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Mosquito hemocyte-mediated immune responses

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Abstract

Hemocytes are a key component of the mosquito immune system that kill pathogens via phagocytic, lytic and melanization pathways. Individual mosquitoes contain between 500 and 4,000 hemocytes, which are divided into three populations named granulocytes, oenocytoids and prohemocytes. Hemocytes can also be divided by their anatomical location with 75% of hemocytes circulating in the hemocoel (circulating hemocytes) and 25% of hemocytes attaching themselves to tissues (sessile hemocytes). Greater than 85% of the hemocytes in adult mosquitoes are granulocytes, which primarily kill pathogens by phagocytosis or lysis. Oenocytoids, on the other hand, are the major producers of the enzymes required for melanization while prohemocytes are small cells that participate in phagocytosis. Both circulating and sessile hemocytes engage in defense against pathogens. The circulatory system of mosquitoes also interacts with hemocytes and facilitates elimination of potential pathogens that enter the hemocoel.

Keywords

Mosquito; immunity; hemocyte; phagocytosis; melanization; lysis

1. Introduction

Like other insects, mosquitoes (family Culicidae) are continuously exposed to different microbes including some that cause disease. The juvenile stages of mosquitoes are aquatic while adults are terrestrial. Potential pathogens infect juvenile and adult mosquitoes by either oral ingestion or entry through the cuticle. The vast majority of mosquito species are also anautogenous, which means that adult females must ingest a blood meal from a vertebrate host to obtain nutrients necessary for the production of eggs. Blood feeding exposes mosquitoes to microorganisms that cause disease in the vertebrate host, and in some

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cases mosquitoes transmit these organisms when they blood feed on a new host. In the case of humans, mosquitoes transmit several major disease-causing organisms including the causative agents of malaria, lymphatic filariasis and dengue fever [1].

The innate immune system of mosquitoes is divided into cellular and humoral components that provide defense against a range of microbes including species that mosquitoes transmit to vertebrates. The cellular arm of the immune system consists of immune cells called hemocytes [2,3], while humoral components refer to soluble molecules in hemolymph such as pattern recognition receptors, antimicrobial peptides, and components of the phenoloxidase cascade [2,4-6]. The line between cellular and humoral immunity is also somewhat subjective given that hemocytes produce many humoral immune molecules, while many humoral molecules play important roles in regulating cellular immune defenses [7-12]. In this review, we summarize our understanding of the biology of hemocytes in mosquitoes including their classification, functions, and interactions with the circulatory system.

2. Hemocyte types

Hemocytes in mosquitoes and other insects form distinct populations that are classified using two non-exclusive criteria. The first classifies hemocytes by morphological, enzymatic, cytochemical and functional characteristics [13,14], while the second divides hemocytes by their anatomical location, with cells in hemolymph being called circulating hemocytes and cells attached to tissues being called sessile hemocytes [15,16]. Recent studies also clearly show that circulating hemocytes can become sessile and vice versa [17,18].

On the basis of light microscopic morphological analyses, studies have divided mosquito hemocytes into populations named plasmatocytes, oenocytoids, granulocytes, granular cells, and adipohemocytes [14,19-25]. Hillyer and Christensen [26] used light microscopy, electron microscopy, enzyme activity assays, and lectin binding assays to classify mosquito hemocytes into granulocytes, oenocytoids, adipohemocytes and thrombocytoids. Functional and comparative studies thereafter concluded that adipohemocytes and thrombocytoids are not hemocytes, but instead are fat body and pericardial cells, respectively, that were inadvertently collected during the hemolymph extraction process [2,27,28]. Castillo et al. [27] then concluded that mosquito hemocytes consist of granulocytes and oenocytoids plus a population of smaller cells called prohemocytes. This latter study also showed that mosquito hemocytes previously referred to as plasmatocytes are not a distinct cell type, but are instead granulocytes. Thus, it is now generally accepted that mosquitoes contain three populations of hemocytes: granulocytes, oenocytoids, and prohemocytes (Figs. 1 and 2).

