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Summary

Cardiovascular disease, in which atherosclerosis is the major underlying cause, is currently the largest cause of death in the world. Atherosclerosis is an inflammatory disease characterized by the formation of arterial lesions over a period of several decades at sites of endothelial cell dysfunction. These lesions are composed of endothelial cells, vascular smooth muscle cells, monocytes/macrophages and T lymphocytes ($CD4^+$). As the lesions progress some can become unstable and prone to disruption, resulting in thrombus formation and possibly a myocardial infarction or stroke depending upon the location. Although the exact triggers for plaque disruption remain unknown, much recent evidence has shown a link between the incidence of myocardial infarction and stroke and a recent respiratory tract infection. Interestingly, many reports have also shown a link between a family of pattern recognition receptors, the Toll-like receptors, and the progression of atherosclerosis, suggesting that infections may play a role in both the progression of atherosclerosis and in inducing the more severe complications associated with the disease.

Keywords: atherosclerosis, infection, inflammation, myocardial infarction, TLR

Introduction

Cardiovascular disease is currently the leading cause of death in the world and is predicted to remain so for many years to come, placing a huge financial burden on the world's health resources [1,2]. The two main causes of death are ischaemic heart disease and cerebrovascular disease, which together accounted for 27·2% of all deaths in high-income countries, and 21·3% of all deaths in low- or middle-income countries in 2001. The majority of these deaths are caused by underlying atherosclerosis, where disruption of arterial plaques within the coronary or carotid arteries prevents or severely reduces blood flow to the target organ.

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease, characterized by the formation of lesions in the large- and medium-sized arteries, often at sites of disturbed blood flow. These arterial lesions, which can form over a period of several decades, are composed of endothelial cells, vascular smooth muscle cells (VSMCs), T lymphocytes ($CD4^+$) and monocytes/macrophages. The currently accepted response-to-injury hypothesis for the development of atherosclerosis suggests that these lesions or plaques form at sites of endothelial cell dysfunction with subsequent monocyte recruitment [3]. There are many causes of endothelial cell

