The study of immune responses in *Drosophila* has already yielded significant results with impacts on our understanding of vertebrate immunity, such as the characterization of the Toll receptor. Several recent papers have focused on the humoral response to damage signals rather than pathogens, particularly damage signals from tumour-like tissues generated by loss of cell polarity or chromosomal instability. Both the triggers that generate this sterile inflammation and the systemic and local effects of it are only just beginning to be characterized in *Drosophila*. Here we review the molecular mechanisms that are known that give rise to the recruitment of *Drosophila* phagocytes, called hemocytes, as well as the signals, such as TNFα, that stimulated hemocytes emit at sites of perceived damage. The signalling consequences of inflammation, such as the activation of JNK, and the potential for modifying this response are also discussed.

1. Introduction

The inflammatory response to infection by pathogens has been intensively studied for many years both in humans and in all the major model organisms. More recently, there has been increasing interest in understanding the situations in which inflammation arises without an external pathogen [1]. These include almost any stimulus that gives tissue damage, such as burns, as well as autoimmune disease, atherosclerosis, stroke, and cancer. The molecular details of these self-induced inflammatory responses are now becoming clearer, though there appears to be a wide variety of triggers and outcomes that range from beneficial to lethal [2]. To make sense of the complexity and sort out causes from effects, model organisms amenable to genetic manipulation can be extremely useful. In this review we will focus on recent progress in understanding the causes and effects of sterile inflammation in *Drosophila*, which has many advantages for this kind of work.

The immune system in *Drosophila* is relatively simple: they lack adaptive immunity but have a robust innate immune system that has many functional and molecular similarities to that of vertebrates [3]. The immune cells in flies are collectively described as hemocytes; in normal animals they consist primarily of plasmatocytes with a phagocytic role as well as some crystal cells for melanization and clotting [4, 5]. The humoral innate immune response includes several antimicrobial peptides (AMPs), which can be produced from most epithelia, and upon infection are generated at high levels from the fat body, the equivalent of the vertebrate liver [6]. In addition there are a range of extracellular signalling molecules that are used to identify the presence of pathogens and trigger an inflammatory response [5]. These triggers include well known factors such as components of bacterial cell walls but also less well understood mechanisms such as an extracellular protease cleavage cascade that results in the activation of the IL-1R-like receptor Toll and the NFκB pathway. Although the inflammatory response in *Drosophila* lacks several features of vertebrate inflammation, such as heat, redness, and extravasation of leucocytes, some signalling pathways regulating the response are conserved and indeed were discovered in *Drosophila*. In both insects and mammals there is the recruitment of immune cells to the affected site and the release of chemicals and peptides intended to damage pathogens; this is the process we are describing as inflammation.

It has become clear that, in the case of sterile inflammation, although there is no pathogen present, the inflammatory response is often similar to that seen in infection, and many
of the same pathways are used [1, 5]. The inflammatory triggers, however, are not from a pathogen and must be generated by changes to normal cells that expose altered or mislocalized self-molecules to the immune system to generate a damage signal. These signals, known as damage-associated molecular patterns (DAMPs), are currently the subject of intensive research and may include extracellular chromatin, ATP, cytoskeletal molecules, and mitochondrial components [2]. In vertebrates, these are detected by diverse receptors including the many Toll-like receptors (TLrs), but in flies the situation is likely to be less complex. In the next section we will examine the types of cellular damage that can give rise to sterile inflammation during larval life in Drosophila and the molecular triggers involved.

2. Sources of Sterile Inflammation

Triggers in Drosophila

Many DAMPs are the normal molecules of the cytoplasm or nucleus that become immunogenic when exposed to extracellular environment. For example, in case of necrosis, nuclear or mitochondrial DNA is released into the extracellular environment and acts as a DAMP. Other DAMPs identified in vertebrates include high mobility group box 1 (HMGB1), reactive oxygen species (ROS), cytoskeletal molecules, nucleotides (e.g., ATP) and nucleosides (e.g., adenosine), uric acid, phosphatidylserine (PS), heat shock proteins (HSPs), hyaluronan, heparan, syndecan, and probably others which are still unidentified [2]. Some of them (e.g., nucleotides) are conserved between species and also shared by all types of tissue injuries [7].