Granulocytes are the most abundant hemocyte type, comprising 80-95% of the circulating hemocyte population. These cells are polymorphic and their cytoplasm contains varying amounts of membrane-delimited vesicles [26,29]. Granulocytes in circulation measure approximately 9 μm in diameter, but ex vivo these cells readily attach to foreign surfaces such as glass and spread to diameters that may exceed 35 μm. Granulocytes are distinguished from oenocytoids by their strong acid phosphatase activity, the presence of the

chitin binding serine protease SP22D, and the strong binding of the lectins wheat germ agglutinin, *Helix pomatia* agglutinin and *Galnathus nivales* lectin [26,27,29]. Granulocytes are also highly phagocytic, whereas oenocytoids are not [28-32].

Oenocytoids comprise ≤10% of the circulating hemocyte population. They are round cells that measure approximately 9 μm in diameter but do not readily spread on foreign surfaces. They contain an eccentric nucleus and a homogenous cytoplasm, but their defining characteristic is that they are the major producers of phenoloxidase [26,27,29], which is the rate-limiting enzyme in the melanization immune pathway [4].

Prohemocytes comprise ≤10% of the circulating hemocyte population and are spherical cells that measure 4-6 μm in diameter. They are characterized by a high nuclear to cytoplasm ratio, and have been hypothesized to function as progenitor cells [27]. However, a recent study showed that these small hemocytes are phagocytic, and that they may arise from the asymmetric division of granulocytes [16]. This suggests that prohemocytes are fate restricted and not multipotent stem cells.

From an anatomical perspective, approximately 75% of the hemocytes in adult mosquitoes are in circulation while 25% are sessile (Fig. 2) [16]. Sessile hemocytes are distributed throughout the abdominal wall, the thoracic wall, the head, the maxillary palps, the legs, the midgut and the Malpighian tubules [16,17,33,34]. Of these locations, the vast majority of sessile hemocytes (65-78%) are present on the abdominal wall, including the tracheoles and the outer surface of the heart [16,17]. Similar to the hemocytes in circulation the vast majority of sessile hemocytes are also granulocytes.

3. Circulating hemocyte numbers

The number of circulating hemocytes in adult mosquitoes decreases with age [27,28,35,36], increases after blood feeding [37,38], and depending on the mosquito-pathogen combination, may increase, decrease or remain the same following infection [10,16,28,35,36,39,40]. While these trends are clear, the total number of hemocytes in circulation has recently received a significant amount of attention (Fig. 2). Studies conducted by multiple laboratories over a period of three decades have all shown that adult mosquitoes contain between 500 and 4,000 circulating hemocytes [10,16,27,28,35,37-39,41], with *Anopheles gambiae* containing more hemocytes than *Aedes aegypti* [27,39]. However, a recent paper on *A. gambiae* reported that mosquitoes contain between 25,000 and 40,000 circulating hemocytes [40], but a different research group using identical methodology was unable to replicate this finding [37]. A range of 500-4,000 circulating hemocytes in adult mosquitoes is also in agreement with the density of circulating hemocytes reported in other insect species. For example, adult *D. melanogaster* contain between 1000 and 2000 circulating hemocytes, adult *Glossina morsitans* contain 500-900 hemocytes per μl of hemolymph, and several other dipteran, lepidopteran and orthopteran insects contain hemocyte densities in this same range [42-47].

Studies reporting that mosquitoes contain between 500 and 4,000 circulating hemocytes roughly divide them into 80-95% granulocytes, ≤10% oenocytoids and ≤10% prohemocytes [26,27,29]. Studies that have recognized additional hemocytes types in mosquitoes also

report that oenocytoids and prohemocytes comprise only a small proportion of the total hemocytes in circulation [23-25,36]. Collectively, these data agree with data from *D. melanogaster*, several species of Lepidoptera and other arthropods, where consistently only a small proportion of circulating hemocytes are identified as oenocytoids (called crystal cells in *Drosophila*) and prohemocytes [15,43,48]. However, these patterns differ strongly from Rodrigues et al. [40], which in addition to reporting that *A. gambiae* adults contain between 25,000 and 40,000 circulating hemocytes, also divide them into 2% granulocytes, 38% oenocytoids and 60% prohemocytes. Given these differences, it is likely the number of circulating hemocytes in mosquitoes will continue to be scrutinized.

