Expression and a novel function of filamin-240 in lamellocyte development in *Drosophila*

PhD thesis

Florentina Rus

Supervisor: Dr. István Andó, PhD, DSc

Biological Research Center of the Hungarian Academy of Sciences
Institute of Genetics

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Abbreviations

AEC- 3-Amino-9-ethyl-carbazole

BSA-Bovine Serum Albumin

DAPI-4', 6-diamino-2-phenylindole dihydrochloride

Dif-Dorsal-related immunity factor

dw- distillated water

ECM-extracellular matrix components

ECL-enhanced chemiluminescence

FADD- Fas-associated death domain

FACS-Fluorescein Activated Cell sorter

FCS- fetal calf serum

FITC- Fluorescein Isothyocianate

GNBP1-Gram-negative bacteria binding protein 1

GTP-guanosine 5'-triphosphate

Ig-immunoglobulin

IL-inyterleukin

IPTG-isopropyl beta-D-thiogalactoside

IRAK-interleukin-1 receptor associated kinase

JAK/STAT-Janus Kinase/ Signal transducer and activator of transcription

JNK-Jun-N-Terminal Kinase

LPS-lipopolyssacharide

MAP- mitogen-activated protein

MEKK-Mitogen activated kinase kinase kinase

NK-natural killer

RIP- receptor interacting protein

RNAi-RNA-interference

PBS- Phosphate Buffered-Saline

PGRP- peptydoglycan-recognititon protein

PGN-peptydoglycan

PIC-Preotease Inhibitor mixture

PMSF-Phenyl-Methyl-Sulfonyl-Fluoride

PTU- 1-phenol-2-thiourea

PVF2-PDGF- and VEGF-related factor 2

SAPK-stress activated protein kinase

SDS- Sodium Deoxycolate

TAK1-TGF-β activates kinase 1

TBS-Tris-Buffer-Saline

TIR-Toll-interleukin-1 receptor

TLR-Toll -like receptor

TNF-tumor necrosis factor

TRAF-TNF receptor-associated factor

WASP -Wiskott-Aldrich syndrome protein

1. Introduction

1.1. Immunity

We are all living in a potentially hostile world filled with a wide variety of pathogens or abnormally developing tissue, including tumors. In order to protect themselves from the harmful invaders and to maintain body integrity, man and animals have evolved a remarkable body defense system, called the immune system. The immune system consists of a complex network of immune cells and molecules and is generally divided into *innate* and *adaptive immunity*. Vertebrates, constituting a very small fraction of the species on earth, defend themselves against infections by both the innate and adaptive immune systems. Invertebrates lack the adaptive immune response, defending only by the innate immune responses [1, 2].

1.2. Innate immunity

Innate immunity is an evolutionarily ancient system and it is the first line of defense against bacterial and viral infection for all multicellular organisms. Its hallmarks are the recognition of microorganisms by germline-encoded, genetically not rearranged receptors, and rapid effector mechanisms that involve phagocytosis, activation of proteolytic cascades and synthesis of potent antimicrobial peptides. In mammals innate immune recognition is required not only for activation of immediate antimicrobial defense mechanisms but also for the stimulation of the adaptive immune response, through the production of costimulatory molecules and the maturation of antigen presenting cells [3, 4]. Innate immune recognition by the germline-encoded receptors is much more rapid than the adaptive immune response, which relies on somatic recombination, selection and proliferation to generate antigen-specific receptors. A weakness of the innate immune system is that it has no memory and because the receptor-repertoire of the innate immune response is very limited.

Having no adaptive immune response, *Drosophila,* rely entirely on innate immune responses. Microbial recognition and signal transduction can be studied without the need to compensate for the effects of strong adaptive immune responses. The mechanisms used by mammals and flies to respond to pathogens are highly conserved [5], thus, determining the mechanisms involved in the innate immune response in *Drosophila* will help in the understanding of innate immunity in mammals.

The innate immune defense system of *Drosophila* consists of humoral and cellular reactions, which provide resistance to pathogens.

1.2.1. Humoral reactions

The humoral reactions include the almost immediate induction of proteolytic cascades, which lead to localized melanization, blood coagulation and opsonisation of pathogens at the site of the injury. Moreover, they involve the rapid synthesis of potent antimicrobial peptides in the fat body, which are then secreted into the hemolymph, the insect analogue of mammalian blood. Fat body is a monolayer sheet of adipose cells, analogue of the mammalian liver [6, 7], which originates from the mesoderm during embryogenesis and becomes immunocompetent only at the larval stage [8], when increases in size by polyploidization and its immunocompetence increases under the control of the ecdysone [9, 10]. In the early adult stage, the larval fat body desegregates and is replaced by a new adult type fat body [11].

1.2.1.1. Melanization refers to deposition of melanin and is a rapid, highly localized defense. Melanization contributes to wound clotting and encapsulation of wasp eggs, and produces toxic intermediates including reactive oxygen species (ROS). Phenoloxidase, which catalyzes melanin production, is maintained as an inactive zymogen and is activated by a cascade of serine proteases [12]. The proteases are related to those of the human complement system. The activated phenoloxidase oxidizes tyrosine and other phenols into reactive compounds that crosslink proteins and polymerize into melanin, which is deposited around parasites and wounded tissue. A mutant lacking hemolymph phenoloxidase is sensitized to infection and is vulnerable to death from wounds [13, 14].

1.2.1.2. Antimicrobial peptides

So far, several different classes of anti-microbial peptides (e.g. attacin, cecropin, defensin, diptericin, drosocin, drosomycin, and metchikowin) have been identified; their expression is regulated at the transcriptional level in response to immune challenge [15]. Some of these antimicrobial peptides are directed against fungi (drosomycin, and metchikowin), some against Gram-positive (defensin) and some to Gram-negative (attacin, cecropin, diptericin, drosocin) bacteria [16]. Within few hours of an infection, these peptides reach micromolar concentrations or higher in hemolymph, which becomes a very hostile environment for the intruders [16].

1.2.2. Cellular reactions

In *Drosophila* the cellular immunity is based on blood cells (hemocytes), that are freely circulating through the body cavities or are sessile, being associated with various tissues and organs [17]. In *Drosophila* larvae four classes of hemocytes can be distinguished morphologically: the plasmatocytes, the podocytes, the crystal cells and the lamellocytes.

Plasmatocytes-are the predominant cell type at all developmental stages and represent approximately 95% of all hemocytes. They are small (8-10 μm in diameter) and rounded cells, which contribute to the host defense by engulfing microorganisms and apoptotic cells. The ability of plasmatocytes to recognize apoptotic cells is mediated by a transmembrane protein, called, Croquemort (Crq), which is a member of the CD36 family of receptors, which also recognize apoptotic cells in mammals [18].

The plasmatocytes cytoplasm contains lysosomes and endoplasmic reticulum in abundance, which is consistent with their phagocytic and secretory functions [19]. Plasmatocytes secrete some ECM proteins, crititcal for many morphogenetic processes [20 21], and also, antimicrobial peptides, such as cecropin, drosomycin, diptericin [22, 23, 24]. Based on their function, the plasmatocytes are considered the counterparts of mammalian phagocytes (neutrophils and macrophages).

Podocytes are characterized by their pseudopodia-like extensions. They are phagocytic and are of similar size to plasmatocytes [19]. However it is not clear if these cells represent distinct lineages or rather differentiated forms of plasmatocytes.

The *crystal cells* represent a small proportion (less than 5%) of the total larval blood cells. They have a similar morphology with the plasmatocytes but somewhat larger in size (10-12μm). *Crystal cells* are nonphagocytic cells characterized by the presence of crystalline inclusions believed to contain prophenoloxidase enzymes, involved in melanin deposition in wounds and around foreign objects [25]. Melanin and its biosynthetic products, such as hydrogen peroxide and nitric oxide are directly toxic to microorganisms [26, 19]. Crystal cells can readily disrupt and deliver their content into the hemolymph where the zymogens can be activated.

The *lamellocyte* represent a unique population of hemocytes, both in terms of morphology and function. They are large (up to 60 μm or more), flat cells, involved in encapsulation and neutralization of intruders or of abnormally developed tissues, too large to be engulfed by plasmatocytes. These cells are non-phagocytic and their cytoplasmic constituents are relatively sparse. Lamellocytes are essentially absent in healthy uninfected larvae but they may appear in

low numbers at the time of metamorphosis. However, immune induction by the parasitization with the Hymenopteran wasp, *Leptopilina boulardi,* initiates the rapid differentiation of lamellocytes, which subsequently adhere to, surround the egg capsule, which begins to melanize, thereby walling it off inside the larvae [19].

Hematopoiesis in *Drosophila* occurs in two phases during development. In the embryo, hemocytes differentiate from the head mesoderm and are first identifiable during embryonic stage 5 by their expression of *serpent, a GATA transcription factor,* required for normal hematopoiesis [19]. Two classes of hemocytes have been found in the embryo: plasmatocytes or macrophages that eliminate the microorganisms and apoptotic cells and crystal cells, which differentiate near the anterior region of the gut. Their role in the embryo is unknown. Towards the end of embryogenesis, the precursors of the lymph glands form in the lateral mesoderm, and migrate dorsally. The lymph glands are considered to be the major source of hemocytes in *Drosophila* larvae [25]. They are composed of four to six lobes that are located along the dorsal vessel. In the posterior lobes they contain mainly undifferentiated precursor cells, called prohemocytes. The anterior lobes contain abundant fully differentiated hemocytes [25]. In larvae, plasmatocytes remain the most abundant cell type in the circulation, but the crystal cells and lamellocytes are also present [17]. In adults, the plasmatocytes are the only cell type present in hemolymph; they derive from embryonic mesoderm and from the larval lymph gland [7]. Components of the transcriptional regulation of larval hematopoiesis have been described. The *glial cells missing* (*gcm*) determines plasmatocytes fate, whereas *lozenge* (*lz*) and *Notch* control the formation of crystal cells [27, 28, 29]. The *serpent* gene is required for normal hematopoiesis [30] and *PVF2* was found to affect hemocyte proliferation [31]. Also, genes that regulate the cellular immune response have been identified. A hemocyte surface protein encoded by *Hemese* gene may play a modulatory role [32]. The product of *Notch* gene is required for normal cellular response [28].

The lamellocytes have an unclear hematopoietic origin. It was proposed that lamellocytes develop directly from plasmatocytes but there is no strong evidence for this. One population of lamellocytes has been found to derive from precursor cells within the anterior lobes of the lymph glands, from where they may be released [33]. It is known that the lamellocyte differentiation is induced by particles which are too large phagocytosed by plasmatocytes [26] or by large wounds [34], by a so far unidentified signaling and induction mechanism. We tested whether experimental wounding itself, comparable to that induced by oviposition, could trigger normal lamellocyte differentiation.

To understand the signaling mechanisms and development of blood cells in *Drosophila* we have defined molecular markers expressed in blood cell subsets using monoclonal antibodies developed in our laboratory. The lamellocytes develop from small (8-10μ), round stem cells and become large (40-50μ) flattened cells capable of binding and encapsulating large foreign particles. The development of lamellocytes from stem cells to effector cells involves two major steps. The small stem cells differentiate to the immediate precursors of the fully differentiated large cells. This step requires cell division and is terminated by the expression of the L1 antigen. Second, the terminal differentiation of the immediate precursors to the large flattened cells. This process does not require cell division but is characterized by expression of molecular markers defined in our laboratory L1, L2, L4, L5 and L6 (unpublished data) (Fig.1).