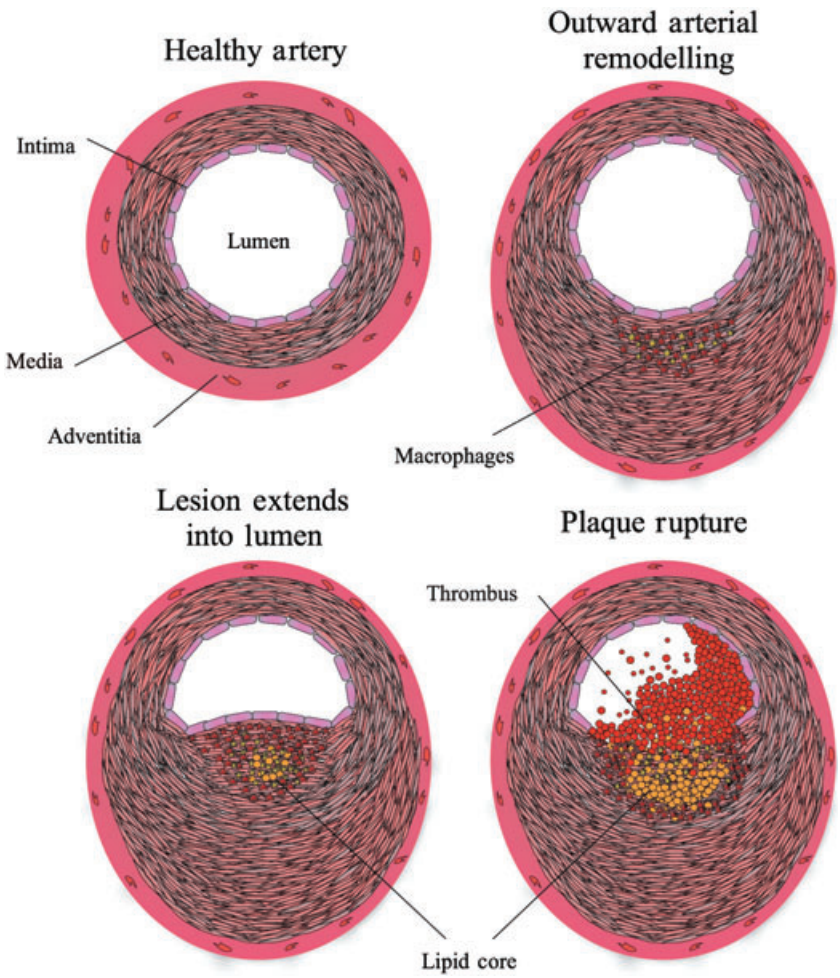

Fig. 1. The progression of atherosclerosis from healthy artery to rupture of the plaque and thrombus formation. (a) Structure of a healthy artery; (b) thickening of the media due increased smooth muscle cell and macrophage invasion, although the lumen size is maintained by outward arterial remodelling; (c) when a critical point is reached the growing plaque extends into the lumen, reducing the blood flow; (d) plaque rupture exposes the prothrombotic necrotic/lipid core to the blood, inducing thrombus formation.

dysfunction, such as the uptake of modified forms of low-density lipoprotein (LDL) [4], disturbed blood flow [5] and exposure to proinflammatory cytokines or pathogenic stimuli [6–8]. As the disease progresses, more inflammatory and smooth muscle cells are recruited to the growing lesion where they proliferate, further enhancing the size of the lesion. Outward arterial remodelling is associated with maintenance of the lumen size until a critical point is reached and the lesion begins to extend into the lumen [9], reducing blood flow. Mature plaques can become unstable, with rupture or erosion resulting in intravascular thrombosis and subsequent vascular occlusion, causing a myocardial infarction or stroke (Fig. 1).

The inflammatory nature of atherosclerosis

During the past two decades work conducted by many laboratories, including our own, has defined atherosclerosis as a chronic inflammatory disease [3,10,11]. Indeed, increased circulating concentrations of C-reactive protein (CRP), an acute phase protein whose release is indicative of an inflammatory response, is predictive of future cardiovascular events [12–14]. A major risk factor for atherosclerosis is elevated levels of modified forms of LDL, which induce a variety of proinflammatory effects in the various cell types involved in atherosclerosis. These modified forms of LDL are formed when high plasma concentrations of native LDL induce its own transport across the endothelium into the subendothelial space and intima. Here it is retained through its interaction with matrix components [15] and undergoes modifications catalysed by products released from endothelial cells, VSMCs and macrophages [16]. Modified forms of LDL induce proinflammatory effects by binding to scavenger receptors such as CD36 and class A macrophage scavenger receptor (SR-A) on macrophages, and the lectin-like oxidized low-density lipoprotein scavenger receptor (LOX-1) on endothelial cells, macrophages, VSMCs and platelets [17].

During the initiation of atherosclerosis, the initial tethering of monocytes and T lymphocytes to the area of endothelial cell dysfunction is mediated through endothelial cell CD62P and CD62E interacting with their respective carbohydrate ligands [18]. More stable interactions between the monocytes or T lymphocytes and the endothelium are mediated through vascular cell adhesion molecule 1 (VCAM-1) binding its integrin ligand $\alpha 4\beta 1$ (VLA-4) on the inflamma-

tory cell [19,20]. Tightly bound monocytes and T lymphocytes can then enter the intima and subendothelial space by diapedesis at junctions between the endothelial cells. Following their transmigration into the subendothelial space and intima, monocytes differentiate into macrophages and up-regulate their scavenger receptor expression, allowing them to take up modified LDL [21]. This uptake leads to formation of lipoprotein-derived cholesterol and cholesterol esters leading to the development of foam cells, which along with T lymphocytes form the earliest type of lesion, the fatty streak [22].

As the atherosclerotic lesion progresses VSMCs migrate from the media into the intima, under the control of growth factors such as platelet-derived growth factor and insulin-like growth factor released by activated macrophages and endothelial cells [23]. Once within the intima, the VSMCs are able to proliferate and synthesize large amounts of extracellular matrix, increasing both the size and the stability of the plaque. This proliferation is controlled by other growth factors, including fibroblast growth factor, which is also released from activated macrophages and endothelial cells [24].