4. Hematopoiesis

Hemocytes in mosquitoes have mostly been studied in adults, whereas studies in *D. melanogaster* and Lepidoptera have examined the origin, proliferation and maintenance of hemocyte populations in all life stages. In *D. melanogaster*, hemocytes originate during embryogenesis from head mesoderm [49]. During the larval stages of *Drosophila* and Lepidoptera, these embryonic hemocytes, both in circulation and in sessile form, replicate by autonomous cell division [48,50-52]. Additional hemocytes in larvae are produced from hematopoietic organs called lymph glands that derive from thoracic mesoderm, with the majority of these hemocytes being released into the hemocoel during the larva to pupa transition [3,53]. The lymph gland degenerates in the pupa, which leaves circulating and sessile hemocytes as the only possible sources for the production of new hemocytes. In adult *Drosophila*, hemocytes are not thought to replicate, resulting in an age-dependent decrease in hemocyte numbers [15].

No hematopoietic organs have been identified in adult mosquitoes, but hemocyte numbers clearly increase in response to infection by certain organisms and following a blood meal [10,16,35,37-39]. This increase in hemocyte numbers is primarily due to mitosis by circulating hemocytes and not the release of sessile hemocytes into circulation [16]. Furthermore, at least two pathways drive hemocyte replication: insulin-like signaling and Ras-MAPK signaling [37,38].

5. Phagocytosis by hemocytes

In insects, hemocyte-mediated immune responses include phagocytosis, encapsulation and nodulation. Phagocytosis is an evolutionarily conserved process that hemocytes exhibit in response to bacteria and other small foreign entities (Fig. 1). Here, the foreign body is recognized by humoral pattern recognition receptors (PRRs) that function as opsonins, and/or PRRs on the surface of hemocytes. The foreign body is then internalized into a membrane-delimited phagosome, the phagosome fuses with a lysosome, and the microbe is hydrolytically digested. Encapsulation and nodulation, in contrast, involve the irreversible binding of multiple hemocytes to the surface of large foreign objects or bacterial aggregates, respectively, to form a multicellular sheath [54]. Hemocytes from Lepidoptera and many other insects readily phagocytose a diversity of single cell microbes like bacteria and protozoa, while also forming capsules around multicellular parasites like nematodes and parasitoid wasps.

Mosquito hemocytes also phagocytose a range of bacteria and other small foreign entities yet have rarely been reported to form cellular capsules. Mosquito granulocytes, and to a lesser extent prohemocytes, are highly phagocytic and initiate this process within 5 min of pathogen exposure [16,29,32]. Up to 95% of circulating hemocytes are phagocytic, and it has been estimated that some hemocytes can phagocytose hundreds of bacteria within 24 h of infection [28,30]. This immune process is effective in the sequestration of bacteria, yeast, *Plasmodium* and small inanimate particles [25,29-32,55], and is carried out by both the circulating and sessile populations [16,17]. The strong effectiveness of the phagocytic response in adult mosquitoes has also been linked to their small body size [56].

Several mosquito PRRs are involved in regulating phagocytosis. The most studied of these factors is the complement-like protein, thioester containing protein 1 (TEP1). Following proteolytic activation, TEP1 opsonizes bacteria and targets them for phagocytosis [57]. Other PRRs with roles in phagocytosis include additional members of the TEP protein family (TEP3, TEP4), leucine rich repeat containing proteins (LRRs; LRIM1), fibrinogen-related proteins (FBN8), and DSCAM, which is a hypervariable immunoglobulin that can be transpliced into over 31,000 variants [58-60]. Although some of these proteins opsonize pathogens, others likely act upstream of the opsonization response (e.g., LRIM1 [61]). For some pathogens, deposition of melanin onto the surface of the foreign entity is also required prior to the initiation of phagocytosis [29,31,32].

Transmembrane receptors that are expressed on the surface of hemocytes and are implicated as PRRs include a β integrin (BINT2), a peptidoglycan recognition protein (PGRPLC), a low-density lipoprotein receptor-related protein (LRP1), and a protein containing both zinc finger and LITAF domains [59,60]. These factors may recognize pathogens directly or recognize pathogens after they have been opsonized by humoral factors. Finally, the intracellular proteins CED2, CED5 and CED6 regulate the internalization of pathogens. TEP1-, TEP3-, LRIM1- and LRP1-mediated phagocytosis occurs through the CED6 pathway, whereas TEP4- and BINT2-mediated phagocytosis occurs through the CED2/CED5 pathway [60].

