Biofilms and chronic wound inflammation

In contrast to the commonly accepted hypothesis of host-centred pathology, it is possible that surface bacteria, not host dysfunction, cause the chronicity and perpetual inflammation associated with chronic non-healing wounds

pro-inflammatory cytokines; proteases; host-immune response; host inflammation

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The host immune system is governed by three principles: universality, tolerance and appropriateness (Box 1). Chronic wounds are 'stuck' in a persistent inflammatory state that disobeys these principles.[1-5] All chronic wounds contain elevated pro-inflammatory cytokines, high protease levels (matrix metalloprotease and elastase) and excessive neutrophils. Attempts to manage them from a host-centred perspective have yielded only small, incremental improvements in healing outcomes.[6] This paper proposes this may be explained by the presence of a wound biofilm and hypotheses on how it develops.

Hypothesis

Although host factors such as abnormal white blood cell function, diabetes or venous insufficiency contribute to the onset of a chronic wound, we suggest that host dysfunction rarely prevents healing directly. Rather, the impotence of the initial immune response allows bacteria to establish a biofilm community in the wound. This community is difficult to eradicate and so perpetuates inflammation, which promotes chronicity. Chronic cutaneous wounds have specific biochemical and cellular similarities in spite of their dissimilar aetiologies. The commonality may be the presence of biofilm.[7,8]

Biofilm infections are only partially responsive to antibiotics.[9] Primary signs of infection, such as erythema, swelling, heat and pain, are often incompletely expressed because much of the antigenic portions of the bacteria (which induce the immune response) are shrouded by the biofilm matrix.

Secondary signs of infection — exudate, stalled healing and undulating exacerbations (waxing and waning signs of infection) — predominate. Clinical manifestations of this undulation are increased exudate, maceration and tenderness one week, following by a quiescent or 'stable' state for several weeks.

Host immunity is impotent against biofilm infections because white blood cells,[10] antibodies[11] and complement[12] are often unsuccessful in resolving the biofilm challenge.[13]

Biofilm and host interactions

Up to 90% of cells in the human body are attached or surface-associated bacteria.[14] These bacteria are generally natural, commensal and often mutualistic (mutually dependent) with the host and occur on most external host surfaces such as the skin,[15] gastrointestinal tract and mouth.[14] Disruptions or dramatic shifts in the constituents of these communities are associated with diseased states. Often, rather than the well-understood infection with a pathogenic bacterium, the change to a diseased state represents the phenotypic and genotypic reorganisation of commensal bacterial populations, such that they convert from commensal interactions with the host to pathogenic interactions. The chronic wound biofilm is one of the most interesting and accessible reservoirs for pathogenic bacteria that might otherwise be characterised as commensal organisms.[7,8]

A wound biofilm hypothesis needs to explain the almost steady-state interaction between the pathogen and host, and to account for several chronic wound phenomena. We propose the following two components in our hypothesis:

- The biofilm community interacts with host tissue, resulting in stable attachment, sustainable nutrition and a parasitic relationship (Figs 1 and 2)

Box 1. Three laws of immunity

Universality

An immense diversity of targeted immune responses can be generated towards almost any potential challenge (up to 10^{15} different antibody receptor combinations are possible)

Tolerance

Major barriers prevent or limit responses to self and non-pathogens (eg, only one in seven T cells pass the 'tolerance' test and are allowed into the body's circulation)

Appropriateness

Responses only target 'pathogens'. The magnitude of the response is appropriate to the threat, and an effort is made to not hurt the host (eg, commensals are 'neglected' by the immune system and local infections do not cause a systemic response)

Box 2. Definition of terms

Adaptive immune response — initiated when the host immune system recognises a microorganism as a previously encountered pathogen. This enables the adaptive immune response to develop an immune response against the pathogen. As the response is specific to the target, its action is more effective than that of the general innate response

Adhesins — cell wall-anchored molecules on bacteria that bind tightly to specific locations (epitopes) in the host

Innate immune response — unlike the adaptive immune system, the innate immune elements does not recognise a pathogen, although it is generally able to distinguish foreign invaders from self. The innate immune response acts against invading microorganisms in the same manner despite repeated exposures. As such, the innate response does not adapt or improve its effectiveness. There are three basic components of the innate immunity: barriers such as the skin and mucosa, non-specific chemicals such as lysozymes and complement, and non-specific effector cells such as neutrophils and macrophages

Planktonic — used to describe single-celled, non-attached, free-floating bacteria

Predation — a predatory behaviour that seeks to kill and devour the food source. This is not a sustainable source of nutrition (as it kills the host)

Pro-inflammatory cytokines — initial molecules mainly secreted by innate immune cells to direct the local immune response. They function as a tightly regulated system that rapidly recognises the threat and responds appropriately

Quorum sensing — bacteria continually secrete molecules that communicate information on population density. When enough signal is present to make a critical density of bacteria (quorum), the molecules coordinate their gene expression. This produces a biofilm community incorporating different gene expression and function (differentiation). Quorum sensing is responsible for phenotypic diversity and some of the genotypic diversity seen in wound biofilms

Virulence factors — bacteria have specialised proteins or molecules that enable them to develop and maintain a niche in the host organism. These factors often damage the host, promote attachment and elicit or slow the immune response. If a bacterial gene promotes or enhances a microorganism's ability to cause disease, it can be defined as a virulence factor

- The host tissue develops conflicting immune and tissue repair mechanisms, demonstrated by a localised hyper-inflammatory response, which is unable to overwhelm the biofilm. This immune response facilitates chronic infection. The host's interaction with the biofilm provides the necessary nutrients to sustain the bacterial community in a steady state among perpetual host inflammation.