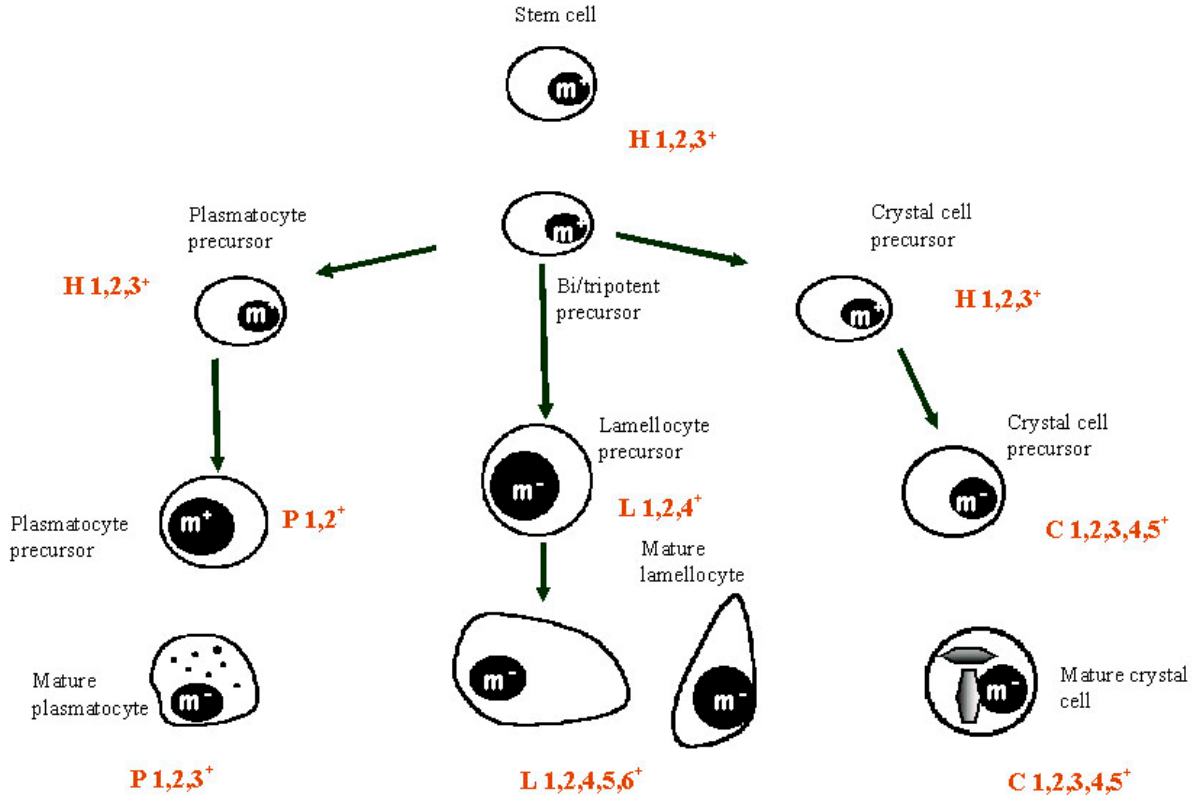

Figure 1. Hemocyte lineages in *Drosophila*

In *Drosophil*a, the mechanisms of cellular immune reactions include: phagocytosis and encapsulation.

1.2.2.1. Phagocytosis is a process that involves binding and internalization of pathogens or damaged tissues by individual cells and it is essential for host defense in higher eukaryotes. It is initiated upon contact and recognition of non-self or altered or apoptotic cells [35].

It was demonstrated that the phagocytosing of microbes by *Drosophila* hemocytes is very similar to the phagocytosis by mammalian macrophages, so phagocytosis is likely a highly conserved process from insects to human. Among hemocytes, the plasmatocytes are the phagocytic macrophage-like cells; most bacteria are phagocytosed by the blood cells within few minutes [36, 37]. Phagocytosis starts with the attachment of the plasmatocyte to the foreign particle. After the establishment of a tight interaction between them the cytoskeleton of the plasmatocyte undergoes changes, resulting in pseudopode formation that reaches out and engulf the bacteria [35]. The engulfed targets are captured within the phagosomes, which fuse with lysosomes, forming the phagolysosomes. In the phagolysosomes, the lysosomal enzymes, reactive oxygen species and nitric oxide come in contact with the foreign particles and destroy them [38]. The phagocytosis process requires rearangements of the actin cytoskeleton. The process of actin remodeling and vesicle trafficking are likely to be conserved between invertebrates and vertebrates [35].

1.2.2.2. Encapsulation

Encapsulation is an essential immune reaction in *Drosophila* where a capsule of overlapping layers of hemocytes is formed around the invaders, which are too large to be phagocytosed such as egg or larvae of parasitic insects [25, 39].

Two types of hemocytes contribute to the process of encapsulation: lamellocytes, which adhere to and enclose the parasite in a capsule and the crystal cells, which contribute to the melanization of this cellular capsule (Fig.2) [40]. Within the capsule, the parasite is eventually killed by the local production of cytotoxic free radicals, quinones or semiquinones [41, 42].

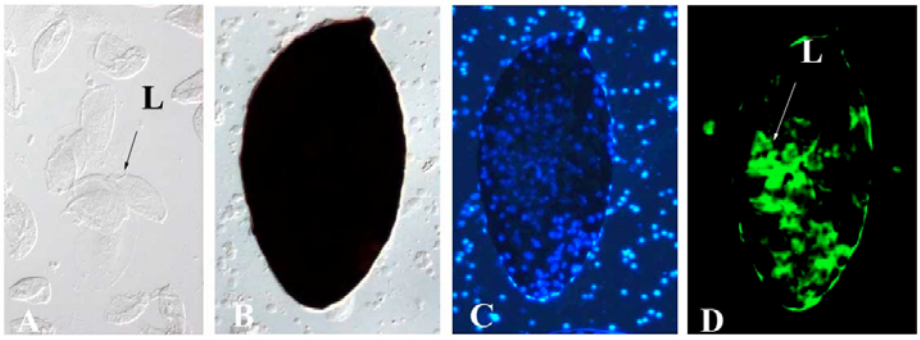

Figure 2. Encapsulation of a wasp egg by lamellocytes. The lamellocytes (fig.1A) are flattened cells, involved in neutralization of intruders, which are too large to be phagocytated. Parasitization by the Hymenopteran wasp, *Leptopilina boulardi*, initiates in *Drosophila* larvae, a rapid differentiation of lamellocytes, which subsequently adhere to and surround the egg capsule (fig1B-D). The adhered lamellocytes were stained with L1-FITC antibody (green). The nuclei were visualized with DAPI (blue). L-lamellocyte. Magnification 100x.

In some cases the encapsulation reaction is directed against the organism itself. In many mutations in *Drosophila,* melanotic tumors can be observed as black masses of tissues [43]. During the tumor development, the hemocytes form a capsule around the self-tissue and deposit melanin in it [44].

1.2.3. Activation of signaling pathways in humoral and cellular responses

In *Drosophila melanogaster*, three signaling pathways signal the presence of microbes and mediate humoral and cellular responses: the Toll pathway, the Imd pathway and JAK/STAT pathway.

1.2.3.1. Toll pathway

Toll was originally identified as a transmembrane receptor required for the establishment of dorso-ventral polarity in the developing *Drosophila* embryo [45]. Later it was discovered that Toll pathway mediates the induction of drosomycin and other antibacterial peptide genes in the fat body in response to fungal and Gram-positive bacterial infection. [46, 47, 48]. However, even Gram-negative bacteria can lead to a weak induction of this pathway [49, 50]. The *Drosophila* Toll signaling pathway shows remarkable similarity to the mammalian IL-1 pathway, which leads to activation of NF-κB, a transcription factor responsible for many aspects of inflammatory and immune responses. The *Drosophila* Toll does not directly recognize pathogens, as do the mammalian TLRs. The ligand for *Drosophila* Toll is a circulating endogenous protein, Spätzle, which is proteolytically activated by a serine protease cascade in response to infection [49, 50] (Fig.3). Recognition of pathogens by receptors acting upstream of Toll is required to cleave and activate Spätzle. The cleaved Spätzle binds to the leucine rich ectodomain of the transmembrane receptor Toll. This ligand/receptor interaction activates the cytoplasmic serine/threonine kinase Pelle, via MyD88 and the adaptor protein Tube. Activation of Pelle promotes degradation of the ankirin repeat protein Cactus, homolog to the mammalian IκB protein, which associates with the Rel-type transcription factor Dif (in humoral response) or Dorsal (in embryonic development) in the cytoplasm. Cactus dissociate from Dif or Dorsal by its phosphorylation [51]. The kinase responsible for Cactus phosphorylation is at present unknown. Once Cactus is degraded in response to Toll-mediated signal, Dif/Dorsal is free to translocate to the nucleus, where it regulates transcription of specific target genes [52]. The most specific target gene of the Toll pathway is drosomycin, but

Toll pathway also contributes to the induction of *cecropins* and *attacins* in concert with the Imd pathway.

The *Drosophila* Toll receptor was also reported to interact with filamin. Yeast two-hybrid screens identified the C-terminal part of filamin to interact with Toll. It has been proposed a model in which filamin associates with Tube and Toll receptor. In this way, filamin is bringing the receptor and intracellular signaling complex into close proximity at the cell surface [53].

Toll pathway is suggested to be involved in cellular defense and hematopoiesis. This was based on finding that mutations in Cactus or Toll, or constitutive expression of Dorsal can induce lamellocyte differentiation and cause the formation of melanotic masses [54], similar to the cellular response to parasites [55, 46].

1.2.3.2. Imd pathway is induced by Gram-negative bacterial infection, and it's activation leads to the expression of the gene encoding antibacterial petide, diptericin, through activation of Relish protein [56] (Fig.3). Although this pathway shares similarities with the human TNF pathway, there is no receptor homolog found in *Drosophila*. It has been identified by a mutation in the *immune deficiency* (imd) gene of *Drosophila* [46]. The exact molecular components from the Gram-negative bacteria that activate the Imd pathway remain controversial. It has been demonstrated that LPS do not stimulate Imd pathway and Gram-negative peptidoglycan was found to be recognized by PGRP-LC and activate Imd pathway [57]. The *imd* gene encodes a protein with a death domain that has stronger similarities to the mammalian RIP protein (a TNF-receptor interacting protein) [58]. Relish is a protein with an NF-κB (Rel homology) domain and an inhibitory IκB domain, similar in structure to mammalian p100 and p105. Downstream of Imd, the pathway branches into two paths, which to the cleavage of Relish. DREDD can associate with dFADD and with Relish, and it has been proposed that this caspase directly cleaves Relish [59, 60]. Also, a mitogen-activated protein 3 (MAP3) kinase homologous to TAK1 (TGF-β activates kinase 1) has been found to be involved in Imd pathway [61]. Maturation of Relish after Gram-negative bacterial infection is also dependent on a protein complex homologous to the mammalian signalosome, comprising dIKK-β and dIKK-γ [62, 63, 64]. This complex is activated by dTAK1 and directs phosphorylation of Relish, which precedes its proteolytic cleavage [65]. Besides being an important kinase in the Imd/Relish pathway, dTAK1 also activates the JNK pathway, which is not required for antimicrobial peptide gene expression but perhaps is a component for the activation of the cellular immune response and the stress response [66, 67]. Relish carries

inhibitory ankirin-repeat sequences at the carboxy-terminal region [49, 68]. In unstimulated cells, Relish is present in the cytoplasm as a full-length precursor protein and soon after infection, Relish is cleaved, allowing the physical dissociation between the ankyrin repeats and the Rel domain which is then freed to translocate to the nucleus and activates antimicrobial gene expression, whereas the C-terminal IκB domain remains in cytoplasm [69].

1.2.3.3. JAK/STAT pathway is an evolutionarily conserved, intracellular signaling pathway that plays a central role during hematopoiesis, cell movement, cell fate determination, and regulation of the cellular immune response [70; 71]. During cell migration, the JAK/STAT signaling pathway may connect to elements that regulate cytoskeletal modifications [72].