2.1. Necrotic Cells. Necrosis is the main source of damage signals in many tissue injuries such as tumors, thermal effects, mechanical trauma, ischemia, hypoxia, and apoptosis-mutants. Acidification and the oxidative environment of necrotic cells are thought to cause proinflammatory changes to DAMPs inside and out of the cell. For example, high mobility group box-1 (HMGB1) is a nonhistone, DNA binding protein that has been implicated as a DAMP in vertebrates [2, 8]. As a result of ROS, partially oxidised HMGB1 is released out of the necrotic cells and binds to extracellular mediators of inflammation (such as ssDNA or lipopolysaccharides) and promotes activation of Toll-like receptors [9]. This mechanism has not been studied in detail in Drosophila, but we have found that loss of HMGB1 reduces sterile inflammation (our unpublished results). The release of DNA from necrotic cells may also contribute to a conserved inflammatory response, as Drosophila mutants that block DNaseII function show a humoral response [10] with similarities to vertebrate signalling [11].

Reactive oxygen species are also released from necrotic cells in Drosophila and act as an immediate damage signal which may trigger the recruitment of hemocytes to the injured tissue [12–14]. ROS and TNFα (Eiger) released from necrotic neuronal cells can trigger JNK activation in surrounding cells [12]. The activated JNK pathway triggers apoptosis, hemocyte recruitment, and wound healing [12, 15]. This occurs at least partly by activation of matrix metalloproteinases (MMPs) which can result in the production of DAMPs by digesting basement membrane [16, 17], though their primary function is one of repair [18]. TNFα, phosphatidylserine (PS), and other DAMPs have been shown to enhance the activation of the prophenoloxidase activating system (PAS) at the site of injury. Activation of the PAS melanizes wound clots and other encapsulated tissues or pathogens [19], as well as triggering a systemic response [20].

2.2. Undead Cells. Undead cells, such as cells that fail to apoptosis due to caspase mutations [21], are known to promote the activation of the extracellular protease Persephone as a trigger of the innate immune response [22, 23]. Persephone acts as a sensor in the hemolymph, which informs the insect about the presence of stress, damage, or pathogens [24]. The trigger for Persephone in sterile inflammation is not known, but we speculate that the release of necrotic material (e.g., intracellular proteases) can trigger activation of Persephone and the cleavage cascade that produces a systemic immune response [3, 5, 6, 22]. For example, it is known that some soluble DAMP in the hemolymph is required for the systemic immune response seen in apoptosis mutants [22]. Restraining the systemic activation of the immune proteases are serpins such as Necrotic, which is expressed ubiquitously and helps in establishing a localized signal gradient at the site of damage by damping the overall proteolytic activity in the body. This localization of the signal assists the recruitment of hemocytes [19].

2.3. Wounds. Wounding, in a nonpathogenic environment, promotes a similar activation of the immune response as described above, because wounds contain both necrotic and stressed cells. Sterile wounding in Drosophila is thought to stimulate the pathogen response as a protective measure against expected infection [25, 26], though, at least in adults, the intensity of the response may be less than that for an infection [27]. In sterile wounding, release of DAMPs at the wound site has been proposed to result in activation of Persephone and differentiation of lamellocytes from precursor hemocytes [4, 5, 24, 28]. Lamellocytes are involved in the encapsulation of target tissue (normally degenerating tissues and oversized pathogenic invaders) and then in melanizing them via activation of the phenoloxidase cascade. Basement membrane (BM) disruption acts as a trigger for the immune response in wound regions as well as metastasizing tumors [16, 29, 30]. Laminin is a major component of the BM which acts as a checkpoint for self/nonself and normal/damaged tissue. It acts as an inhibitory ligand for hemocytes [17, 31] and is also important for cell integrity. Cell integrity (cell-cell adhesion and apicobasal polarity) of self-tissues also acts as a determinant for the immune response. Loss of both BM and cell integrity is required to target an otherwise self-tissue for encapsulation by lamellocytes [17].