Wound biofilm: an overview

Many reviews and texts have explored the nature of biofilms.[9,16-20] Here, we attempt only to describe aspects of biofilms as they relate to wound biofilm.

Biofilms do not comprise a particular type or species of bacterial cells. Rather, they are '…groups of bacteria (often multiple genotypes) held together by extracellular polymeric substances (polysaccharides, proteins, DNA), associated with a surface, and resistant to environmental stresses that can overwhelm a lone bacterium.'[16]

Bacteria within biofilm communities are influenced by pheromone systems (quorum sensing — see Box 1 for definition), which can modulate the community's activity and development by directing its components to differentiate into specific roles. In this way, the biofilm structure enhances the community's nutrient-gathering capacity, defence and reproductive abilities.[21] Genotypic and phenotypic diversity within the community improves the fitness of the biofilm by increasing the likelihood that at least a portion of the cells will survive when challenged with chemical, physical or biological stresses.[7]

One of the most important characteristics of a biofilm, which will play an important role in the proposed hypothesis, is that the biofilm community is in constant flux. Competent (viable) fragments of biofilm often slough into the local environment.[22] In addition, up to 30% of the biofilm population can differentiate on a daily basis into a single-cell (planktonic) phenotype. Some of these bacterial 'seeds' survive predation (see Box 1) and begin biofilm formation on uncolonised surfaces or are incorporated into existing biofilms in another location.[23]

Initial attachment

During *de novo* biofilm development, planktonic bacteria initiate irreversible attachment (Fig 1) when the bacterium senses appropriate environmental cues such as nutrients, proximity to a surface, quorum-sensing molecules, pH, salts and host epitopes (the part of an antigen molecule to which an antibody attaches itself). The bacterium's actions are controlled by elegant pathways, defined as two-component systems.[24-26] Numerous transcription regulators coordinate the phenotypic changes that result in attachment, where as many as 800 proteins within a single species alter their expression.[27]

Staphylococcus aureus is an example of the alteration in genetic expression induced during the switch from free-floating to surface-associated physiology. The process of attachment stimulates numerous cell-wall anchored effectors (adhesins), which attach to host epitopes. Such adhesins include:

- Cna, which binds collagen[28]
- ClfA and ClfB, which bind fibrinogen[29]
- FnbpA and FnbpB, which bind fibronectin.[28]

The abundance of receptors targeting host extracellular matrix epitopes and haeme (the ferrous, non-protein part of haemoglobin responsible for oxygen transportation) allows the bacterium to quickly recognise that it is within a favourable host environment.[30] Staphylococci possess a haeme sensory system (HssRS) that alerts the bacterium inside the host and prompts radical changes in the bacterium. A pioneering bacterium such as *Pseudomonas aeruginosa* quickly shifts (10 hours) its metabolism to express a biofilm phenotype as it settles in the host environment[31] (Fig 2).