Cytokines, chemokines and inflammatory lipids in atherosclerosis

Monocyte recruitment to the intima and subendothelial space occurs by chemotaxis, mainly towards monocyte chemoattractant protein-1 (MCP-1/CCL2) [25], although a role for other chemokines such as regulated upon activation normal T cell expressed and secreted (CCL5) [26] and interleukin (IL)-8 (CXCL8) [27] has been suggested. T lymphocytes are recruited by the interferon (IFN)-γ-inducible cytokines inducible protein-10 (IP-10) (CXCL10), monokine induced by IFN-γ (MIG) (CXCL9) and IFN-inducible T cell alpha chemoattractant (I-TAC) (CXCL11) [28].

Uptake of modified LDL by macrophages induces the release of a plethora of proinflammatory mediators including IL-1β, tumour necrosis factor (TNF)-α, IL-6, IL-8, MCP-1 and several growth factors [29]. These mediators recruit further inflammatory cells to the lesion and induce the activation of endothelial cells and VSMCs, leading to the release of more proinflammatory mediators and up-regulation of adhesion molecules. Increased IL-6 enhances the release of acute phase proteins, including CRP, from the liver, enhancing inflammation further [30]. More cells are then recruited to the growing lesion, where they can be activated in this continuing cycle of inflammation.

One further axis with a key role in the progression of atherosclerosis is CD40/CD40L. Both the receptor and the ligand are expressed on many of the cell types associated with atherosclerosis [31]. CD40 is up-regulated on endothelial cells in response to IL-1β, TNF-α and IFN-γ, and interaction with CD40L can induce the expression of VCAM-1 and CD62E and the release of IL-6 and IL-8 [31]. CD40/CD40L interactions also increase the release of IL-1β and TNF-α from monocytes and macrophages [32] and the expression of tissue factor on macrophages, endothelial cells and VSMCs [31].

Recently there has been much interest in the role of lipids in the progression of atherosclerosis, especially in the prostanoids and lipoxins. Prostanoids include prostaglandins, thromboxanes and prostacylins, and are formed from arachidonic acid through a pathway involving cyclooxygenase [33]. Lipoxins are anti-inflammatory eicosanoids also derived from arachidonic acid through a pathway involving lipoxygenases [34]. The role of both cyloxygenases and lipoxygenases in atherosclerosis is complex because of their ability to metabolize arachidonic acid into both pro- and anti-inflammatory mediators, capable of influencing the various stages of the disease [33,35].

The IL-1β is a key mediator involved in the progression of atherosclerosis. Along with its naturally occurring antagonist, IL-1ra, it is expressed in endothelial cells, VSMCs and macrophages. Atherosclerotic plaques express significantly raised levels of IL-1β [36] and IL-1ra [37] compared with control arteries, particularly within the endothelium, and there are increased concentrations of circulating IL-1β in patients suffering from unstable angina [38]. Secretion of IL-1β is regulated tightly and efficient release requires a primary stimulation which induces up-regulation of pro-IL-1β, followed by a second stimulation such as ATP accumulation to induce activation of the inflammasome and processing of pro-IL-1β to the mature biologically active form [39]. IL-1β induces adhesion molecule expression, vascular permeability, leucocyte migration, macrophage activation and VSMC proliferation [40], ultimately driving atherosclerosis and plaque instability.

The IL-1β has been implicated in the progression of atherosclerosis using animal models, with reduced atherosclerosis observed in IL-1β [41] and IL-1 receptor-deficient mice [11], while IL-1ra-deficient mice exhibit increased neointima formation [42]. IL-1Ra administration to Apo E-deficient mice or porcine arterial injury models reduced neointima formation significantly [11,43] as well as reducing vascular remodelling following acute myocardial infarction in the rat [44]. Recently, small-scale human trials using anakinra, the recombinant form of IL-1ra, showed improved vascular and left ventricular function and reduced endothelial nitro-oxidative stress in rheumatoid arthritis patients [45]. The potential for IL-1ra to reduce inflammatory markers after a non-ST elevation myocardial infarction is under investigation in a multi-centre study [46].

Atherosclerotic plaque disruption

The presentation of atherosclerotic disease as myocardial infarction or stroke is caused by disruption of atherosclerotic plaques which can become unstable, and have either rup-

tured their fibrous caps, exposing blood to the prothrombotic contents, or when the surface of the plaque has become eroded, exposing the prothrombotic matrix. Under basal or stable conditions the atherosclerotic plaque is composed of lipid contained within macrophages (now called foam cells) or within a necrotic core, VSMCs which synthesize collagen to give the cap mechanical strength, a few T lymphocytes and an overlying endothelial cell layer [10].