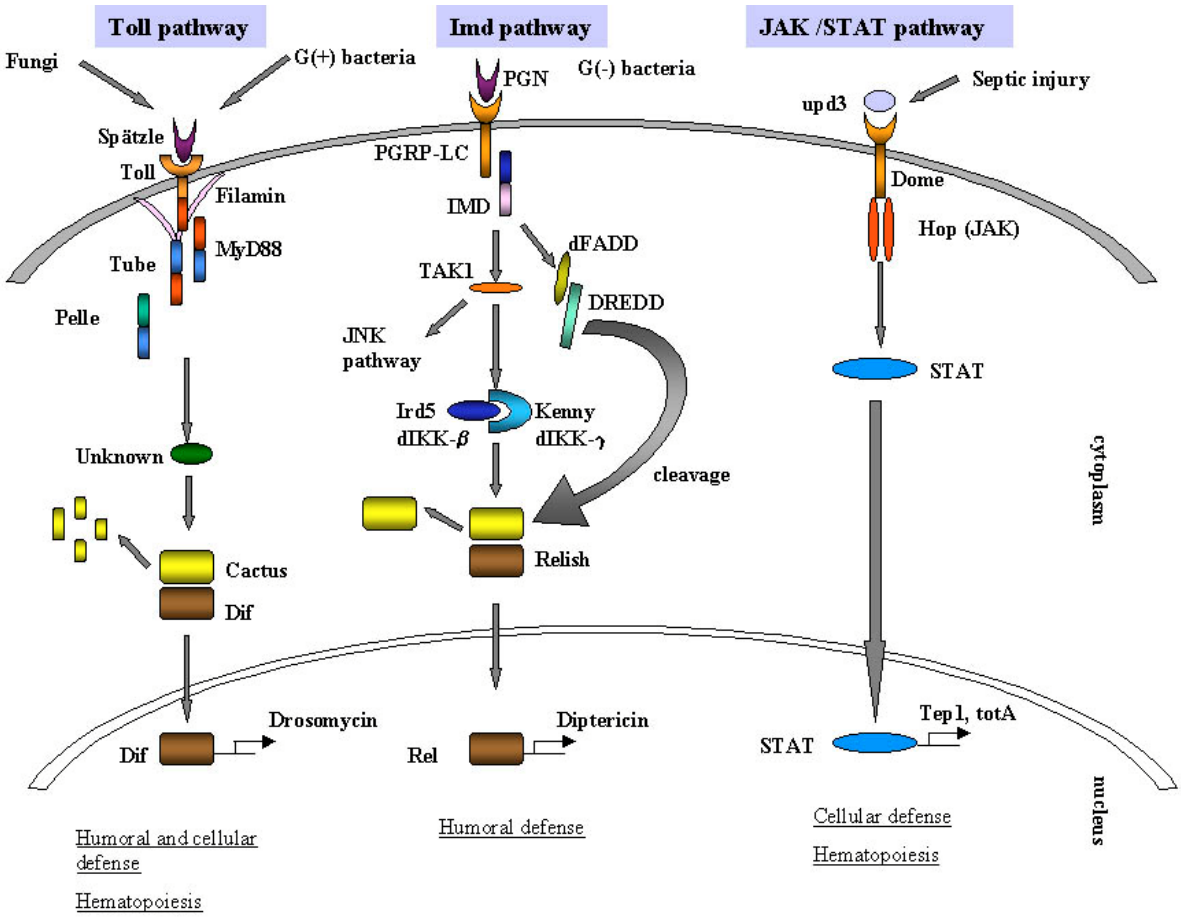

Figure 3. The Toll, Imd and JAK/STAT innate immunity pathways in *Drosophila melanogaster*. The Toll pathway (left) is induced by Gram-positive bacteria and fungi and is involved also in the cellular immune response and hematopoiesis. The Imd pathway (center) is involved in the activation of the humoral response against Gram-negative bacteria. The

JAK/STAT signaling pathway (right) in *Drosophila* plays a role in the regulation of cellular immune response and hematopoiesis.

JAK-STAT signaling in *Drosophila* is required for the induction in the fat body of a number of genes in response to infection, including the stress-induced gene *totA,* as well as *Tep1*, which encodes a thiolester-containing protein that may be an opsonin [73, 74]. The four main components of his pathway are the ligand, unpaired (upd), the receptor domeless (Dome), the JAK (Hopscotch/Hop), and the STAT (STAT92E/Marelle) [75]. Upon septic injury, breakage of the cuticle and epithelium barriers leads to the penetration of bacteria into the hemolymph. These events lead to the activation of *upd3* expression in hemocytes. The Upd3 cytokine is presumably released in hemolymph and it activates transcription of *totA* in the fat body by signaling through the receptor Domeless and the JAK-STAT pathway [74]. The activation of *totA* in *Drosophila* fat body is very similar to some aspects of the mammalian acute-phase response. These signaling events control the activation of genes encoding *Tep1* and the *totA* (Fig.3). The *Tep1* is supposed to promote phagocytosis, whereas the function of the *totA* peptides is unknown**.** It was found that overexpression of *tot A* confers a longer survival of flies subjected to heat stress. It was suggested that the Tot proteins are involved in physical protection/repair of damaged tissue. In general, dependence on JAK-STAT signaling correlates with delayed, transient induction following immune challenge [73]. In addition, constitutive JAK signaling hyperactivates the hemocytes [73]. In addition to its role in the production of humoral factors in fat body, the JAK/STAT pathway is also probably involved in the cellular immune response taking place in the lymph glands. It have been suggested that the activation of JAK/STAT pathway might be responsible for lamellocyte differentiation upon wasp infestation. The events that lead to differentiation have not been determined [72].

1.2.4. The involvement of the actin cytoskeleton in cellular immune reactions

In this work we have cloned and characterized a *Drosophila* blood cell antigen expressed in lamellocytes, and showed that it corresponds to *Drosophila* Filamin-240, an actin-binding protein. Therefore, it is need to describe briefly what it is known so far about the role of the actin cytoskeleton in *Drosophila* cellular immune responses.

The cytoskeleton is a dynamic complex network of protein filaments that extends throughout the cytoplasm. It consists of three types of filaments that are fundamental for the spatial organization of the cells: intermediate filaments, microtubules and actin filaments. Among

these, the actin filaments are flexible structures, organized in a variety of linear bundles, two-dimensional networks and three-dimensional gels. Although actin filaments are dispersed throughout the cell, they are most highly concentrated in the cortex, under the plasma membrane. The actin cytoskeleton plays a central role in many cell functions such as the maintenance of cell shape, cell division, adhesion, motility, signal transduction and protein sorting [76]. A cellular immune response such as phagocytosis requires rearrangement of the actin cytoskeleton, and a myriad of actin regulatory proteins orchestrate this rearrangement [77]. To produce outward protrusions such as pseudopodia and lamellipodia, the actin cytoskeleton must be continually remodelled in response to intracellular and external stimuli [78]. In *Drosophila* hemocytes, the control of actin dynamics during phagocytosis is achieved by a variety actin binding proteins [79]. For example profilin, a protein, involved in the regulation of actin polymerization, was found to have a negative regulatory role in phagocytosis. This was suggested on the basis that in a profilin mutant, called *chicadee* (*chic*) was found an increased phagocytosis [80]. D-SCAR and D-WASp proteins have been also found to be required for phagocytosis by *Drosophila* hemocytes [80]. They activate and regulate Arp2/3 complex-mediated nucleation of actin filament formation and, together with filamin, play an important role in actin filament branching and dynamics [80-85]. It was observed that in hemocytes lacking D-SCAR, the kinetics and/or frequency of Arp2/3 complex-dependent actin branching and nucleation may decrease, and the dynamic balance between the nucleation of new filaments and the elongation of existing filaments may be altered, resulting in decreased phagocytosis. The hemocytes lacking D-SCAR have dramatically altered morphology and contained numerous F-actin-rich spikes [80]. A microarray study of a *Drosophila* mbn-2 cell line has revealed up-regulation of several genes (*spire*, *singed*, *inscutable*, and *enabled*) with different functions in cytoskeletal organization [79].

The single known function of lamellocytes is to participate in the encapsulation reaction against intruders that exceed the size limit for phagocytosis. So far, was found that rearrangement of microtubules accompanies the modification of lamellocyte shape from a disc to bipolar form during the cellular immune response [86]. The cellular reaction as well as the terminal differentiation of lamellocytes, must involve extensive rearrangements of the actin network as well, but the actin binding proteins and their function in these processes remain to be identified.

2. Aim of study

In contrast to the well studied regulation of antimicrobial peptide production, the humoral responses, very little is known about the molecular mechanisms of the cellular immune response in *Drosophila* and the origin and differentiation of hemocytes. For an understanding of the recognition, signaling and development of hemocytes in the different cellular compartments of the *Drosophila* immune system, there is a need for molecular markers that are expressed in a cell type specific manner. Our laboratory created a library of hemocyte-specific monoclonal antibodies and used it to define specific marker molecules restricted to hemocytes. Some antibodies recognize antigens, expressed on all blood cells; other are restricted to subset of blood cells, such as the plasmatocytes, the lamellocytes or the crystal cells. The antibodies have been clustered on the basis of their reactivity with hemocytes and the biochemical features of the identified antigen (Table 1).

We expect that identified molecules specifically expressed on different hemocyte subsets are involved either in the immune function of the corresponding blood cell type or may play a role in hematopoiesis. So far has been cloned and characterized the gene for the L2 pan-hemocyte antigen, Hemese, by using a reverse genetic approach. The gene encodes for a transmembrane protein expressed on all circulating blood cells and is involved in the regulation of blood cell development [32]. Also, the gene for the plasmatocyte specific antigen P1, has been identified as a transmembrane receptor with several EGF repeats. The gene is located in the cluster of genes for structurally related molecules, possibly involved in the regulation of blood cell development and/or function (manuscript, in preparation). The definition and use of further hemocyte markers could help us to identify blood cell antigens, to define their genes and to study their structure and function. Finally, molecular and genetic analysis could elucidate the mechanisms of hemocyte development and activation in immune responses.

Table 1

Hemocyte antigen clusters (CDs) in *Drosophila melanogaster*

Cluster	Cell type	M.W.[+]	Hybridoma clone
H1	all hemocytes and embryonic macrophages	135-160	H11
H2	all hemocytes	30-60	1.2
H3	all hemocytes	16	4A12
P1a	mature plasmatocytes	100-110	N1
P1b	mature plasmatocytes	100-110	N47
P3	plasmatocyte subset	*	8B1
L1a	lamellocytes	16	H10
L1b	lamellocytes	16	7A6
L1c	lamellocytes	16	29D4
L2	lamellocytes	44	31A4
L4	lamellocyte subset	82-86	1F12
L5	lamellocyte subset	85-100, 240^	4B8
L6	lamellocyte subset	96	H3
C1	crystal cells and their precursors	84	12F6
C2	crystal cells and their precursors	*	21D3
C3	crystal cells and their precursors	*	10D2
C4	mature crystal cells	100	9C8
C5	mature crystal cells	66,135	1.19
Ad1	all hemocytes in the adult	10	7C8

[+] kDa, non reducing conditions

* conformation – dependent epitope

^ reduced

3. Results

3.1. The physical requirement for lamellocyte development

To test the effect of mechanical injury on lamellocyte development we punctured second instar larvae and at the same time we performed a wasp infestation with *Leptopilina boulardi.* Two hours after mechanical injury or wasp-infestation a melanotic spot appeared at the site of the lesion. We quantified hemocyte induction by determining the number of all hemocytes (Fig.4A) and of lamellocytes (Fig.4B) 48, 72, 96 and 120 hours after immuno-induction or wounding. Lamellocytes appeared in the circulation in both the sterile wounded and in the infested animals but not in the untreated controls. Also, the total hemocyte number was elevated both in the sterile wounded and in the wasp infested groups, as compared to the un-induced control. The total hemocyte counts and the number of the lamellocytes were the same in the infested and in the sterile-wounded groups ($p>0.3$). The first L1 positive cells appeared 48 h after induction, their number peaked at 72 and 96 hours and they were not detectable in the white pupa, 120 hours after infestation.