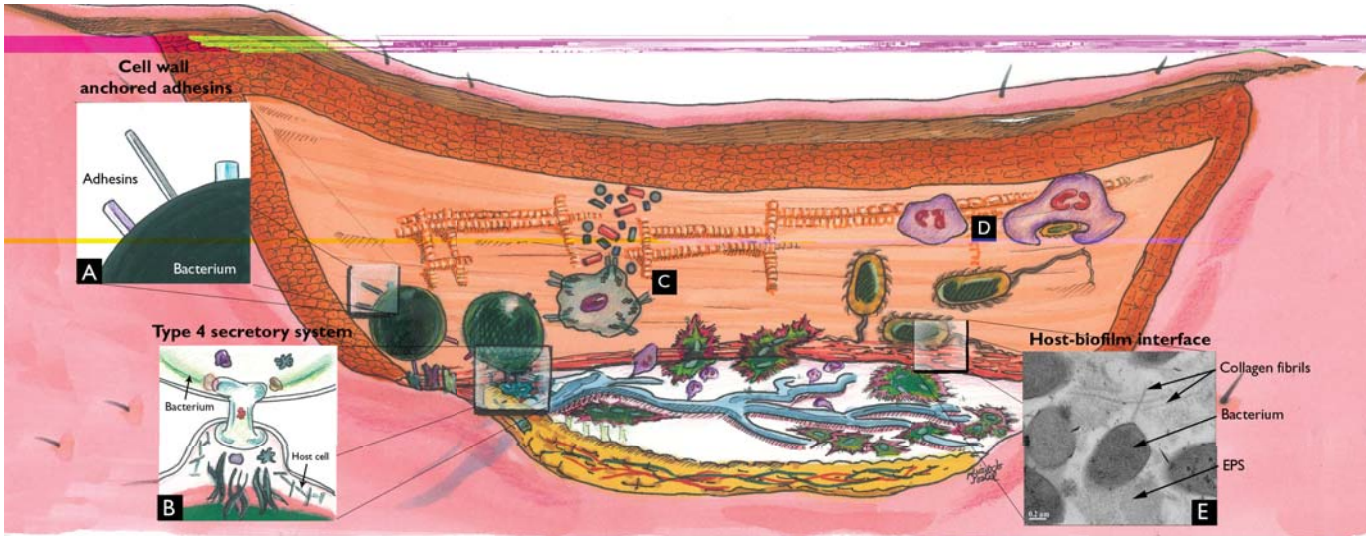

Fig 1. Bacterial attachment. This is the first committed step a bacterium takes to form a biofilm. Bacteria use several mechanisms to attach to specific molecular patterns (epitopes) in the host extracellular matrix. Most bacteria possess cell wall-anchored adhesins that bind to specific epitopes in collagen, fibronectin, vitronectin and other extracellular matrix components (a). Some bacteria inject effector proteins into host cells. This polymerises actin filaments, producing a 'pedestal' to which the bacteria can attach indefinitely (b). This provides a firm attachment site. To attach, planktonic bacteria must avoid macrophages and neutrophils (c and d). Macrophages and neutrophils have receptors that recognise pathogen-associated molecular patterns (PAMP) and in turn activate the phagocytes to clear single-cell bacteria. If it can evade the innate immune system for a short time, *Pseudomonas aeruginosa* can identify an acceptable site for binding with its flagellum (e). Following the initial binding, the cell will rotate 90 degrees so that its type IV pili can firmly adhere to the host epitopes. To prevent biofilm formation, the host immune system must respond forcefully and rapidly to prevent bacterial attachment. Once the biofilm is established, the host's immunity may be unable to overcome the infection without medical intervention

Sustained nutrition

Once firmly attached on a stable substrate, the cells require nutrition to grow and reproduce. By upregulating virulence factors, a host-associated biofilm can expand its territory by necrosing host tissues and acquiring nutrients from them. When this process occurs rapidly, it may present clinically as necrotising fasciitis.[32] However, the speed with which necrotising fasciitis kills its host is unusually rapid, and we suggest this disease is more representative of bacterial predation than the bacterial parasitism, the latter being commonly observed in chronic wound infections.

Typically, necrotising fasciitis does not occur. Instead, the wound size appears to be controlled. This may be because a chemical communication system within the biofilm (quorum sensing) interacts with host signals, or due to the host's ability to 'wall off' the infection (as in tuberculosis).[33]

Once the biofilm community is established, it can adjust virulence factors.[34] Downregulation of virulence factors suggests that an established wound biofilm does not rely on locally necrosed host tissue as its primary source of nutrition. It must therefore derive nutrients from other sustainable sources. We suggest this sustainable nutrition is delivered to the biofilm via the host's circulatory system in the form of nutrient-rich plasma and iron from lysed red blood cells. Clinically, this nutrient source is observed as exudate.

This suggestion is supported by Schaber et al., who observed biofilm organising around capillary basement membranes within hours of an infection in a burned-mouse model (Fig 2f).[35] Using traditional theories of infection, it is counterintuitive that bacteria should seek the host vasculature as a primary source of attachment because this places them in close proximity to white blood cells and antibodies. However, bacteria encased within the biofilm matrix are largely unperturbed by the host's immunity. We suggest that the wound biofilm can sustain its nutrient source by manipulating capillary transudate (gradual discharge of fluid through a membrane's pores), thereby effectively controlling nutrient flow by dictating host-mediated inflammation.

Host-immune response: an overview

Historically, the model system for the study of the host immune response to bacterial infection has been in the context of free-swimming, community-independent bacteria in acute clinical situations. By examining the immune system response to bacteria that are free-living, free-moving and naked to host defences (not in a biofilm), science and medicine