6. Hemocyte-mediated humoral responses: melanization and lysis

Hemocytes produce factors with roles in humoral immune responses (Fig 1). These include components of the melanization response, which is primarily regulated by a network of proteases referred to as the phenoloxidase (PO) cascade. Activation of the PO cascade results in conversion of tyrosine to melanin [4]. Associated cross-linking of proteins also results in the deposition of melanin on the surface of pathogens, which in the mosquito literature is often referred to as melanotic encapsulation [4,62]. Mosquito hemocytes produce several factors implicated in the melanization response including multiple phenoloxidases, phenylalanine hydroxylase, dopachrome conversion enzyme, serine proteases, serine protease inhibitors and C-type lectins [7-12]. These enzymes together with PRRs are also considered essential in formation of melanotic capsules [4,26,27,29,63-68]. In some insects, oenocytoids lyse upon immune challenge to release PO and other components of the PO cascade that lack signal peptides for secretion. In contrast, it remains unclear how PO is released from mosquito hemocytes.

Mosquito hemocytes also produce cytotoxic effector molecules. For example, hemocyte-produced Tep1 binds to *Plasmodium* parasites and targets them for killing [57,61,69,70]. Tep1 exists in hemolymph as part of a protein complex composed of Tep1 and the leucine-rich repeat containing proteins, LRIM1 and APL1. *Plasmodium* infection causes dissociation of the complex, freeing Tep1 to bind and lyse pathogens. Other lytic factors, such as antimicrobial peptides and reactive oxygen and nitrogen intermediates are also produced by hemocytes [7-12,68]. Production of nitric oxide, for example, increases in hemocytes following infection, and is involved in the killing of bacteria and malaria parasites [71,72]. Lastly, cytotoxic intermediates generated by the PO cascade directly kill viruses in mosquitoes and other insects [73,74].

7. Wound healing

Some insects close external wounds by means of a clotting reaction called coagulation [75]. In mosquitoes, the coagulation response includes: (1) the lysis of granulocytes at the wound site, (2) the deposition of melanin, (3) the binding of additional hemocytes, and (4) the regeneration of the epidermis [76]. However, other than detecting hemocyte-produced prophenoloxidase at wound sites [77], nothing is known about other molecules mosquito hemocytes produce during the wound healing response.

8. Hemocytes and hemolymph circulation

Hemocytes are propelled throughout the hemocoel by the mosquito's circulatory system (Fig. 3). Anatomically, the mosquito circulatory system consists of hemolymph (hemocytes plus plasma), the hemocoel, and a series of muscular pumps. The primary pump is the dorsal vessel, which is a tube-like structure that extends along the dorsal midline of the insect and is divided into a thoracic aorta and an abdominal heart [78]. Hemolymph enters the dorsal vessel through paired valves, called ostia, which are located at the thoraco-abdominal junction and at the anterior portion of each abdominal segment. Once inside the dorsal vessel, hemolymph is sequentially propelled in both the anterograde (toward the head) and retrograde (toward the posterior abdomen) directions until it is discharged into the hemocoel through excurrent openings located at the distal ends of the insect. Hemolymph then flows back toward the ostia, at which point it re-enters the heart and completes its circulatory cycle [78,79].

Pathogens that enter the hemocoel can be encountered by either hemocytes in circulation or by sessile hemocytes surveying the hemolymph as it flows by them. Sessile hemocytes have the highest probability of encountering pathogens when they are located in areas of high hemolymph flow. While sessile hemocytes are scattered throughout the hemocoel, the only location they consistently aggregate in mosquitoes is the surface of the heart [16,17]. Sessile hemocytes flanking regions surrounding the ostia are called periostial hemocytes, and rapidly phagocytose bacteria and malarial parasites within seconds of entry into the hemocoel. As an infection progresses, circulating hemocytes also move into periostial regions, where they bind the cardiac musculature and each other, and amplify the phagocytosis response. Periostial hemocyte aggregation occurs in a time- and infection dose-dependent manner, and once this process is triggered, the number of periostial

hemocytes remains elevated for the mosquito's lifetime [17]. The periostial regions are the only locations of the hemocoel where circulating pathogens accumulate, and are also the only locations where hemocytes aggregate in response to infection [16,17]. This immune response represents a co-adaptation of the mosquito circulatory and immune systems.