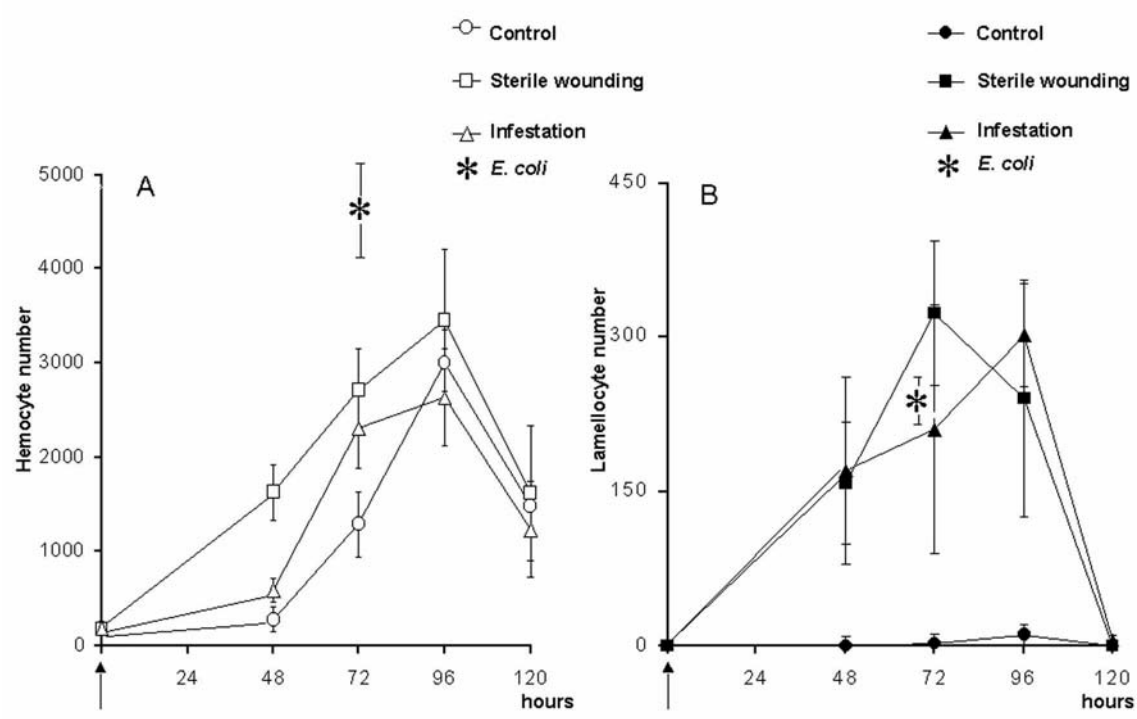

Figure 4. Kinetics of the hemocyte response following infestation, sterile wounding and infection by *E. coli* bacteria. Blood cells were isolated at times indicated and adhered. The total blood cell count (A) and the number of lamellocytes (B) are showed. The bars represent the standard errors of the mean.

In natural environment rupture of the cuticle may be accompanied by penetration of bacteria into the body cavity through the wound. We injected live *E.coli* with the micro-capillary into second stage larvae and counted lamellocytes and all hemocytes, in order to test whether or not, infection via the wound canal would have an effect on lamellocyte induction. The results have shown no difference in the lamellocyte counts between the sterile-punctured and the *E.coli*-injected groups (Fig.4), however the total hemocyte number was slightly, but significantly higher ($p<0.01$) in the *E.coli* injected individuals (Fig 4A).
The morphological characteristics, the kinetics of the lamellocytes induced by sterile wounding were the same as in those induced by parasitic wasp infestation (data not shown).

3.2. Identification of a lamellocyte-specific antigen by 4B8 antibody

We have identified an antigen by the aid of 4B8 monoclonal antibody, as being expressed in the majority of lamellocytes in a hemocyte overproducing *l(3)mbn-1* mutant larvae [87] (Fig. 5-A-B) as well as following immune induction of Oregon-R larvae by the parasitic wasp *Leptopilina boulardi* (Fig.5C-D). The antigen recognized by the antibody was detectable on permeabilized hemocytes, but not on native, live cells (data not shown) demonstrating that it was expressed in the cytoplasm.

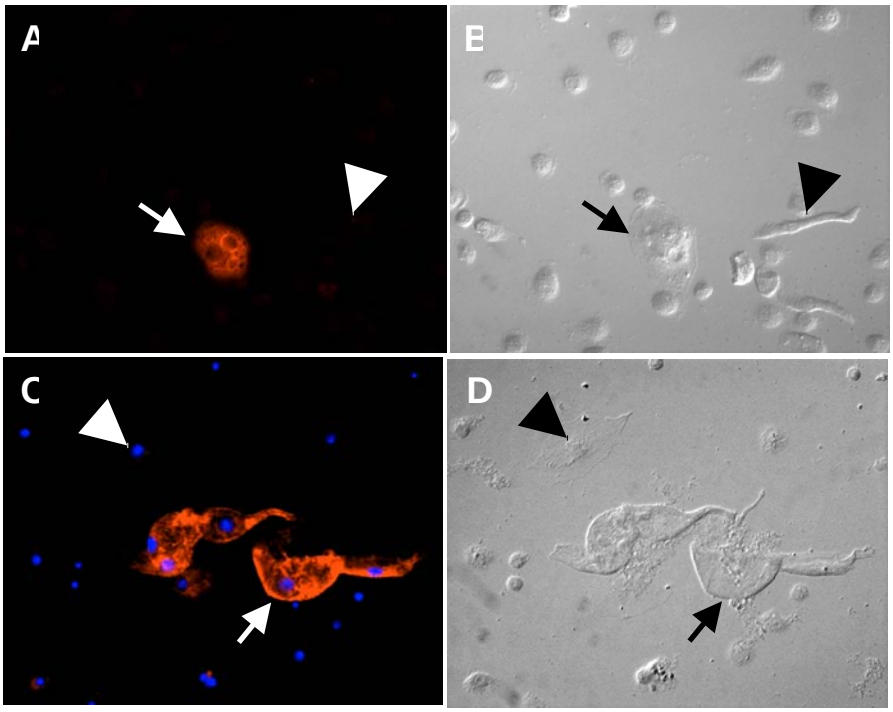

Figure 5. The 4B8 antibody reacts with lamellocytes.
Immunofluorescent detection of the recognized antigen in circulating hemocytes of *l(3)mbn-1* larvae (A) and wasp infested Oregon-R larvae (C). Figures (B) and (D) are the corresponding Nomarski pictures. Red fluorescence is 4B8 staining, whereas the blue, DAPI-fluorescence, marks the nuclei. Lamellocytes expressing or non-expressing this antigen are marked with arrows and arrowheads, respectively. Magnification 400x.

Western blot analysis of extracts from *l(3)mbn-1* homozygous larvae yielded two major protein bands, corresponding to 90 and 240 kDa (Fig. 6A-lane 1). In extracts from the wild type Oregon-R or the hemocyte deficient *dom*^{1} homozygous larvae, one, 90 kDa protein band was found (Fig. 6A-lane 2; A-lane 3). In hemocyte extracts prepared from the *l(3)mbn-1* homozygous mutants or from the wasp infested Oregon-R larvae, a predominant 240 kDa protein was expressed (Fig.6B-lane 1, B-lane 2), whereas the expression of the 90 kDa protein is under the level of detection or is very low. In the extracts derived from hemocytes of the non-infested Oregon-R larvae the expression of these proteins is near the threshold of detection (Fig.6B-lane3). Thus, both the cellular expression pattern of the antigen and the Western blot analysis both confirmed that the expression of the 240 kDa protein seen by the 4B8 antibody is restricted to lamellocytes.

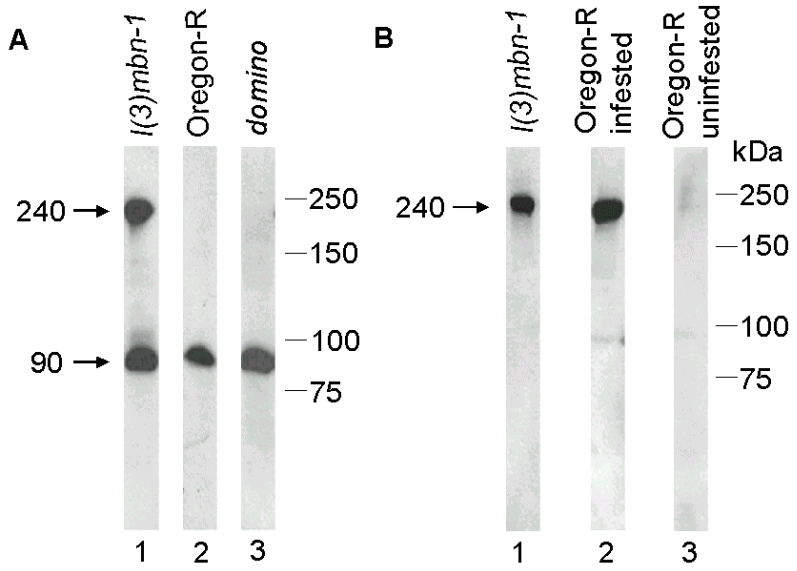

Figure 6. Biochemical characterization of the protein recognized by mAb 4B8.
Western blot analysis on whole larval (A) and hemocyte extracts (B). In extracts from the *l(3)mbn-1* homozygous mutant larvae, two major protein bands corresponding to molecular masses of 90 and 240 kDa (A-lane 1) are seen. In extracts from the wild type Oregon-R (A-lane 2) or the hemocyte deficient *dom*^{1} homozygous mutant larvae (A-lane 3), only the 90 kDa protein band is present. In the hemocyte extracts of *l(3)mbn-1* (B-lane1) or in that of wasp infested Oregon-R larvae (B-lane 2) a predominant expression of the 240 kDa protein is seen. Slot B-lane 3 is uninfested Oregon-R control.

3.3. Expression cloning of the gene coding for the antigen recognized by 4B8 mAb

To get insight into the structure and function of the recognized molecule, first we screened a *Drosophila* cDNA expression library [32] with the 4B8 antibody. Seven clones were isolated and analysed further. The sequence analysis of the five positive clones defined eight exons of the *Drosophila cheerio* gene, the gene encoding for the *Drosophila* filamin (Table 2). Mapping of the isolated cDNA sequences to the filamin domain showed that they corresponded to the "filamin-folding domain", a consensus motif profile generated from the 20 existing filamin repeats (Fig.7).

Table 2.

L5 positive clones and their corresponding exons on the *cheerio* gene

L5 positive clones	***cheerio* locus**
2/1 (101-215)	Exon 10
(219-278)	Exon 11
2/2	No homology
2/3 (413-592)	Exon 7
5/1 (102-333)	Exon 2
(333-478)	Exon 3
(498-806)	Exon 4
8/1 (89-386)	Exon15
9/1 (99-212)	Exon14
(213-829)	Exon 15
11/1	No homology

Mapping sequences found in isolated cDNAc to filamin domain structure

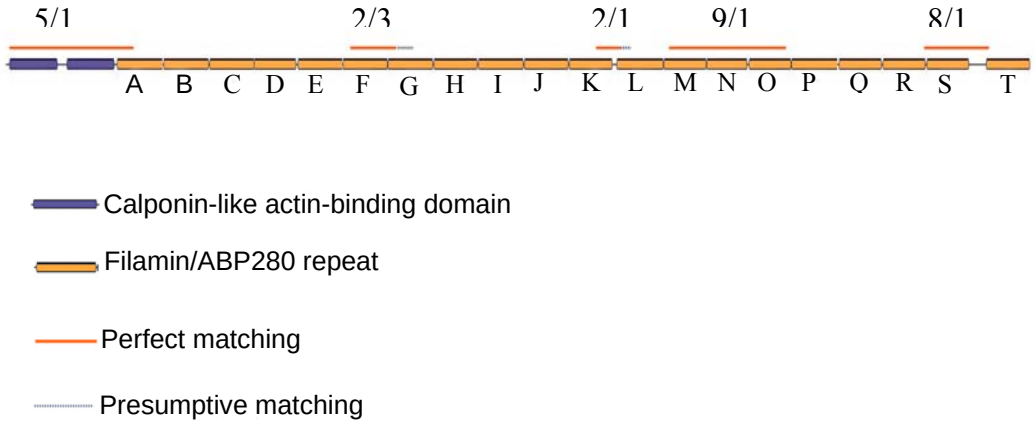

Figure 7. Mapping of the isolated cDNA sequences to the filamin domain structure. Five of the isolated cDNA sequences correspond to the filamin repeats of the rod region and one cDNA fragment has sequences which correspond to the actin binding domain of the filamin protein.