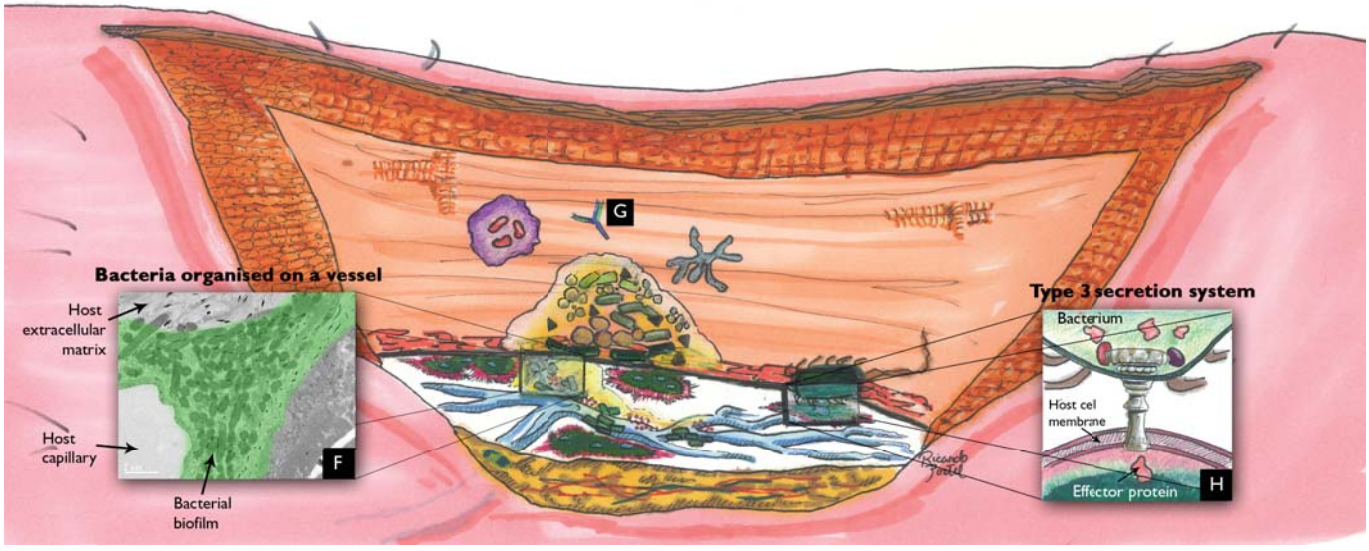

Fig 2. Community establishment: once the bacterium is attached and has performed several cell divisions, it forms a microcolony. The microcolony, along with other species/strains, embeds itself in a common matrix. Very early in the infection, the bacteria selectively organise within certain tissues, such as the basement membranes of vessels in the wound bed (f). The matrix, which is composed of self-secreted polymers including polysaccharides, proteins and DNA, as well as host components, produces a barrier that is recalcitrant to white blood cells, opsonisation and all other host immune strategies (g). Some bacteria possess the type 3 secretion system apparatus that allows translocation of as many as 40 different effector proteins, which can commandeer host cell function (h)

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have described how humans easily overwhelm these unprotected pathogens. However, when it encounters chronic biofilm infections, the immune system faces bacteria with different behaviours, phenotypes and defences. It is necessary to consider the immune system's reaction to bacteria and apply that understanding to chronic infections.

The host has highly developed methods of recognising pathogens. Not only can the host distinguish whether a bacterium it encounters is a risk, but it also seems capable of identifying which type of pathogen it has encountered. This pattern-recognition system, which is comprised of different pattern recognition receptors (PRRs), recognises a large variety of bacterial fragments — and is termed pathogen-associated molecular pattern (PAMP).[36]

To recognise pathogens, the host uses a family of proteins known as Toll-like receptors, which not only recognise PAMP but also initiate the host innate immune responses through nuclear factor Kappa B (NF-kB). This innate immune response is considered the first line of defence against invading pathogens.

Once Toll-like receptors have recognised the presence of a pathogen, neutrophils are rapidly mobilised and recruited to combat the bacterial invaders. The immune system uses a complex system of pro-inflammatory cytokines, including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), interferon-gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-8 (IL-8) and interleukin-6 (IL-6).

In concert with other pro-inflammatory cytokines, IL-8 closely regulates the recruitment and activation of neutrophils — highly destructive first responders. Interleukin-8 is a chemotactic factor that stimulates the CXCR1 receptor (a large transmembrane receptor specific for IL-8) on neutrophils. This stimulation causes neutrophil migration and activation of oxidative and proteolytic enzymes. Recruiting cytokines such as IL-8 also upregulate bactericidal activity in macrophages, dendritic cells and mast cells, producing the characteristic innate immune response.[37]

As the innate response progresses, the bacterial invaders are labelled with complement and further processed by the immune system (opsonisation), allowing the newly recruited phagocytes to recognise the target. Neutrophils bind and engulf the bacteria, and ultimately digest them.[38] These neutrophils are in turn digested by macrophages to prevent neutrophil-derived proteases from extensively damaging the host.

When functioning properly, the innate system is a quick, non-specific responder that overcomes potential pathogens. But when the system is compromised by host comorbidities, bacteria gain an advantage that can prove difficult to reverse.

The immune system, once attacked, develops long-term defences against the invading pathogens,

known as adaptive immunity (acquired immune response). In this way, the host can recognise and remember pathogens, and mount much stronger, less self-destructive attacks when confronted with the bacteria in the future. This system acts in concert with the innate system to increase the efficiency of the killing response through the recruitment of cell-mediated and antibody responses.