Disruption of the plaque involves a proinflammatory switch within the cells of the plaque. This switch matches well with a T helper type 1 proinflammatory phenotype when IFN-γ can activate an M1 phenotype in macrophages and causes inhibition of VSMC proliferation [47]. Macrophages within an activated or vulnerable plaque (vulnerable to rupture or causing thrombosis) express matrix metalloproteinases capable of digesting the fibrous cap [48,49], as well as tissue factor, on their cell surface [50]. Tissue factor is the essential co-factor for activation of the coagulation cascade and will produce thrombin upon exposure to blood following rupture.

Plaque erosion may be responsible for up to 30% of clinical presentations of atherosclerosis [51]. Here the cap of the plaque becomes eroded in such a way that subendothelial matrix is exposed to blood and produces thrombin, presumably because of expression of platelet adhesion molecules and tissue factor. The mechanism of plaque erosion is unknown, but apoptotic endothelial cells are known to express tissue factor [52]. The pathophysiological inducer of endothelial cell apoptosis is unknown.

Infections can increase the risk of myocardial infarction and stroke

Although many of the factors that predispose atherosclerotic plaques to rupture have been identified, the exact triggers for plaque disruption are as yet unknown. An early indication that infections may play a role in altering plaque stability was suggested by the seasonal distribution of myocardial infarctions. Analysis of data from 1400 hospitals in the United States of more than 250 000 cases of acute myocardial infarction reported that there was a 53% increase in hospital administrations because of myocardial infarction in the winter months compared with the summer [53]. This work confirmed earlier observations from a previous smaller study [54]. These results led to the hypothesis that severe respiratory tract infections, which occur at higher frequencies during the winter months, may play a role in inducing plaque disruption. Evidence supporting this hypothesis was provided by numerous reports showing that vaccination against influenza had a positive effect on reducing the incidences of ischaemic events [55]. One small study of 218 patients who had suffered a myocardial infarction reported lower levels of influenza vaccinations within the group that suffered further myocardial infarctions during the period of study, with vaccination associated with a 67% reduction in subsequent myocardial infarctions [56]. Similarly, it was also reported that there was a lower percentage of influenza vaccination among patients suffering out-of-hospital primary cardiac arrest [57], and among patients who had suffered an ischaemic stroke [58], when compared with the control groups. A large-scale study on the effects of influenza vaccination on ischaemic disease used the data from three large managed-care organizations of more than 280 000 patients who were aged at least 65 years. This study found that vaccination reduced the risk of stroke by 16–23% and the risk of cardiac disease by 19% [59]. These data imply clearly that influenza vaccination has the potential to prevent many deaths each year from myocardial infarction and stroke, with one report suggesting that up to 90 000 deaths could be prevented each year in the United States alone [55].

Additional evidence for the role of infection in inducing myocardial infarctions and strokes is provided by several studies showing an increased risk of these events following severe respiratory tract infections. The first of these analysed the United Kingdom General Practice Research Database (UKGP) and reported a transient but substantial increase [odds ratio (OR) 3·0] in the risk of myocardial infarction between 1 and 10 days after a reported respiratory tract infection, with this risk returning to normal after a period of several weeks [60]. Two more recent larger studies have confirmed the link between respiratory tract infections and myocardial infarctions. Again using the UKGP database, one research group analysed the records of more than 125 000 patients who had suffered a first or subsequent myocardial infarction or stroke. Patients who had a systemic respiratory tract infection had an increased risk of suffering their first myocardial infarction (OR 4·95) and stroke (OR 3·19) for the period of 3 days after the diagnosis, with the risk gradually falling and returning to normal after several weeks [61]. There was also an increased risk of a subsequent myocardial infarction (OR 3·14) and stroke (OR 2·57) during the 3-day period after diagnosis of a systemic respiratory infection. This study also found an increase in the risk of myocardial infarction or stroke following a urinary tract infection, although this was smaller than that following a respiratory tract infection, and has not been a universal finding [61]. Other work using the IMS Disease Analyser Mediplus database (IMS) studied the records of more than 20 000 patients who had suffered a first myocardial infarction or stroke [62]. This study showed an increased risk of myocardial infarction (OR 3·75) and stroke (OR 4·07) for the period of 3 days after a respiratory tract infection, which again returned to normal after several weeks. Further evidence for the role of infections in myocardial infarction was provided by analysis of autopsy-confirmed deaths in St Petersburg, Russia, from 1993 to 2000. For every year studied, the peak rate of deaths from myocardial infarction occurred during the influenza epidemic and peak acute respiratory disease activity [63].