3.4. The antigen recognized by 4B8 mAb corresponds to the *Drosophila* Filamin-240

We have found that immunostaining with 4B8 antibody and a polyclonal antibody to the last 810 C-terminal residues of filamin [88] overlaps in lamellocytes of *l(3)mbn-1* homozygous larvae (Fig.8 A-D) and also in hemocytes isolated from parasitic wasp infested Oregon-R larvae (data not shown).

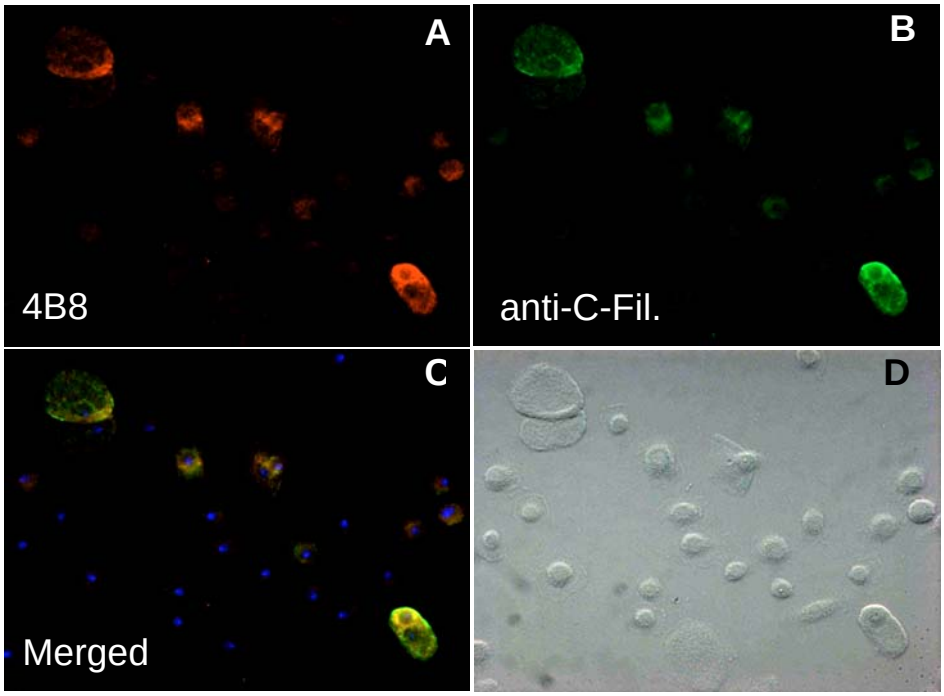

Figure 8. Cellular co-localization of the epitopes of the antigen recognized by the 4B8 and the polyclonal anti-filamin antibodies (A-D). Immunofluorescence analysis of *l(3)mbn-1* hemocytes. The 4B8 and the polyclonal antibody-reactive lamellocytes are visualized with red (A) and green (B) fluorescence respectively. Merged image (C) shows co-localisation as yellow colour. Nomarski image (D) of the same field. Magnification 400x.

As it was reported that Filamin-240 is expressed in the ring canals of Drosophila ovaries [88] we also stained ovaries to confirm that the 4B8 antibody reacts with ring canals. It was found that, similarly to the polyclonal antibody (Fig.2 E, G) 4B8 reacted with the ring canals and this staining was not present in ovaries of the *cher*^{1} homozygous mutant, which lack Filamin-240 (Fig.9 A-D).

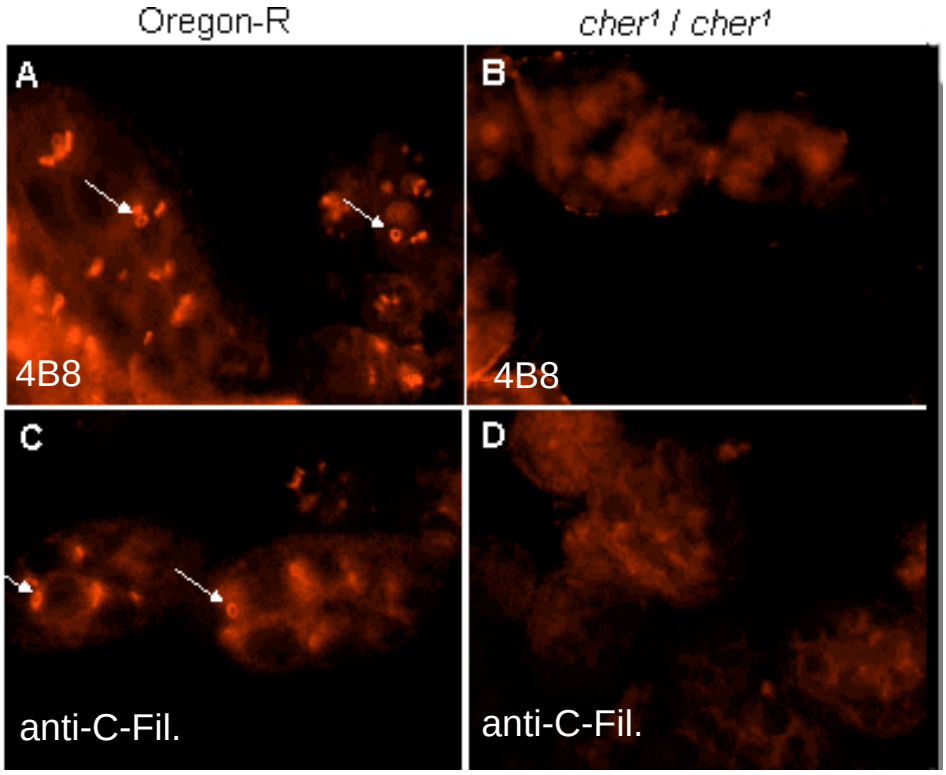

Figure 9. Expression of the antigen recognized by 4B8 (A, B) and the polyclonal antibody (C, D) in the ovaries of Oregon-R (A, C) and *cher1* homozygous (B, D) adult females. Ring canal staining is seen by both antibodies (A, C) in Oregon-R, while no staining is seen in ovaries of *cher1* homozygotes. Arrows indicate the stained ring canals.

Hemocyte extracts were used in Western blot analysis to see whether mAb 4B8 and the polyclonal anti-filamin antibodies indeed recognize the same protein (Fig.10A). The results showed the expression of both the 90 and the 240 kDa bands with the predominant expression of the high molecular weight protein. An immunoprecipitated material, isolated with the 4B8 antibody was also analyzed to reveal whether the isolated material contained proteins, detectable with both the 4B8 and the polyclonal antibodies (Fig.10B). The Western blot analysis of the immunoprecipitated antigen showed the presence of both the 90 and 240 kDa filamin bands, confirming that the proteins are Filamin-90 and Filamin-240 (Fig.10B-lane 1 and lane-2).

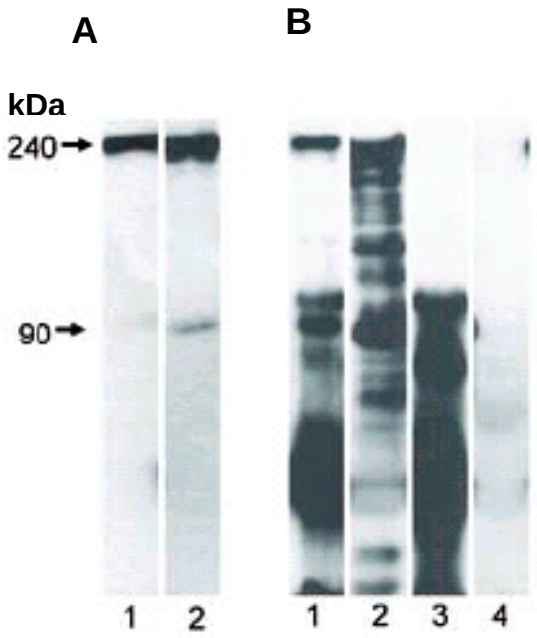

Figure 10. Western blot analysis of hemocyte extracts from of *l(3)mbn-1* homozygous larvae (A) with 4B8 antibody (lane-1) and anti-C-fil (lane-2) antibodies. Both antibodies reveal the the 90 and the 240 kDa bands, with predominant expression of the 240 kDa protein. Western blot analysis of the protein isolated with the 4B8 antibody (B) with 4B8 (lane-1) and anti-C-fil (lane-2) antibodies. Both antibodies reveal the 90 and the 240 kDa bands. Lanes 3 and 4 are the corresponding IgG controls.

3.5. Filamin-240 is expressed differentially in induced lamellocytes

Originally it was described that a loss of function allele of the *cheerio* gene, *cher*^{1}, due to the lack of Filamin-240, is defective in ring canal assembly, and as a consequence, has female sterility [88]. As the initial results revealed that the isoforms have a cell-type specific expression among hemocytes, we have studied Filamin-240 expression upon lamellocyte-induction followed by infestation with *Leptopilina boulardi in vivo,* in *cher*^{1} homozygous larvae and also in *cher*^{1} heterozygous larvae as a control. Hemocytes prepared from the non infested (Fig.11A, D) and from wasp-infested (Fig.11G, J) *cher*^{1} homozygous larvae did not react with mAb 4B8 and the 240 kDa band was not detected by Western blot analysis of the hemocytes of non infested (Fig.11M-lane1) or infested larvae (Fig.11M-lane2). In hemocytes of uninfested heterozygous larvae Filamin-240 is not expressed (Fig.11B, E and M-lane 3), however lamellocytes of wasp infested heterozygous larvae were stained (Fig.11H, K) and the staining correlated with expression of Filamin-240, as detected by Western blot analysis (Fig.11 M-lane 4).

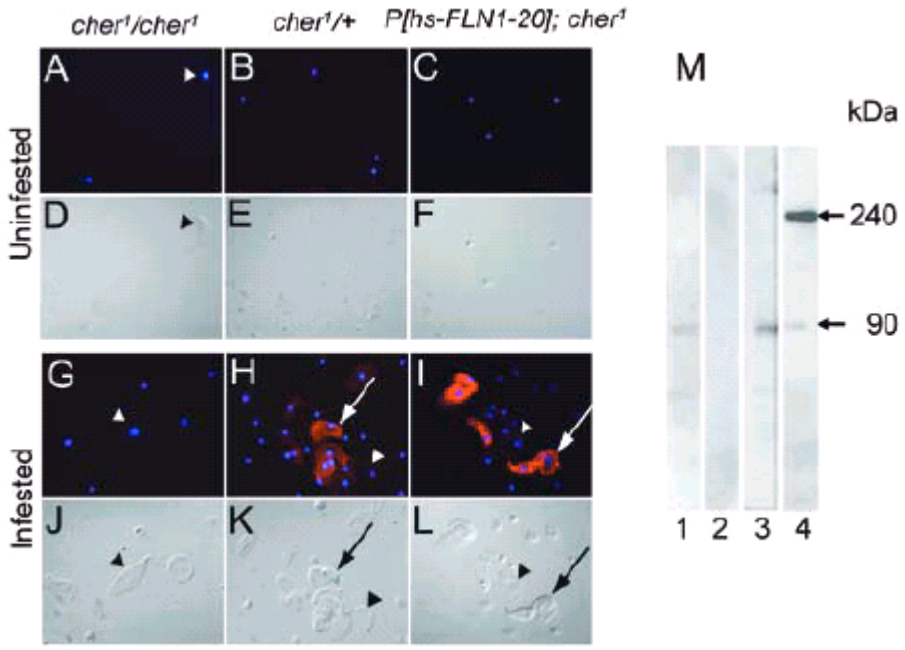

Figure 11. Lamellocytes express the Filamin-240. Immunofluorescent (A-L) and Western blot analysis (M) of hemocytes. Filamin-240 expression is visualized by 4B8 antibody staining in circulating hemocytes of uninfested (A-F) and infested (G-L) *cher*^{1} homozygous and heterozygous and *P[hs-FLN1-20]; cher*^{1} rescued larvae. The Filamin-240 is not expressed in the uninfested and infested *cher1* homozygous (A and G), in the uninfested heterozygote (B) and in uninfested *P[hs-FLN1-20]; cher*^{1} rescued larvae (C), but it is expressed in the lamellocytes from infested *cher1* heterozygous (H) and infested *P[hs-FLN1-20]; cher*^{1} rescued larvae (I). Figures D, E, F, J, K and L are the corresponding Nomarski images. Red fluorescence is 4B8 staining, blue fluorescence marks the nuclei. Lamellocytes expressing or non-expressing Filamin-240 are marked with arrows and arrowheads, respectively. Magnification: 400x. Western blot analysis (M) reveals that Filamin-240 is not expressed in the hemocyte extracts from uninfested (lane 1) and infested (lane 2) *cher*^{1} homozygotes, and in hemocyte extracts from uninfested *cher*^{1} heterozygous larvae (lane 3). In the hemocyte extracts from infested *cher*^{1} heterozygotes (lane 4) the Filamin-240 is expressed.