Biofilm hijacks the host immune response

Chronic wounds are held in a persistent inflammatory state, similar to inflammatory patterns seen in other chronic infections such as cystic fibrosis, periodontal disease and inflammatory bowel disease, many of which have been linked to biofilm.[39,40] We hypothesise that the wound biofilm establishes a persistent nutrient source by commandeering the host immune response to achieve a steady state of hyper-inflammation, which is out of the host's control. We will describe how wound biofilms can use generalised mechanisms, rather than specific virulence factors, to hijack the host immune response and improve its own fitness and maintain its niche.

Hyper-inflammation

An almost universal physiological characteristic of chronic wounds is that pro-inflammatory cytokines are highly upregulated.[41,42] These can persist indefinitely at concentrations 1000 times above the normal level.[2,43]

One pathway for inducing pro-inflammatory cytokines is associated with pathogen-associated molecular patterns. Two primary Toll-like receptors associated with pathogen recognition are TLR4 and TLR2, which recognise both Gram-negative bacterial lipopolysaccharide and Gram-positive bacteria teichoic acids.[41] The TLR2 receptor also responds to degraded fragments of CXCR1 receptor.

The wound biofilm can overwhelm these receptors by generating an overabundance of substrate (a substance acted upon by enzymes) and effectively overrunning the expression of pro-inflammatory cytokines, which is normally tightly controlled in a healthy, innate (Th1) immune response.

This state of perpetual inflammation in a chronic wound is commonly considered a host abnormality. It is thought the host is deficient in switching from

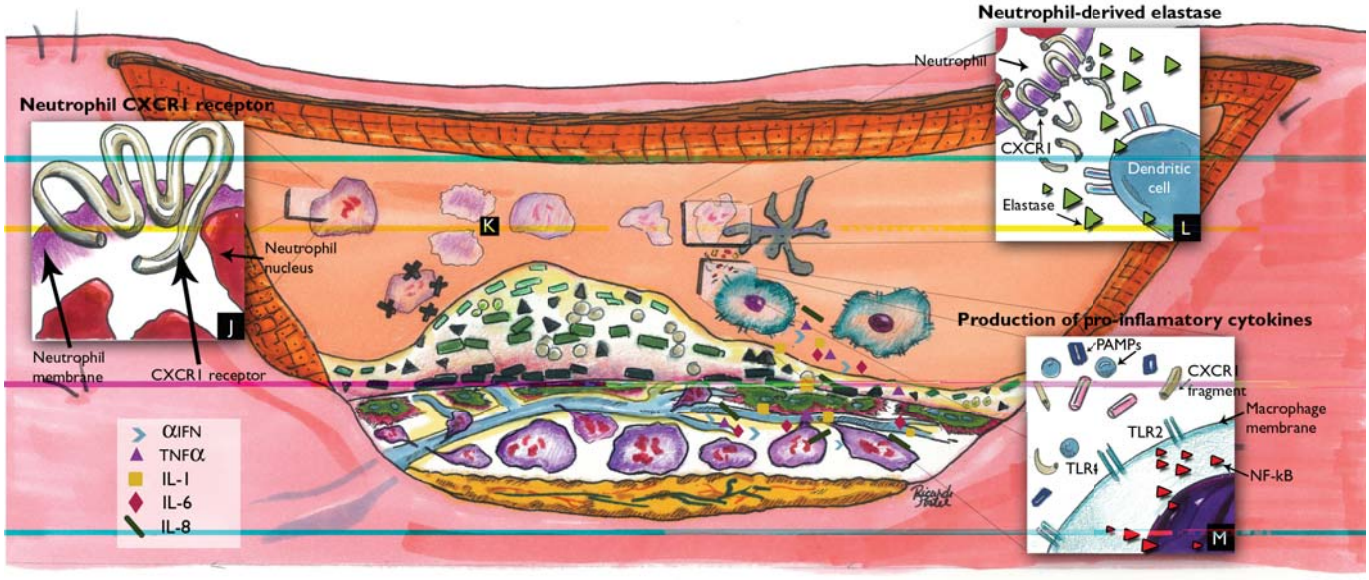

Fig 3. Biofilm maintenance: once the bacteria have attached, established a protected community, organised themselves within the tissue and subverted host cellular function, they maintain a sustainable inflammatory niche by manipulating the host immune response. Chronic wounds contain excessive neutrophils. Interleukin-8 receptor (CXCR-1), which is bound within the neutrophil's membranes, activates the neutrophil and causes it to migrate into the wound (j). Many bacteria possess virulence factors that kill neutrophils (k). If they are not engulfed by macrophages, the dying neutrophils release large amounts of elastase into the environment. This neutrophil-derived elastase degrades the CXCR-1 receptor on neutrophils, causing them to stagnate and preventing them from being properly phagocytised by macrophages. The fragments of the CXCR-1 receptor stimulate Toll-like receptor-2 (TLR2) on dendritic cells and macrophages to produce a massive release of pro-inflammatory cytokines (l). The action of pathogen-associated molecular patterns (PAMP) and fragments of CXCR-1 work through a nuclear factor Kappa B pathway (NF-kB) to release pro-inflammatory cytokines (m). Tissue inhibitors of matrix metalloproteases (TIMPs) produced by the wound bed cells are deficient and do not downregulate the production of pro-inflammatory cytokines. The proinflammatory cytokines create gaps in the endothelial cells of the capillaries, allowing neutrophils to migrate through the vessel wall into the wound bed (diapedesis). This completes a sustainable cycle in which bacteria invoke excessive neutrophil migration and cause persistent inflammation

