wild-type and PKM2-deficient neutrophils activated with Zy/op for 30 min determined by flow cytometry. Representative histogram and graph bar showing MFI for 2-NBDG. n = 6. c, Pyruvate Kinase (PK) activity in wild-type and PKM2-deficient neutrophils activated with Zy/op for 1h determined by colorimetric assay. n = 5. d, Pyruvate production by wild-type and PKM2-deficient neutrophils activated with Zy/op for 1h determined by colorimetric assay. n = 3. e, Lactate production by wild-type and PKM2-deficient neutrophils and by human neutrophils pre-treated with oxalate (3 mM) for 1h and activated with Zy/op for 1h determined by colorimetric assay. n = 5. f, Glycolytic metabolites from <sup>13</sup>C-glucose tracing in wild-type and PKM2-deficient neutrophils activated with Zy/op for 1h. The y-axis refers to the height of the chromatography peak obtained by LC-MS. n = 3. Error bars are mean ± SEM. p values were determined by one-way ANOVA followed by Tukey’s post hoc test. *, p < 0.05.

Fig. 5 | PKM2 modulates ROS production in neutrophils by a TPI-dependent mechanism. a, Illustration showing the glycolytic pathway and the steps where the drugs inhibit glycolysis. b, ROS production by wild-type neutrophils treated with 5-PDR (30 µM) or propranolol (30 µM) for 1h and then activated with Zy/op (100 µg/mL) for 1h. n = 3. c, Immunoblot analysis of p47-phox phosphorylation and p47-phox expression in wild-type (Pkm2<sup>+/+</sup>) and PKM2-deficient (Pkm2<sup>Δ/Δ</sup>) activated with Zy/op for 30 min. d, ROS production by wild-type and PKM2-deficient neutrophils and activated with Zy/op or PMA (50 nM) for 1h. n = 6. e, ROS production by wild-type and PKM2-deficient neutrophils and human neutrophils pre-treated with oxalate (3 mM) for 1h and then activated with Zy/op for 1h in the presence or not of OAG (10 µM). n = 3 and 6. f, ROS production by wild-type neutrophils pre-treated with oxalate or PEP (1 mM) for 1h and then activated with Zy/op for 1h. n = 3. g, DHAP production by wild-type neutrophils pre-treated with PEP for 1h and activated with Zy/op for 1h determined by a fluorometric assay. n = 3. h, G3P production by wild-type neutrophils pre-treated with PEP for 1h and activated with Zy/op for 1h determined by colorimetric assay. n = 4. i, TPI activity in wild-type neutrophils pre-treated with PEP for 1h and activated with Zy/op for 1h determined by colorimetric assay. n = 3. j, Immunoblot analysis of p47-phox phosphorylation and p47-phox expression in wild-type neutrophils pre-treated with oxalate, PEP, 5-PDR or propranolol for 1h and in wild-type and PKM2-deficient neutrophils. Cells were activated with Zy/op for 1h. k, TPI activity in wild-type and PKM2-deficient neutrophils and in human neutrophils pre-treated with oxalate for 1h and activated with Zy/op for 1h determined by colorimetric assay. n = 3 and 5. l, ROS production by scramble, PKM2-<sup>Δ</sup> or TPI-<sup>Δ</sup> deficient NB4 cells activated with Zy/op for 1h. n = 3. m, Working hypothesis. When PKM2 is absent or inhibited, PEP accumulates and inhibits TPI activity, decreasing DHAP accumulation and, consequently, DAG production, impairing PKC activation and then ROS production. Error bars are mean ± SEM. p values were determined by...
two-tailed unpaired Student’s t-test (I, k) or one-way ANOVA followed by Tukey’s post hoc test (b, d-h, l). *, p < 0.05.

Fig. 6 | PKM2-deficiency in myeloid cells impairs host defence against S. aureus infection. a, Schematic protocol of peritonitis infection with S. aureus. b, Bacterial load in the blood 18h after challenge in Pkm2<sup>fl/fl</sup> and Pkm2<sup>ΔLyz2</sup> mice. n = 7 and 5. c, Bacterial load in the exudate 18h after challenge in Pkm2<sup>fl/fl</sup> and Pkm2<sup>ΔLyz2</sup> mice. n = 7 and 5. d, Neutrophils cell counts 18h after challenge in Pkm2<sup>fl/fl</sup> and Pkm2<sup>ΔLyz2</sup> mice. n = 7 and 5. e, Mononuclear cell counts 18h after challenge in Pkm2<sup>fl/fl</sup> and Pkm2<sup>ΔLyz2</sup> mice. n = 7 and 5. f-h, Concentration of IL-6, CXCL2, and CCL2 in the plasma and exudate 18h after S. aureus challenge in Pkm2<sup>fl/fl</sup> and Pkm2<sup>ΔLyz2</sup> mice. n = 7 and 5. i, Schematic protocol of localised cutaneous infection with S. aureus. j, Mean of total lesion size (cm<sup>2</sup>) ± the standard error of the mean (SEM) evaluated for 11 days. n = 10. k, Representative pictures of the dorsal skin lesions in the back of Pkm2<sup>fl/fl</sup> and Pkm2<sup>ΔLyz2</sup> mice evaluated for 11 days. l, Bacterial load in the wound two days after challenge in Pkm2<sup>fl/fl</sup> and Pkm2<sup>ΔLyz2</sup> mice. n = 9. m, Myeloperoxidase activity evaluated in the wound two days after S. aureus challenge in Pkm2<sup>fl/fl</sup> and Pkm2<sup>ΔLyz2</sup> mice. n = 9. n-p, Concentration of CXCL1, TNF-α and IL-6 in the wound two days after S. aureus challenge in Pkm2<sup>fl/fl</sup> and Pkm2<sup>ΔLyz2</sup> mice. n = 9. Error bars are mean ± SEM. p values were determined by one-way ANOVA followed by Tukey’s post hoc test. *, p < 0.05.