2.4. Tumors. Tumor interactions with the immune system are typically required for their growth and metastasis [4]. Tumour growth generates signals that have been linked
to hemocyte proliferation and recruitment [32–34]. In *Drosophila*, tumor cells activate TNFα, Pvr/Pvr, and the Toll pathway to trigger the systemic immune response (see below). The loss of apicobasal cell polarity often seen in malignant outgrowth also induces recruitment of hemocytes and encapsulation [33]. Triggering mechanisms have not been explored in detail, but expression of an oncogene (RasVT12) in *Drosophila* showed hyperplastic growth and increased expression of metalloproteinases [34, 35]. This is relevant because increased expression of MMPs causes degradation of basement membrane, which leads to inflammation [16, 34]. In addition, exposure of phosphatidylserine on the surface of RasVT12 mutant cells [34] can trigger the prophenoloxidase activating system which gives melanisation and encapsulation [19].

Finally, cancer cells often exhibit a high rate of genetic change due to chromosomal instability (CIN). CIN can provide variability and adaptability but at the cost of generating ROS and cellular stress which often results in cell death [36, 37]. Release of cellular debris from CIN tissue gives both localized and systemic activation of the Toll pathway (our unpublished data). CIN also leads to DNA damage [36–38]. Unrepaired DNA damage in *Drosophila* elicits an innate immune response which leads to systemic activation of JAK/STAT signaling, hemocyte proliferation, and melanization [16, 39, 40].

### 3. Effects of the Inflammatory Response

Inflammation in *Drosophila* typically results in the production of antimicrobial peptides and the recruitment of hemocytes [4, 5, 41]. Antimicrobial peptides are not known to have strong effects on the organism in the absence of a pathogen, though they can potentially affect neural tissue [42] and promote autoimmunity [43]. Recruitment of hemocytes, on the other hand, has profound implications for the tissue involved as well as for the animal as a whole.

A population of hemocytes constantly circulates in the haemolymph, having access to the basal surface of most organs and tissues. As described above, there are a number of signals released by damaged or aberrant tissues that lead to the accumulation of hemocytes at the site, in a process that is thought to involve capture of passing hemocytes rather than active migration, at least in the larva [29]. In a sterile wound, both plasmatocytes and crystal cells gather, degranulating to release clotting factors as well as a range of signalling molecules [5]. These signals include the TNFα homolog Eiger and the cytokines Unpaired-3 and Spaetzle (Figure 1), showing clear similarity to vertebrate sterile inflammation [2].

#### 3.1. Cytokine Signaling

The production of the NGFβ homolog Spaetzle [44] by hemocytes is primarily thought to drive systemic rather than local immune responses in *Drosophila*. For example, activation of the Spaetzle receptor Toll just in hemocytes does not improve immune responses [45]. Instead, the principal immune effect of the Spaetzle signal is seen in the fat body [33], equivalent to the vertebrate liver, which responds by becoming the primary source of antimicrobial peptides [46, 47]. Recent work has shown, in response to tissue dysplasia, that Spaetzle activating Toll in the fat body is also needed to drive TNFα mediated cell death in the aberrant tissue [33]. Spaetzle is a highly regulated signal, being secreted as a proprotein that requires protease cleavage in order to be active. A wide range of extracellular proteases that are either produced by bacteria or activated by bacterial molecules are known to generate active cleaved Spaetzle during infections [5]. In sterile inflammation, activation of the protease Persephone is probably required [22, 23], though how it is regulated is not known. We speculate that the same necrotic cell death that attracts hemocytes can release normally intracellular proteases that trigger Persephone and the cleavage cascade that produces active Spaetzle. The molecular pathway by which the Spaetzle receptor Toll activates the humoral immune response has been analysed in detail and closely parallels the vertebrate pathway [3, 5, 6]. Still relatively unknown, however, are the transcriptional outputs of this pathway in response to DAMPs, beyond a handful of antimicrobial peptides. We do not know, for example, what targets of NFκB might be relevant for the fat body and Toll-dependent death of tumour tissue [33]. Presumably this is mediated by the fat body signalling to increase the release of TNFα on the tumour by hemocytes, but the molecules used are not known. Spaetzle is also implicated in cell competition, where it activates signalling via Toll-like receptors to kill relatively unfit cells [48]. The source of Spz and the involvement of hemocytes in this process have not yet been determined.