3.6. *Cheerio* is a suppressor of lamellocyte development

As the expression of Filamin-240 is restricted to lamellocytes among blood cells next we asked whether or not this isoform could be involved in regulation of lamellocyte development. Analysis of the blood cell phenotype of the loss-of-function, *cher*^{1} homozygous mutant revealed that lamellocytes appear in the circulation without any immune challenge (Fig.4A and G), which is further increased following infestation with the parasitoid wasp (Fig.4B and G). This phenotype is correlated with the lack of immunostaining with 4B8 antibody in lamellocytes of uninfested (Fig.3 A and D) and infested (Fig. 3 G and J) and the lack of the 240 kDa band in larval extracts (Fig. 4 J). In control experiments wild type Oregon-R larvae, which expressed the 240 kDa protein (Fig 4.K) had no lamellocytes in the circulation (Fig 4.C and H) and following infestation the number of lamellocytes (Fig. 4D and H) was significantly

less than that in the *cher*^{1} homozygotes. On the basis of these data a specific enhancing effect of the mutation on the number of lamellocytes is observed therefore we propose that *Drosophila* Filamin-240 is a suppressor of lamellocyte development. To confirm that the lamellocyte overproducing phenotype was the result of the specific mutation in the *cheerio* gene we rescued the mutant phenotype by introducing the full-length transgene (*P[hs-FLN1-20]*), encoding the *Drosophila* Filamin-240, into the *cher*^{1} homozygous mutant lacking the 240 kDa isoform (Fig. 4 J). Introduction of the full length transcript into *cher*^{1} results in the rescue-stock *P[hs-FLN1-20];cher*^{1}, with the same genetic background as *cher*^{1}. The rescued larvae expressed Filamin-240 as revealed by Western blotting (Fig.4 L), similarly to the induced Oregon-R wild type larvae (Fig.4K). In the hemolymph of the rescued larvae (Fig.4E), similarly to the wild type Oregon-R (Fig.4C), we have found no lamellocytes; in addition after parasitic wasp infestation, a comparable lamellocyte count was found in these two groups (Fig.4D,F,H,I) which however never reached the number of lamellocytes seen in the *cher1* homozygotes. Comparison of the *cher*^{1} homozygotes with the rescue stock shows that there are 10 times more lamellocytes in the *cher*^{1} homozygotes (lamellocyte number: 2003) than in the *P[hs-FLN1-20];cher*^{1} stock (lamellocyte number: 227), while there is no significant difference between the total cell numbers (*cher*^{1} homozygotes total cell number: 3634 vs. *P[hs-FLN1-20];cher*^{1} total cell number: 3292, p=0,3) showing that the effect is specific to lamellocytes. In spite of that, the wild-type Oregon-R is an independent stock with a different genetic background; the lamellocyte number is comparable to that of the rescue stock. This stock has initially less blood cells than the other two stocks and also fewer blood cells than the other two stocks, however, upon infestation the proportional increase is the same as in the other two stocks. Also, we found the same pattern of Filamin-240 expression by immunofluorescence analysis in uninfested (Fig.3C, F) and wasp infested *P[hs-FLN1-20];cher*^{1} hemocytes (Fig.3I, L) as in the wild type Oregon-R blood cells where fully differentiated lamellocytes express Filamin-240.

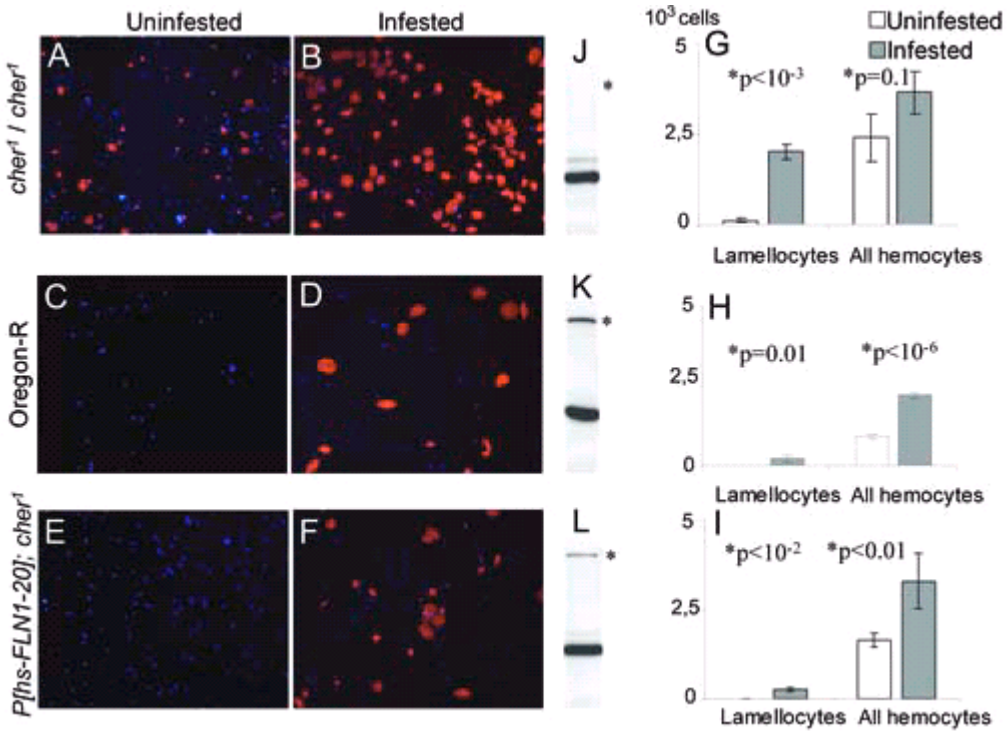

Figure 12. Analysis of blood cells in *cher*^{1} homozygote (A, B, G), Oregon-R (C, D, H) and *P[hs-FLN1-20]; cher*^{1} rescued larvae (E, F, I) upon wasp infestation. Circulating hemocytes of uninfested and wasp infested larvae of *cher*^{1} homozygotes (A and B), Oregon-R (C and D) and *P[hs-FLN1-20]; cher*^{1} rescued larvae (E and F). The lamellocytes are visualized by immunofluorescent staining with L1 antibody (red), the nuclei are stained with DAPI (blue). Differential hemocyte counts (G-I) show that regardless parasitic infestation the number of lamellocytes and all hemocytes in the *cher*^{1} homozygote larvae (G) is higher than in the wild type Oregon-R larvae (H). In the *P[hs-FLN1-20]; cher*^{1} rescued larvae (I) the lamellocyte number is restored. The error bars represent the standard error of the mean. Western blot analysis (J-L) shows that Filamin-240 is not present in the whole larval extracts of infested *cher*^{1} homozygotes (J). In whole larval extracts from infested Oregon-R (K) and from infested *P[hs-FLN1-20]; cher*^{1} rescued mutant (L), the Filamin-240 is detected (the position of the 240 kDa protein is shown by asterix)

Thus the analysis of the rescue experiments shows that introduction of the full length cheerio cDNA into the *cher*^{1} homozygotes results in the expression of Filamin-240 in lamellocytes as well in suppression of lamellocyte counts. We conclude therefore that in the rescue-starin, *P[hs-FLN1-20];cher*^{1}, filamin undergoes a normal post-transcriptional modificaton in lamellocytes as it does in the ovaries [101], and functions in blood cells of *Drosophila* as a suppressor of lamellocyte development.

3.7. Other actin-binding proteins are expressed in different subsets of hemocytes

Since, filamin is not the only cross-linking protein present in the actin cytoskeleton, we extended our work investigating the expression of other actin-binding proteins in *Drosophila* hemocytes and we found that some of them are expressed in a hemocyte subset-specific manner (Fig.15). Singed protein was expressed in a subpopulation of lamellocyte, while hts was found in a subpopulation of small, rounded hemocytes (Fig.15-A, B, D, E). Kelch, another actin-binding protein was expressed in plasmatocytes (Fig.15-C, F).

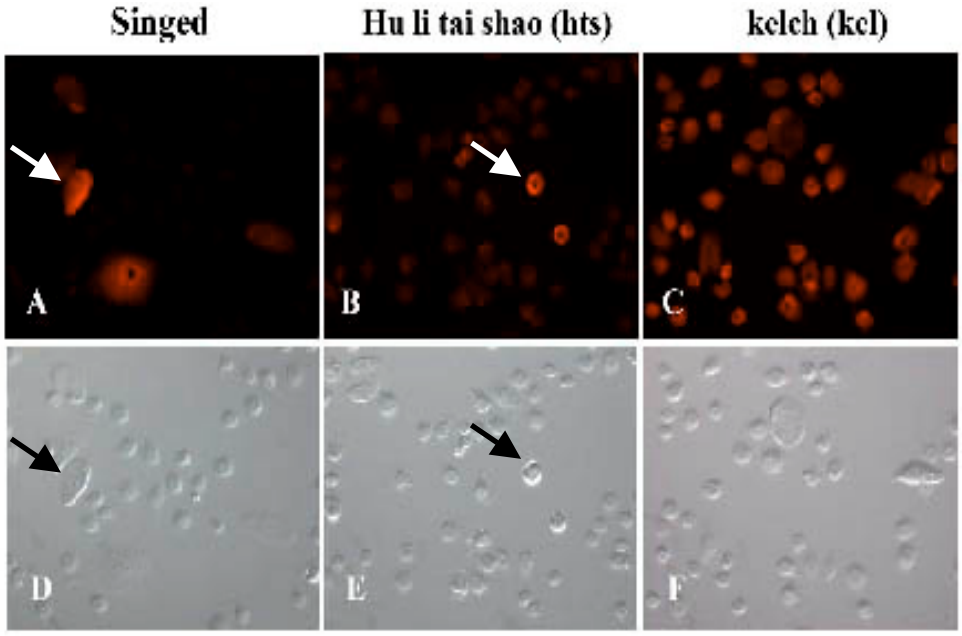

Figure 15. The expression of other actin-binding proteins in different subsets of hemocytes. Immunocytochemical analysis of *Singed*, (A) *hts* (B) and *kel*(C) expression in circulating hemocytes isolated from third-instar larva of overproducing hemocyte *l(3)-mbn-1* mutant. Magnification 400x. The cells were stained with anti-*Sn* mAb, anti-*hts* mAb, anti-*kel* mAb and visualized with Streptavidin-Cy3 complex (red). The nuclei were detected with DAPI. (D)(E)(F) Nomarski images.

4. Discussion

It is known that bacterial challenge does not induce lamellocyte differentiation, but objects placed in the larval hemocel that are too large to be phagocytated can artificially induce this.

We have found that sterile wounding of the cuticle is a sufficient trigger for lamellocyte development. The lamellocytes induced by sterile wounding express all the so far identified immunological markers of lamellocytes, and the kinetics of lamellocyte induction is the same as in those induced by parasitic infestation. Our results suggest that the ruptured basal lamina itself could be the minimal and sufficient single signal for normal lamellocyte development and cellular immune response in *Drosophila*.