Although evidence for the role of infections in increasing the risk of myocardial infarction and stroke is growing, the

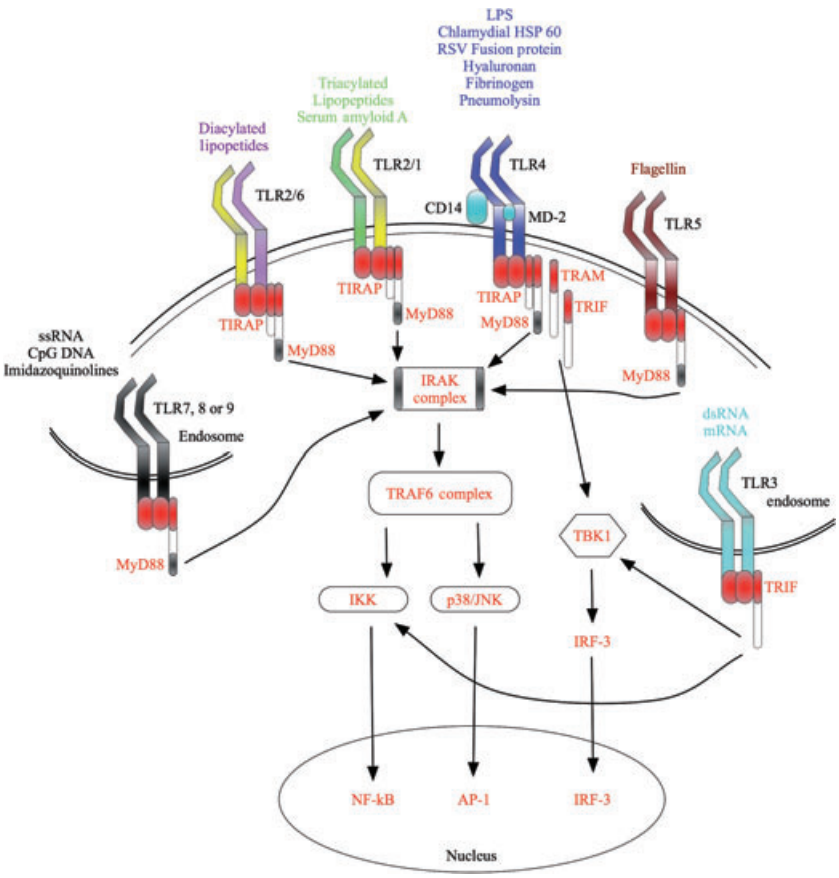

Fig. 2. Toll-like receptor (TLR) activation. TLRs are activated by a number of different agonists, and are expressed either on the cell membrane or intracellularly on the membranes of endosomes. The extracellular receptors TLR-1, -2, -4, -5 and -6 respond predominantly to bacterial products as well as some endogenous agonists, whereas the intracellular receptors TLR-3, -7, -8 and -9 respond to nucleotides of viral and bacterial origin. The figure also shows a very simplified TLR signalling pathway, showing how many structurally different agonists can induce activation of similar pathways.

mechanisms by which this occurs remain unclear. Recent evidence suggests that the inflammation caused by a systemic infection may alter the structure of the plaque, causing an increase in the number of inflammatory cells and therefore making the plaque more prone to disruption. Whether this is through infection and activation of cells within the plaque, activation of cells which then enter the plaque or through the release of inflammatory mediators which activate cells within and around the plaque is unknown. Indeed, Apo E-deficient mice infected with influenza had increased numbers of inflammatory cells and smooth muscle cells in the subendothelial infiltrate, and increased superficial platelet aggregation when compared with uninfected Apo E-deficient mice [64]. Other studies have reported an alteration in human plaque structure in response to infections. Autopsy samples of atherosclerotic plaques from patients who were diagnosed with an acute systemic infection were analysed and compared with those who had no infection. This revealed increased numbers of macrophages in the adventitia, more T lymphocytes in the adventitia and periadventitial fat and more dendritic cells in the intima and media of those patients who had suffered an infection [65]. T lymphocytes isolated from atherosclerotic plaques were more responsive to influenza A virus than those purified from peripheral blood of the same donor, suggesting a mechanism of influenza-induced plaque inflammation [66]. It therefore seems as though infections may alter the structure of the atherosclerotic plaque, causing an increase in the number of inflammatory cells and reducing the stability.