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an innate immune to an adaptive immune response.

The traditional theory of inflammation predicts that the underlying cause of the perpetual inflammation is the dysfunctional innate response, mediated by T-helper 1 cells and their release of pro-inflammatory cytokines. It would therefore be an appropriate therapeutic strategy to direct the host towards a Th2 (adaptive) response in the wound, which would decrease the level of pro-inflammatory cytokines expression.[44] However, there is little evidence that a Th2 environment in the wound is any more successful in healing the wound.

In contrast to the traditional theory of wound inflammation, we hypothesise that, in a chronic wound, the biofilm hijacks the host immune response. The host immune response is not 'broken' (host-centred pathology), but rather the host defences are commandeered by the entrenched pathogenic biofilm (bacteria-centred pathology).

Hijacking mechanisms

There is an emerging understanding that 'bacterial pathogens express a large array of virulence factors that dampen and/or reorient both [the] innate and adaptive immune response'.[45] Studies have demonstrated that bacteria can upregulate pro-inflammatory cytokines in order to manipulate the host's innate immune response. For example, Shigella expresses a plasma virulence gene that increases expression of pro-inflammatory cytokines.[46] Quorum-sensing molecules from *Pseudomonas aeruginosa* can act directly on host cells to induce expression of pro-inflammatory cytokines.[47] *Staphylococcus aureus* (and other pathogens) express 'modulins' and superantigens (enterotoxins, toxic shock syndrome toxin-1), which can induce massive and sustainable release of pro-inflammatory cytokines.[48] Additionally, the type III secretion systems of pathogens inject protein effectors into host immune cells, dampening phagocytosis and reducing the lethal actions of neutrophils, thereby increasing the release of pro-inflammatory cytokines into the wound (Fig 3).[49]

Our hypothesis does not focus on specific virulence genes, but on general bacterial strategies that turn the host immune response against the host.

General mechanisms of inducing host inflammation

Biofilms continually release planktonic 'seeds' of bacteria from the safety of the biofilm matrix, which can 'bait' the immune system (via PAMP recognition mechanisms) and continually recruit the host's inflammatory response. The biofilm can harvest nutrients from the host exudate that accompanies the inflammatory response. In this way, the sacrifice of a few bacteria promotes the survival of the community through continual nutrient acquisition. Suppressing the host response with steroids or physically removing the bacterial load through debridement may reduce the nutrients available to the bacteria.

A major component of Gram-negative bacteria cell walls is lipopolysaccharide, which decreases neutrophils' response to IL-8 and fouls phosphatidyl serine.[50] Through these pathways, lipopolysaccharide is a potent inducer of continued chemotaxis of neutrophils to the site of the wound biofilm. Thus, the biofilm does not need specific pathogens with powerful virulence factors to maintain an inflammatory response, but only requires an accessory population of Gram-negative bacteria that release lipopolysaccharide. Biofilms also constantly release lipopolysaccharide and membrane vesicles made up of lipopolysaccharide (of previously unknown function) into the surrounding environment.[51,52]

As well as releasing planktonic cells and cell-wall components into the local environment, the biofilm releases its own genetic material (bacterial DNA) into the surrounding matrix.[53] This also stimulates inflammatory recruitment in the localised tissue.[54]

Thus, unlike the single pathogen, biofilms have an arsenal of generalised tools that can stimulate, maintain and ultimately control an inflammatory state. Meanwhile, the heart of the biofilm remains intact and unabated by the host response.

Manipulating neutrophils

Individual bacterial populations such as *Pseudomonas aeruginosa* (which often populate wound biofilms) have developed virulence factors, such as pyocyanin, that prevent the removal of neutrophils.[55] However, wound biofilms can use generalised mechanisms to produce similar results.

The literature indicates that excess neutrophils accumulate at the site of chronic wounds[2,56] and many other chronic infections.[57-61] Interleukin-8 in and around the wound biofilm promotes chemotaxis of neutrophils by binding their CXCR1 and CXCR2 receptors (Fig 3j).[62] However, as stated above, bacteria use lipopolysaccharide and other factors to degrade these receptors.[50,63,64] In addition, lipopolysaccharide can interfere with the neutrophils' membrane-associated phosphatidylserine.