Using expression cloning, we found that the monoclonal 4B8 antibody recognizes the protein Filamin-240. Filamins are a family of high molecular mass cytoskeletal proteins that organize filamentous actin in networks and stress fibres, anchor various transmembrane proteins to the active cytoskeleton and provide a scaffold for a wide range of cytoplasmic signaling proteins [89].

They are long, flexible cross-linking proteins, which allow fibres to have a perpendicular orientation found in networks (Fig.16). Filamin protein works like a clip to connect the filament at the crossover points. The actin network formed by filamin is required for cells to extend lamelipodia that help them to crawl across solid surface [76].

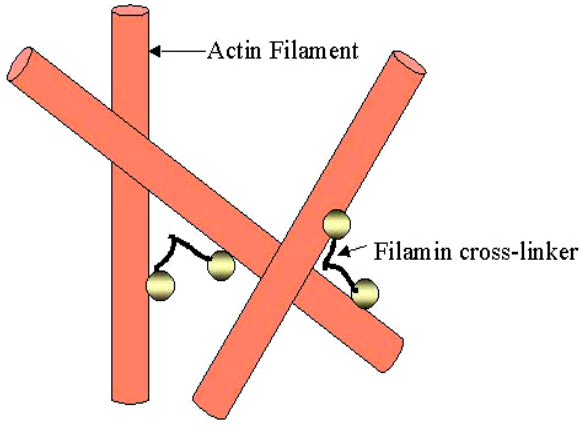

Figure 16. Actin filaments cross-linked by filamin protein

Filamin is the most widely distributed mammalian member of a protein family, also expressed in birds and insects with paralogues in lower eukaryotes [90].

In human three filamin genes, encoding filamin proteins have been identified: FLNA, FLNB and FLNC, which by alternative splicing give rise to several isoforms. Human filamins bind to transmembrane proteins, such as integrins, [91] the glycoprotein Ib/V/IX complex [92],

dystrophin-glycoprotein complex [93] and integrate signaling pathways by binding to kinases such as MEKK [94] or to small GTPases: Rac, Ral A, Rho Cdc 42 [95]. In platelets filamin attaches the actin cytoskeleton to the transmembrane glycophorin Ib/V/IX complex, which binds on the exterior to clotting factors, permitting communication between extracellular matrix and cytoskeleton. As a result the platelet can tighten the clot by contracting [76]. Also, human filamin binds to TRAF2 and play a role in TNF receptor and TRAF2-mediated activation of SAPK and Toll receptor-mediated activation of NF-κB. Human melanoma cells deficient in filamin fail to activate NF-κB in response to TNF or when it is transfected with constitutively active TLR4 or TRAF6 [96]. Mouse, chicken, *Drosophila, Dictioselyum, Entamoeba histolytica* orthologues of human filamin have also been identified [89]. *Drosophila* filamin exists in four isoforms, (FlyBase accession numbers: FBpp0088478, FBpp0088480, FBpp0088965, FBpp0003455) as a result of alternative splicing, but available ESTs indicate that still others may exist. The domain organization of *Drosophila* Filamin-240 is identical to other filamins: an N-terminal a-actinin-like actinbinding domain, followed by 20 filamin repeats, which are interrupted by two linker sequences, called hinges, which confer flexibility to the molecule. The hinge 1 region divides the protein into rod I and rod II.. At the carboxy-terminal region there is the dimerization domain [88, 97] (Fig.17). The region of highest similarity between *Drosophila* and human filamins is in the actin-binding domain (70% identity).

It has been established that Filamin-240 associates with the plasma membrane of the ring canal in Drosophila ovaries and attaches the actin scaffold to the membrane. Also, it contributes to forming parallel bundles of actin filaments in the inner rim of the ring canals.

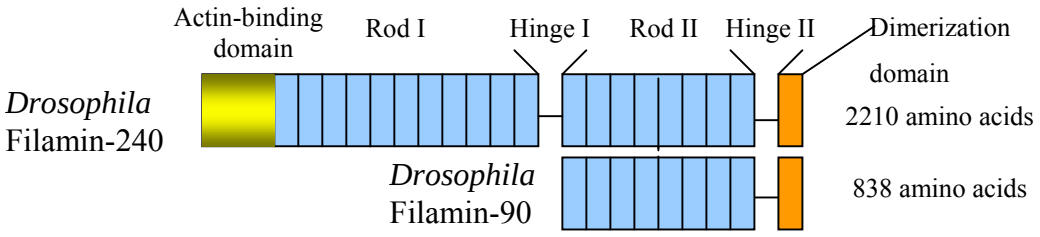

Figure 17. Domain organization of *Drosophila* Filamin-240 and 90. The length is indicated to the right of each protein.

Mutations in fruit fly Filamin-240 causes a reduced and defective organization and stabilization of actin in the ring canal structures and as a consequence are formed small and nonviable eggs. The shorter form, Filamin-90, lacks the actin-binding domain and rod I, and is broadly expressed. The function of this isoform has not been identified yet [88]. Among the four existing isoforms of filamin arisen by post-transcriptional modification, we have detected Filamin-240 and Filamin-90 using a monoclonal antibody, 4B8. Biological activity so far has been assigned to Filamin-240, containing the acting binding domain; the lack of this isoform results in female sterility [88]. Here we show that this isoform is expressed in a subset of blood cells, the lamellocytes too. *Drosophila* filamin was found to interact with Toll and Tube, confirming its role in Toll signaling pathway [53] and also with transmembrane endoprotease presenilin, showing that overexpression of filamin could suppress dominant phenotypes produced by presenilin overexpression on notch signaling during development [98]. The interactions of filamin with Toll, Tube, presenilin suggest that filamin provides a scaffold for a variety of signal transduction complexes. We have identified *Drosophila* Filamin-240 as being expressed differentially in lamellocytes. The results suggest that Filamin-240 is a suppressor of lamellocyte development.

Dynamic reorganization of the actin cytoskeleton transforms cell shapes and generates the forces necessary for cell locomotion and cell division. The leading edge of motile cells contains three-dimensional orthogonal networks of short filaments overlapping in X-, T- or Y-shaped junctions. Filamin has been detected at such junctions in rabbit macrophage, human platelet and tumor cell cytoskeletons [90]. Cultured human melanoma cell lines that do not express filamin are more easily deformed than other melanoma cell lines that contain this protein. The filamin-null cells are unable to migrate across porous filters in response to chemoattractants. Under conditions expected to promote cell locomotion, the filamin-deficient cells exhibit continuous circumferential blebbing. These results implied that the lack of filamin leads to aberrant actin filament organization, incapable of maintaining surface stability or supporting locomotion [99]. Also, *Dictyostelium* motility requires filamin; motile *Dictyostelium* amoebas that lack filamin are unable to support pseudopodia and as a result, their locomotion is affected. [100]. In *Drosophila*, reduced expression of filamin in follicle cells causes defects in encapsulation of germline cysts and in the migration of the border cells through the germline cyst [101].

We found that the encapsulation reaction which occurs as a response to parasitic was infestation was not affected in *cher*^{1} mutant. This suggests the filamin is not involved in this function of lamellocytes.

Lamellocytes undergo massive differentiation in response to infection caused by parasites and pathogens [102]. Cell shape transformation requires dynamic reorganization of the actin cytoskeleton. It was found previously that rearrangement of microtubules accompanies the modification of lamellocyte shape from a disc to bipolar form during the cellular immune response [36]. The terminal differentiation of lamellocytes, flattening must involve dynamic reorganization of the actin cytoskeleton and Filamin-240 is involved in this process. So far, it was found that Drosophila filamin interacts with Toll receptor [53] and mutantions in Toll can induce lamellocyte differentiation [54]. By attaching the cytoskeleton to the Toll signaling pathway it is possible that Filamin-240 enables the dynamic rearrangements of the actin cytoskeleton required for lamellocyte differentiation. Further, it would be interesting to investigate how the actin cytoskeleton functions in the Toll pathway and how the interaction of Filamin-240 with Toll receptor may be regulated.

Also, we found that *singed* protein, the homologue of fascin, known to be required for actin filament bundle formation in the cytoplasm of nurse cells during *Drosophila* oogenesis, [103] is also expressed in a subpopulation of lamellocytes. It was suggested that in human fascin protein play a role in extending the membranes, which is need it for cell motility or for interactions with other types of cells [104]. In *Drosophila*, *singed* may be involved in the formation of membrane extending, which are needed for lamellocyte motility during the encapsulation process. *hts* (hu-li-tai-shao) protein, involved in assembly and function of ring canals in the nurse cells of *Drosophila* ovaries, [105, 106] was found expressed in a subpopulation of small round hemocytes. *Kelch* protein, known to hold the actin bundles together during the expansion of the ring canals, [107] is expressed in plasmatocytes, too. Further, it would be interesting to study the role of these actin-binding proteins in *Drosophila* hemocytes. Also, other questions remain to be answered about filamin function in *Drosophila*. Since filamin is known to interact with and be required for the proper function of some receptors in mammalian systems, it would be interesting to find the interacting partners of *Drosophila* filamin and its role in the signaling pathways.

5. Summary

Lamellocytes are only occasionally observed in healthy larvae. They appear in the circulation as a result of parasitic wasp infestation.

Relatively little is known about the physical requirements for induction of lamellocyte differentiation and regulation of development. First I have studied physical requirements, then cloned a gene involved in regulation of this blood cell type.

Previous studies have shown that an attack and oviposition by parasitic wasp *Leptopilina boulardi* induces a vigorous cellular immune response in *Drosophila melanogaster* larvae, manifested by the differentiation of lamellocytes. We found that sterile wounding, comparable to that occurring during oviposition, induces normal lamellocyte development. The morphology and the kinetics of the lamellocytes induced by sterile wounding were the same as in those induced by parasitic infestation. Our data indicate that mechanical damage of the cuticle and subsequent disruption of the basal lamina is a minimal and sufficient single signal for normal lamellocyte development in the course of the cellular immune response of *Drosophila*.

In this work I used a molecular marker with specific expression in *Drosophila* lamellocytes to understand the differentiation and function of this cell type. By the aid of this marker we have cloned and characterized a novel *Drosophila* blood cell antigen. The protein corresponds to *Drosophila* Filamin-240, an actin-network specific protein, detected so far in the ring canals of the ovaries. *Drosophila* filamin exists in four isoforms, as a result of alternative splicing, but available ESTs indicate that still others may exist. So far, it was found that mutations in fruit fly Filamin-240 causes a reduced and defective organization and stabilization of actin in the ring canal structures and as a consequence are formed small and nonviable eggs. The shorter form, Filamin-90, lacks the actin-binding domain and rod I, and is broadly expressed. The function of this isoform has not been identified yet. Among the four existing isoforms of filamin arisen by post-transcriptional modification, we have detected Filamin-240 and Filamin-90 using a monoclonal antibody, 4B8. Biological activity so far has been assigned to Filamin-240, containing the acting binding domain; the lack of this isoform results in female sterility. Here we show that this isoform is expressed also in a subset of blood cells, the lamellocytes.

In the *cher*^{1} homozygous larvae, which lack Filamin-240 protein, we observed that a vigorous lamellocyte differentiation occurs which is further enhanced upon *in vivo* immune challenge by a parasitic wasp, *Leptopilina boulardi*. By introducing a full-length transgene encoding the *Drosophila* Filamin-240 protein into the *cher*^{1} filamin deficient homozygous mutant, the

mutant blood cell phenotype was rescued. These data demonstrate that the expression of Filamin-240 is strictly lamellocyte-specific in *Drosophila* blood cells and that the protein is a suppressor of lamellocyte development.

Lamellocytes undergo massive differentiation in response to infection caused by parasites and pathogens. Cell shape transformation requires dynamic reorganization of the actin cytoskeleton. It was found previously that rearrangement of microtubules accompanies the modification of lamellocyte shape from a disc to bipolar form during the cellular immune response. The terminal differentiation of lamellocytes, flattening must involve dynamic reorganization of the actin cytoskeleton and Filamin-240 is involved in this process. So far, it was found that Drosophila filamin interacts with Toll receptor and mutantions in Toll can induce lamellocyte differentiation. By attaching the cytoskeleton to the Toll signaling pathway it is possible that Filamin-240 enables the dynamic rearrangements of the actin cytoskeleton required for lamellocyte differentiation. Further, it would be interesting to investigate how the actin cytoskeleton functions in the Toll pathway and how the interaction of Filamin-240 with Toll receptor may be regulated.