Toll-like receptors in atherosclerosis

With atherosclerosis defined clearly as an inflammatory disease, and the suggested link between infections and ischaemic events, many groups have shown a link between many infectious organisms such as *Chlamydia pneumoniae*, *Porphyromonas gingivalis*, *Helicobacter pylori* and cytomegalovirus, and the progression of atherosclerosis. The roles for these organisms in atherosclerosis have been extensively reviewed elsewhere [67–71]. Recently, interest in the role of Toll-like receptors (TLRs) in the progression of atherosclerosis has increased. TLRs are a family of pattern recognition receptors that function to enable responses to a wide range of both pathogen-associated molecular patterns and damage-associated molecular patterns [72–88] (Fig. 2), and there is evidence of activation of their signalling pathways within the plaque [89]. TLRs are expressed on all cell types associated with atherosclerotic plaques, such as monocytes [90], endothelial cells [91], VSMCs [74] and platelets [92]. TLR expression is up-regulated on endothelial cells exposed to endogenous mediators of inflammation such as histamine [93,94], and also on macrophages and endothelial cells asso-

ciated with plaques [95]. Similarly, the level of both TLR-2 and TLR-4 mRNA is increased with time in arterial tissue obtained from Apo E-deficient mice, and TLR-2 and TLR-4 expression on circulating monocytes is increased at 40 weeks in Apo E-deficient mice compared with controls [96]. Modified forms of LDL can enhance macrophage TLR-4 expression in atherosclerotic plaques [97], and agonists of TLR-3 can inhibit macrophage efflux of cholesterol [98], increasing foam cell formation. Animal studies have confirmed a role for TLRs in atherosclerosis, with atherosclerosis-prone mice deficient in TLR-2 or TLR-4 showing reduced atherosclerosis development when compared with those animals with functional receptors [99,100]. Recently, *C. pneumoniae*-induced enhancement of the progression of atherosclerosis in Apo E-deficient mice was shown to be dependent upon TLR-2, TLR-4 and MyD88 [101].

The TLR-4 is the receptor for lipopolysaccharide (LPS), and many studies have shown that this may influence the progression of atherosclerosis. In the Bruneck study, which looked at the number and size of atherosclerotic plaques in the carotid artery over a 5-year period, it was found that circulating LPS was a risk factor for atherosclerosis progression in smokers and patients with a chronic infection [102]. Similarly, two studies reported that weekly injections of LPS into rabbits fed on a high cholesterol diet enhanced atherosclerosis progression [103,104] and that LPS treatment of the adventia enhanced cellular migration into the media and intima, enhancing lesion size [105]. The TLR-2 agonist, Pam_3CSK_4, also enhanced lesion development in Apo E-deficient mice [106].

Conclusions

There is a clear role for inflammation and infections in both the progression of atherosclerosis and in the serious complications resulting from plaque disruption such as myocardial infarction and stroke. Many of the inflammatory mediators involved in mounting an effective inflammatory response are also involved in the exacerbation of atherosclerosis, so it is unsurprising that a family of pattern recognitions receptors, the TLRs, have been implicated in the disease. A key molecule involved in atherosclerosis is IL-1β, and TLR agonists are some of the most potent inducers of IL-1β release known. As we understand more about the complex signalling mechanisms that act to enhance inflammation, then we may be able to target novel, specific pathways that influence strongly the progression of atherosclerosis.

In terms of plaque disruption, it is clear that a severe infection increases the risk of myocardial infarction or stroke dramatically, but more research is required to determine the exact mechanism so that we can intervene therapeutically. Work from our group has indicated that far more complex signalling networks exist between endothelial cells and monocytes in a simple co-culture model of acute inflammation (data in preparation). The challenge is to dampen down the inflammatory response sufficiently around the time of infection to reduce the risk of ischaemic events, while not allowing the pathogen to take advantage of an impaired inflammatory response.

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