Hemocytes also release the IL-6 related cytokine Unpaired-3, which is produced in a feedback response to Unpaired signalling from wounds or tumours [16]. Damaged tissue activates the JNK pathway which increases the transcription of Unpaired, Unpaired-2, and Unpaired-3, which are secreted by the tissue to activate JAK/STAT signalling in hemocytes that have been recruited, as well as from the fat body. JAK/STAT signalling produces more hemocyte secretion of the Unpaired cytokines in a positive feedback loop as well as driving hemocyte proliferation and lamellocyte differentiation [49]. This system resembles a simplified version of the mammalian use of interleukins and JAK/STAT signalling in inflammatory responses [50].

#### 3.2. Tumor Necrosis Factor Signalling

While Spaetzle and Unpaired have systemic effects on hemocyte numbers, the primary effector molecule secreted by hemocytes in sterile inflammation is TNFα (Eiger) [51, 52]. TNFα is clearly secreted by hemocytes that have been recruited to sites of cellular damage [33] and possibly also by unstimulated hemocytes [53, 54]. TNFα signalling through the TNF receptor Wengen has two well described effects: activation of JNK signalling and cell death [52]. Strong and persistent activation of JNK leads to increased transcription of the proapoptotic genes *hid* and *reaper* [55], which TNFα also activates by a parallel pathway involving the TRIp homolog Nopo [56]. Consequently, cell death is a significant feature of normal inflammatory responses in *Drosophila* larvae. However, it
is important to bear in mind that JNK also has many other functions [57], so, for example, if its apoptotic role is blocked, Eiger-JNK signaling can contribute to proliferation and metastasis [58–60]. Furthermore, JNK signalling can be protective in neural tissue, and this ROS-mediated protection by JNK activation is needed to survive even sterile wounds [20].

It is interesting to consider what constitutes the targeting signal of the innate immune response. Hemocytes, as the primary detectors of damage or pathogens, release active Spaetzle to give systemic Toll activation and Unpaired to drive hemocyte proliferation, but this does not explain how the response is focused on the site of infection/damage [53]. It appears that hemocyte recruitment and retention is essential to localize the response. Reacting to the as-yet poorly defined damage signals, hemocytes potentially reinforce their localization by generating more local damage. The release of TNFα causes cell death as well as JNK activation, which drives the secretion of basement membrane proteases such as MMP1, which is sufficient to generate hemocyte-localizing damage, as described above. Furthermore, we have found that local activation of Toll in the target tissue is essential for the normal apoptotic response. In this case, signalling through Toll/NFκB in defective tissue activates JNK to produce MMP1 and recruit hemocytes (our unpublished data). This constitutes a local amplification loop perhaps resembling the vertebrate Toll-like receptor p75 that drives both NFκB and JNK [52].