In a healthy immune response, neutrophils begin apoptosis after engulfing a pathogen. They express proteinaceous surface molecules, such as phosphatidylserine, which macrophages interpret as a request for phagocytosis.[65-67] Macrophages recognise, engulf and then degrade these functionally terminal neutrophils, preventing necrotic disintegration of the neutrophil *in situ*.[66-70]

A localised accumulation of neutrophils at the infected site is a healthy host response, but this accumulation must be accompanied by the orderly elimination of neutrophils by macrophages in order to control the inflammatory response.[71] When bacterial products like lipopolysaccharide interfere with

the phosphatidylserine, the macrophages may not recognise the neutrophils. The latter degrade in the presence of the wound biofilm and release elastase, which interferes with wound healing.

We suggest that neutrophils respond to and are drawn to the presence of the wound biofilm. There they encounter and engulf sacrificial planktonic bacteria, but are also acted upon by virulence factors, which induces an apoptotic cycle.

At this point the neutrophil requests phagocytosis, but the pro-inflammatory cytokines in the wound interfere with the cellular signalling.[70] The neutrophils become trapped and are induced into a delayed apoptotic process[69,72] or self-destruct.[73]

The neutrophils are inefficiently cleared from the site of infection, resulting in localised release of proteolytic enzymes (elastase, metalloproteases and inflammatory mediators) into the wound bed.[72,74,75] Elastase then degrades the neutrophil's CXCR1 receptors, fragments of which stimulate TLR2 receptors to produce pro-inflammatory cytokines.

This completes the cycle that produces the consistent or chronic biochemical pattern of all biofilm disease, especially wound biofilm, and results in the development of unregulated proteases and release of pro-inflammatory cytokine, which damages the host. This mechanism of sustained inflammation does not depend solely on host comorbidity, but rather upon a biofilm infection.

Wound inflammation is perpetuated because the neutrophils disintegrate *in situ*, releasing proteinases and pro-inflammatory cytokines. This causes a continued influx of inflammatory cells and plasma into the locale of the wound biofilm, thereby overwhelming the host's anti-protease defences.[76] This communication breach in the immune system at the site of chronic wounds results in a major protease/antiprotease imbalance,[76] which is common in biofilm diseases.[68]

General mechanisms to maintain chronic inflammation: proteases

Chronic wounds are highly proteolytic. Although bacteria can produce numerous proteases, chronic wounds seem to possess mainly excessive host-derived proteases.[4] Studies have demonstrated that these proteases are elastase, collagenases (MMP8 and MMP1), and gelatinases (MMP2 and MMP9), and mostly originate from the innate immune response.

We suggest that the biofilm causes this host response and that protease activity may therefore be related to biofilm activity. In a vicious cycle, effete neutrophils release proteolytic enzymes (mainly elastase). Macrophages are less able to recognise and remove these cells, thereby promoting necrotic disintegration. The soluble fragments from the host elastase-degraded CXCR1 receptors can stimulate TLR2 receptors, producing additional pro-inflammatory cytokines that feed the inflammatory cycle and recruit additional neutrophils. This perpetual cycle produces and sustains elevated levels of inflammation,[77] which discourages healing.

Proteases and elastase induce apoptosis in other host cell types, including local tissue, that should be involved in the wound repair process.[78] This highly destructive host milieu is even more remarkable for what it is missing — mainly tissue inhibitors of metalloprotease (TIMP). It is unclear if this is secondary to either the bacterial subversion of host wound bed cells responsible for TIMP production or the physical barrier created by the biofilm's tight adherence to the wound bed surface. This is the basis of the protease/anti-protease imbalance seen in chronic infections such as wound biofilm. As we have explained, this state can be induced and maintained using the generalised mechanisms inherent in any biofilm, even biofilms lacking 'well-known' pathogens. The pathological consequence of unopposed protease activity is sustained host tissue damage and chronic inflammation.[79,80]

Interestingly, many of these neutrophil-released proteases may directly benefit the wound biofilm. One example is *Staphylococcus epidermidis*, which requires activated accumulation-associated protein to initiate biofilm formation. Host proteases have been found to proteolytically process accumulation-associated protein, which activates the bacteria's adhesion function.[81,82] Rohde et al. conclude that, 'it is therefore reasonable to assume that *in vivo* effector mechanisms of innate immunity can directly induce protein-dependent *Staphylococcus epidermidis* cell aggregation and biofilm formation, thereby enabling the pathogen to evade clearance by phagocytes'.[81]

We have explored one pathway of how neutrophils are usurped by biofilm to produce inflammation and release excess proteases at the infection site. It is possible that macrophages and dendritic cells that regulate the inflammatory response may also be manipulated by the biofilm. Macrophages and dendritic cells can abandon their regulatory role when confronted with a potent invader and pursue a 'delayed-type hypersensitivity' approach of 'slash and burn' to confront the challenge.[83]

When chronic wounds begin to heal, they sometimes exhibit this behaviour. This is observed clinically as lysing layers of the wound bed, tendon and fragments of bone, and the subsequent removal of these infected fragments by wound contraction, whereby the material moves up to the surface of the wound bed and is expelled. A wound biofilm may block or manipulate this potentially useful defence.