9. Concluding remarks

The current literature clearly indicates that hemocytes are an essential component of the mosquito immune system. However, several aspects of hemocyte biology in mosquitoes remain unclear. First, the vast majority of what we know about hemocyte function derives from only three species: *A. gambiae*, *A. aegypti*, and *Armigeres subalbatus*. However, with an estimated 3,500 species of mosquitoes, it is uncertain how representative these data are for the Culicidae as a whole. Second, while recent papers clearly show that hemocytes are capable of proliferating in adults [16,37,38], virtually nothing is known about the developmental origin of these cells in embryos and/or larvae, nor the regulatory pathways that regulate the differentiation of hemocytes into morphologically and functionally distinct populations. In addition, almost no literature exists at present on the function of mosquito hemocytes during the immature stages. Third, although we have begun to understand the molecular basis of hemocyte-mediated immunity [7-12], these studies have ignored the sessile population of hemocytes, and have failed to distinguish the differential role that granulocytes, oenocytoids and prohemocytes play in the infection response. Fourth, hemocyte-mediated immunity has so far been studied in the context of bacterial, malarial, or filarial nematode infection. However, understanding of how mosquito hemocytes recognize these entities as foreign, the signaling pathways activated in hemocytes, and effector responses produced remain incompletely understood. In addition, while some viruses infect mosquito hemocytes [80], what role if any hemocytes play in antiviral defense is unknown. Emerging molecular and imaging methodologies will undoubtedly continue to illuminate the important role these cells play in controlling infection.

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Highlights

- A major component of the mosquito immune system are hemocytes
- Hemocytes are anatomically divided into circulating and sessile hemocytes
- Hemocytes are functionally divided into granulocytes, oenocytoids and prohemocytes
- Hemocytes kill pathogens via phagocytosis, melanization and lysis

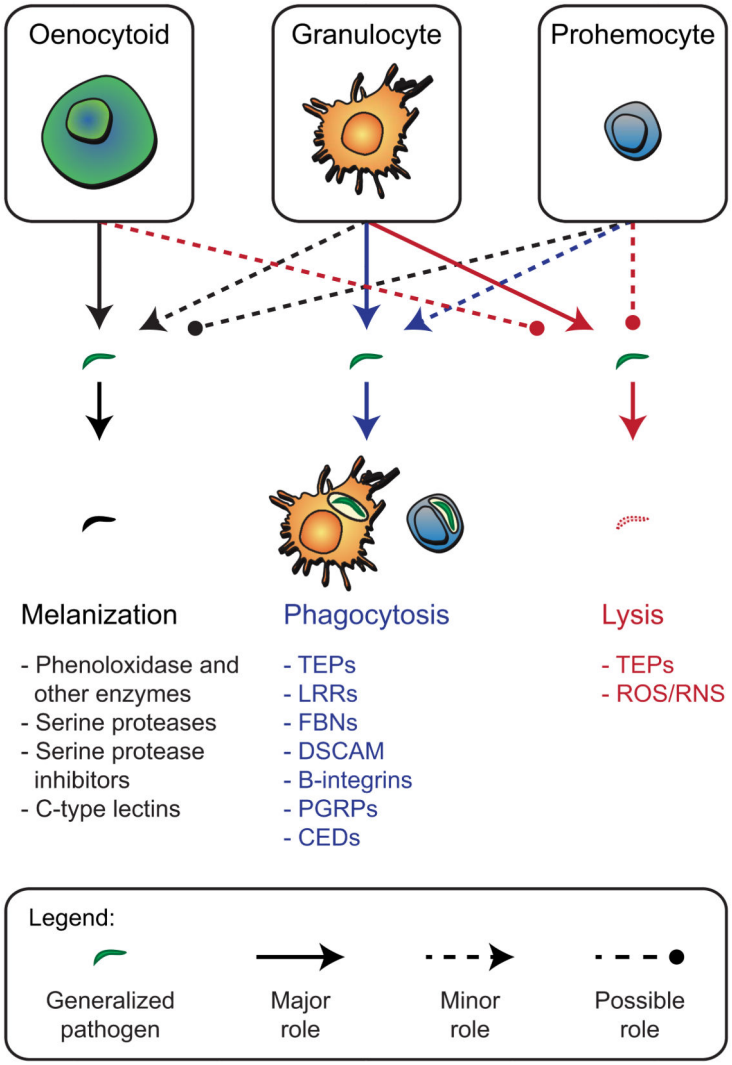

Figure 1.