3.3. Reactive Oxygen Species. The role of reactive oxygen species in sterile inflammation is still unclear, though undoubtedly significant. Several ROS molecules have been implicated in antibacterial responses [3, 61] and in these cases they are typically generated to damage pathogens but also to stimulate hemocytes to generate a systemic response [62]. Less is known about how ROS might act in sterile inflammation: they can be produced by TNFα signalling and contribute to the resulting cell death [63], and they act with or without growth signals to activate JNK signalling in different tissues [20, 64]. The hydrogen peroxide produced by damaged tissue is necessary, at least in Drosophila embryos and zebrafish, for the recruitment of hemocytes or leukocytes [14, 65]. These studies implicate the calcium flash from wounding in triggering activation of the peroxide-generating enzyme DUOX to generate the ROS signal for attracting hemocytes. However, metabolic disruption in tumours or chromosomal instability also produces ROS [36, 66], so the same ROS signal may also be used in the absence of external wounding. As a damage signal, hydrogen peroxide has many advantages: it is readily produced by defective cells, it diffuses through membranes, and it is reactive enough to limit its own diffusion to a few cell diameters [67]. Following the recruitment of hemocytes to an inflammatory site, we expect that the production of TNFα by the hemocytes [33] increases ROS production in nearby cells [63, 68], as yet another positive feedback loop to encourage the death of damaged cells.

ROS production is strongly affected by a range of metabolic controls that are altered by inflammation [3]. In Drosophila this may be mediated by the fat body, which responds to necrosis by activating JNK targets such as FOXO that both increase antioxidant production and drive lipolysis, the normal response to starvation [21]. This energy wasting effect is commonly seen in both infections and cancer in all
organisms, leading to cachexia that has been associated with elevated TNF levels and may respond to anti-inflammatory therapy [69].

4. Implications of Sterile Inflammation Control

It is perhaps surprising that the inflammatory response that is used to clear bacterial infections and to clot wounds should cause hemocytes to secrete TNFα, which damages the host more than the pathogen. Unwanted TNFα can be responsible for debilitating disease, as seen in allergies and autoimmune diseases that respond strongly to anti-TNFα therapies. Nonetheless, TNFα is a valuable protective mechanism, as TNFα inhibitor therapy in humans is associated with increased risk of infections and, significantly, cancer [70]. Experiments in *Drosophila* have underlined the need for localised TNFα production by hemocytes to control the growth of neoplastic tissue [16, 33]. In this context it is worth noting that loss of neutrophils, which share some features with hemocytes, is still an extremely common but obviously undesirable side effect of the front-line human chemotherapies [71]. Not only does neutropenia leave the patient vulnerable to infection, but also reduces the body’s innate immune response to cancer.

While T-cell based immunotherapies are now available [72], effective cancer treatments using innate immune responses have not been developed. This may be partly because tumours must have typically developed some resistance to the innate immune response to have survived and grown to a point where they can be detected [7]. At this point the tumour may well be dependent on proinflammatory signalling, so therapies have been developed instead to combat inflammatory signalling. This stage of tumour development has been modelled in *Drosophila* by expression of active Ras, which can be used to generate tumours that depend on TNFα for invasive outgrowth [54]. These results underline the key role of JNK in modulating outcomes: the innate immune response can activate JNK signalling to kill damaged or infected tissue, but, in cases where cell death is blocked, the same signal promotes outgrowth and proliferation [55, 57, 60]. Clearly caution is needed in any intervention that alters the level of inflammatory response in either direction.

As this review has indicated, there is still a great deal that is unknown about the mechanisms that regulate sterile inflammation. Many inflammatory triggers from damaged tissue are yet to be characterized, particularly in model organisms. Similarly, we know little about the mechanisms that damp the many positive feedback systems to prevent a life-threatening excessive response to tissue damage. However, the signalling pathways and cytokines that mediate inflammation are now becoming relatively well studied and amenable to analysis by the mutagenesis screening approaches that have made *Drosophila* such a valuable tool [73]. With the current intense activity in the field we expect significant improvements in our understanding of sterile inflammation in the near future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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