The exact biochemistry and cellularity observed in a wound will depend on the species constituting the biofilm, expressed virulence factors, host immune competence and yet to be recognised factors. However, a successful pathogenic biofilm will need to

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maintain infusion of nutrient-rich plasma exudate by promoting inflammation and prevent wound healing by maintaining high protease levels. Traditionally, a single pathogen is thought to be capable of causing disease, but it may be possible that species of what may be considered traditionally 'non-pathogenic' bacteria can maintain a chronic infection by working as functionally equivalent pathogroups.

Functional equivalent pathogroups

Functional equivalence dramatically alters the single-pathogen paradigm of infection. Our work on diversity of bacteria in chronic wounds[7,8] strongly indicates the possibility of a 'functional equivalence' theory in relation to wound biofilm.

An increase in genetic diversity within a biofilm is commonly associated with an increase in its ability to withstand environmental stress. Based on what is understood about biofilms to date, a wound biofilm that demonstrates a single predominant pathogenic species should be less robust and easier to suppress than a genetically diverse biofilm.[84] A genetically homogenous infection would be evidenced clinically by an infection that responds quickly and completely to a simple treatment strategy, such as antibiotics. In contrast, wound biofilms require multiple concurrent therapeutic strategies that must be adjusted frequently during the course of healing.[6] The recalcitrance of some wounds to single-treatment strategies may be explained, therefore, by their diversity and the generalised mechanisms of pathogenesis described earlier.

For a diverse community of species to act as a unified pathogenic entity (functional equivalent pathogroups) that produces a persistent infection, it must collectively possess properties that allow genetically homogenous pathogens to be successful. Thus, diverse biofilms must possess a 'functional equivalence' that allows bacteria to attach, organise and maintain a hyper-inflammatory wound environment.

The significance of this concept may be vital to developing improved methods of treating wound biofilms. The hypothesis that 'non-pathogenic' bacterial species might act symbiotically as part of an unified biofilm community to promote a chronic wound biofilm infection is unique. Several pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* may possess all of the functions necessary to initiate and sustain a wound biofilm on their own, but a functionally equivalent diverse population may require several cooperating species to achieve the same degree of infection. One species within the population may attach to host epitopes; a second species may self-secrete or organise host components into a protective matrix; a third species may perpetually release lipopolysaccharide and planktonic seeds, inducing a perpetual inflammatory state; a fourth species may co-aggregate the community. If the appropriate species can coexist, then a genetically diverse biofilm with a pathology that is functionally equivalent or superior to that of a single successful pathogen may be able to form a robust and recalcitrant wound biofilm.

Conclusion

Pathogenic biofilms cause the host to violate the laws of universality, tolerance and appropriateness. The majority of biofilms can (and often do) slowly debilitate their hosts unless the infections are surgically removed.

The principle of universality is mainly exhibited in the adaptive immune system, with its random sequence generators. These almost endless sequences result in T-cell receptors, B-cell receptors and antibodies that can recognise a 'universal' number of antigens. However, a biofilm's colony defences can prevent penetration by white blood cells and antibodies.[85-87] This renders the host immunity's 'universality' irrelevant to biofilm.

Tolerance is not only applied to self-antigens but also to commensal and mutualistic bacteria. Our mutualism with certain bacterial species in permissible host locations is complex but is clearly governed by the principle of tolerance.

Biofilm does not promote the process of tolerance. In fact, it inhibits the host from downregulating its immune response or developing any sort of tolerance to the prolonged presence of an infectious biofilm. Pathogen-associated molecular pattern, superantigens, degradation of phosphatidylserine and CXCR1, seeding cells and generalised bacterial factors (lipopolysaccharide, DNA fragments) perpetuate a destructive level of host inflammation.

The lack of appropriateness is what sets biofilm diseases apart from other infections. Acute infections that have rapidly growing bacteria tend to destroy host tissue through bacteria-derived virulence factors. However, chronic biofilm infections can destroy tissue by using host-originated factors inappropriately. Biofilm may regulate host protease production in a wound to 'balance' the destruction of host tissue with any host healing that is taking place, or the biofilm mechanism may simply arrest healing.

Many wound experts believe the destructive levels of pro-inflammatory cytokines and proteases in chronic wounds are present because of abnormal immune responses. Suggested causes of these dysfunctions include abnormal host genetics and comorbidities such as repetitive trauma, malnutrition or poor perfusion. Currently recognised immune 'abnormalities', such as dysfunctional dendritic cells,[88] aberrant T cells,[89] reduced TIMP[4] and excessive neutrophils,[56] may be better explained by the presence of bacterial pathogens and functionally equivalent pathotypes that organise as wound biofilms that commandeer the host immune response. ■

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