Morphological and functional classification of circulating hemocytes. The upper portion of the figure shows granulocytes, oenocytoids and prohemocytes, while the lower portion lists gene products identified from mosquitoes with roles in three immune defense functions: melanization, phagocytosis, or lysis of pathogens. Solid arrows indicate hemocyte types with major roles in each of these defenses, dotted arrows indicate minor involvement, and dotted lines with circles indicate possible involvement.

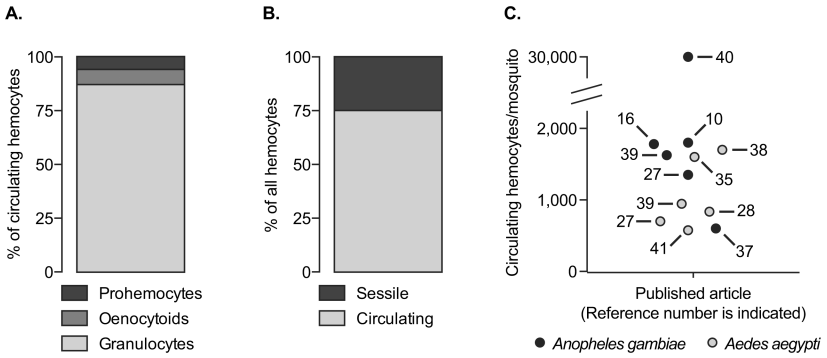

Figure 2.

The abundance of different hemocyte types in mosquitoes. A. Proportional distribution of granulocytes, oenocytoids and prohemocytes in the circulating hemocyte population. The data used for this graph originates primarily from Castillo et. al [27] and Baton et. al [10]. B. Proportional distribution of circulating and sessile hemocytes in the body of 6-day-old *A. gambiae*. These data were adapted from King and Hillyer [16]. C. Average number of total circulating hemocytes in mosquitoes as reported in different published articles. The numbers inside the graph represent the article number in the reference list, and black and grey circles denote *A. gambiae* and *A. aegypti*, respectively. When the article reported data on multiple treatment groups, the hemocyte numbers denote naïve mosquitoes at 3-7 days post-emergence.

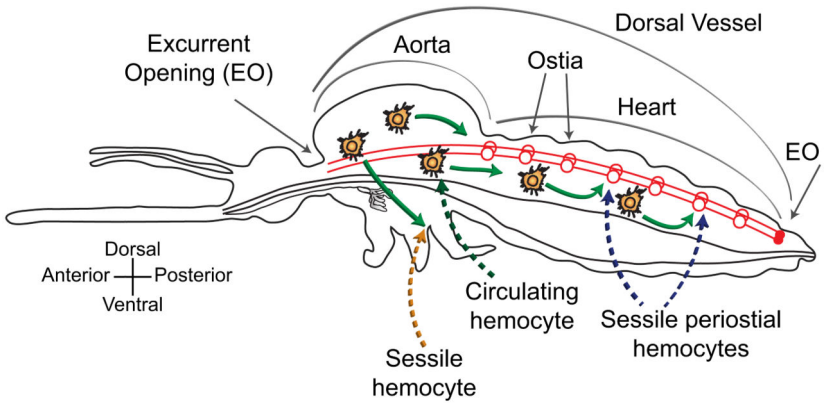

Figure 3.

Hemolymph circulation and hemocyte-based immunity. Hemolymph enters the dorsal vessel (heart and aorta; red lines) through ostia (red circles) and is propelled in anterograde and retrograde directions. Hemocytes either circulate (green arrows showing extracardiac movement during anterograde heart flow) or are sessile. In response to infection, hemocytes aggregate in the regions surround the ostia, thus becoming periostial hemocytes. This diagram is based on the heart physiology study of Glenn et. al [78], and the immunity work of King and Hillyer [16,17].