Also, we found that *singed* protein, the homologue of fascin, known to be required for actin filament bundle formation in the cytoplasm of nurse cells during *Drosophila* oogenesis, is also expressed in a subpopulation of lamellocytes. It was suggested that in human fascin protein play a role in extending the membranes, which is need it for cell motility or for interactions with other types of cells. In *Drosophila*, *singed* may be involved in the formation of membrane extending, which are needed for lamellocyte motility during the encapsulation process. *hts* (hu-li-tai-shao) protein, involved in assembly and function of ring canals in the nurse cells of *Drosophila* ovaries, was found expressed in a subpopulation of small round hemocytes. *Kelch* protein, known to hold the actin bundles together during the expansion of the ring canals is expressed in plasmatocytes, too. Further, it would be interesting to study the role of these actin-binding proteins in *Drosophila* hemocytes. Also, other questions remain to be answered about filamin function in *Drosophila*. Since filamin is known to interact with and be required for the proper function of some receptors in mammalian systems, it would be interesting to find the interacting partners of *Drosophila* filamin and its role in the signaling pathways.

6. Materials and methods

6.1. Chemicals and reagents

-AEC stock solution –1%3-amino-9 ethyl carbazole(Sigma) in DMF

-Acetate buffer-0.2M Na-acetate, pH 4 .6

-PBS-BSA-PBS containing 0.1% BSA

-Phosphate buffered-saline (PBS)-0.13 M NaCl, 7mM Na_2HPO_4, 3mM Na_2HPO_4, pH 7.4

-Ringer solution- 7.5gNaCl, 0.35g KCl, 0.21g $CaCl_2$, 1000 ml dW, pH 7.0

-Ficoll-hypaque solution,r =1.077 (Pharmacia)

-Heat-inactivated fetal calf serum (FCS ;Gibco)

-Schneider insect medium (Sigma)

-T2/T48 antibody directed against the CD45 human leukocyte antigen as a negative control, 0.5mgml^{-1} in 0.15 M NaCl solution (Oravecz, T., Monostori, E., Kurucz, E., Ando, I., 1991, CD3-induced T- cell proliferation and interleukin-2 secretion is modulated by the CD45 antigen.Scand.J.Immunol.34, 531)

-Goat anti-Mouse Immunoglobulin, FITC conjugate (DAKO)

-Goat anti-Mouse Immunoglobulin, biotin conjugate (DAKO)

-Peroxidase conjugated streptavidine (DAKO)

-HRPO conjugated streptavidine (DAKO)

-Fluorolink Cy3 labelled streptavidine (Amersham Pharmacia Biotech)

- anti-Rat immunoglobulin Cy2-conjugated

- PTU(1-phenol-2-thiourea) FLUKA

-DAPI (4', 6-diamino-2-phenylindole dihydrochloride

-TBS containing 0.1% Tween 20 (TBST)

-IPTG (isopropyl beta-D-thiogalactoside, Sigma)

-lysis buffer (50 mM Tris-HCl pH=8.0,150 mM NaCl, 1.0% NP40, 0.5% sodium deoxycolate, 0.1% SDS) containing also protease inhibitors: PMSF (Sigma) and PIC (Boehringer Mannheim)

-Transfer buffer: 25 mM Tris, pH 8.3/192 mM glycine/20% methanol

-ECL-Plus system (Amersham Pharmacia)

6.2. Fly strains

Flies were grown on cornmeal-yeast food at 25°C. We used Oregon-R wild-type stock and the *l(3)mbn-1* strain tumor suppressor, hemocyte overproducing mutant. The *domino* is a P-element induced *Drosophila* mutant devoid of circulating hemocytes and with melanized lymph glands in the third-instar larvae. The *cher*^{1} strain [107] is a filamin deficient female sterile mutant having defects in ring canal assembly and egg chamber morphology. *P[hs-FLN1-20];cher*^{1} rescue stock [108] was obtained by introducing *P[hs-FLN1-20]* (hsp 70 promoter-driven full length *cheerio* cDNA), into *cher*^{1} mutant. Heat shock rescue was accomplished by placing vials containing second stage larvae in a 37^{0}C incubator for half an hour, twice a day, for two days and then the larvae were dissected.

6.3. Preparation of antibodies

Mouse monoclonal antibodies to lamellocytes, 4B8 and L1 were produced as described previously [32]. The filamin specific polyclonal antibody was a gift from Dr. Lynn Cooley, Yale University, U.S. is described in Ref. [88].
The singed, hts and kelch specific monoclonal antibodies were gift from Dr. Matyas Gorjanacz, BRC, Szeged.

6.4. Indirect immunofluorescence and FACS analysis of live hemocytes

Aliquots of 20 µl Schneider's *Drosophila* medium supplemented with 10% FCS, containing 10^{7} hemocytes per ml were placed in wells of a 96-well U-form multiwell plate. Hybridoma supernatant (50 µl) was added to each well, and the plate was incubated for 45 min on ice. After washing the cells three times with ice-cold Schneider's medium, FITC-labeled anti-mouse IgG antibody (Sigma) was added at 1:100 dilution. After 45 min on ice, the hemocytes were washed three times with ice-cold Schneider's medium and the cells were analyzed with a FACS Calibur equipment (Beckton Dickinson) for fluorescence intensity.

6.5. Immunocytochemistry

A total of 20 µl of hemocyte suspension in Drosophila Ringer solution, containig1-phenol-2-thiourea (PTU) (FLUKA) was placed in each spot of a multispot microscope slide (SM-011,

Hendley, Loughton, U.K.). The hemocytes were allowed to settle for 20 min at room temperature and then fixed in acetone for 6 min, rehydrated, and blocked in PBS containing 0.1% BSA (PBS-BSA). After incubation with the primary antibody for 1 h, and three washes with PBS for 5 min each, the samples were incubated with biotin coupled anti-mouse immunoglobulins (DAKO) for 1 h at room temperature. After three washing steps with PBS, 5 min each, the bound antibody was detected by peroxidase-conjugated Streptavidin (DAKO) or by Streptavidin-Cy3 complex (Amersham Pharmacia Biotech). The peroxidase activity was revealed with a chromogen substrate 3-Amino 9ethyl-carbonate (AEC) in 1M acetate buffer pH=4.6 containing 0.02% v/v H_2O_2. The nuclei were stained by DAPI (4',6-diamino-2-phenylindole dihydrochloride). Slides were analyzed with an Axioskop 2 MOT, Carl Zeiss fluorescence microscope, using a camera (Axio Cam) and software (Axiovision 3.1). Differential counting of lamellocytes was based on L1 antigen staining, while total hemocyte number was determined by nuclear staining with DAPI. The significance was determined by Student's *t*-test.

6.6. Immunofluorescence, double staining

Hemocytes were prepared as described in 4.5. Samples were incubated with the 4B8 antibody for 1 h at room temperature and then washed three times with PBS for 5 min each. The samples were then incubated with polyclonal anti-C-Filamin antibody (dilution 1:100) for one hour and biotin coupled anti-mouse immunoglobulin was added for another one hour. After three washing steps with PBS (5 min. each), the bound antibody was detected by Streptavidin-Cy3 complex and Cy2-conjugated anti-rat antibody. In the last staining step DAPI was added to stain nuclei. Slides were analyzed as described in 4.5 Images were processed using Adobe Photoshop version 6.

6.7. Immunostaining of ovaries

Ovaries isolated from wild type Oregon-R and *cher*^{1} females in *Drosophila* Ringer solution, fixed in acetone for 6 minutes, rehydrated and blocked in PBS containing 0.1% BSA. Samples were incubated with the primary antibody overnight, at 4^0C, washed three times with PBS for 5 min each, and the bound antibody was detected by Streptavidin-Cy3 complex (Amersham Pharmacia Biotech).

6.8. Immunostaining of whole larvae

Third stage larvae were dissected and fixed in 2% paraformaldehyde for 20 minutes. The fixed larvae were rehydrated with 0.1% BSA-PBS for 20 minutes. The larvae were then incubated with primary antibody overnight at 4^0C. After three washes with PBS (5 minutes each) the samples were incubated with biotin coupled anti-mouse immunoglobulins for 1 hour at room temperature. The bound antibody was detected by Streptavidin-Cy3 complex. The nuclei were visualized by DAPI staining. Slides were analyzed with Axioskop 2 MOT, Carl Zeiss fluorescence microscope, using a camera (Axio Cam) and software (Axiovision 3.1). Images were processed using Adobe Photoshop version 6.

6.9. Preparation of hemocytes and cell extract

Hemocytes were collected from third stage larvae bleeding into *Drosophila* Ringer solution on ice. After centrifugation cell were extracted into lysis buffer containing also protease inhibitors: PMSF (Sigma) and PIC (Boehringer Mannheim) at 4^0 C for 1 hour. After centrifugation at 12.000g for 15 minutes the supernatant was subjected to Western-blot analysis.

6.10. Immunoprecipitation of antigens

Five milliliters of hybridoma supernatant was added to 50 µl of 20% slurry of Protein G Sepharose and rotated at room temperature for 1 h. After washing the protein G Sepharose–antibody complex three times with PBS, the cell extract was added and immunoprecipitation was carried out overnight at 4°C. Finally, the immunoprecipitates were washed three times in lysis buffer and solubilized in SDS/PAGE sample buffer. The samples were subjected to SDS/PAGE and Western blotting.

6.11. Western blot analysis

Protein extracts were separated by SDS/PAGE. After electrophoresis the proteins were transferred onto nitrocellulose membrane (Hybond-C, Amersham Pharmacia) in transfer buffer. Nonspecific binding on the nitrocellulose membrane was blocked with PBS containing 0.1% Tween 20 (PBST) supplemented with 5% nonfat dry milk for 1 h at room temperature. The blotted proteins were subjected to hybridoma supernatants for 3 h with agitation at room

temperature. The blot was washed with PBST three times for 10 min each and then incubated with HRPO-conjugated anti-mouse antibody (DAKO). After three washes for 10 min each in PBST, the proteins were visualized by the ECL-Plus system (Amersham Pharmacia) according to the manufacturer's instructions.

6.12. Screening of cDNA expression libraries

We used the 4B8 antibody to screen *l(3)mbn-1* larval expression libraries Kurucz et.al, [102]. The XL/1Blue MRF' strain of *E. coli* was cultured in LB medium with 0.2% maltose and 1% 1M $MgSO_4$ at 37^0. At OD_{600} value of ~0.8, the bacteria were centrifuged at 4^0C, 3500 rpm for 25 min. and resuspended in 10mM $MgSO_4$. Prior to the infection with phage, the OD_{600} of bacterial solution was adjusted to ~0.5. The bacteria were mixed in 4:1 ratio with the phage appropriately diluted in SM buffer. The mixture was incubated for 15 minutes at 37^0 C and mixed with top agarose (NZY 0.7%) at 48^0C, then poured over the plates with bottom agar NZY 1.5%. The plates were incubated at 37^0C for 7 h, till the plaques started to appear. Nitrocellulose filters (Amersham Pharmacia Hybond-C Extra) were soaked in a water solution of IPTG (isopropyl beta-D-thiogalactoside, Sigma) (2.4 mg/ml), dried and applied overnight onto the bacterial plates at 37^0C. About $\approx 8 \times 10^4$ plaques were transferred onto nitrocellulose filters and probed with 4B8 antibody for 2 h with agitation at room temperature and then overnight at 4^0C. HRPO-conjugated secondary antibody (Amersham Pharmacia) was applied for 1 hour at room temperature and the reaction was visualized with the ECL-Plus system (Amersham Pharmacia). The positive plaques were collected and re-screened twice.

6.13. Filamin cDNA clones and sequence analysis

Plasmid DNA was prepared from positive clones of the pBK-CMV phagemid vector derived from the ZAP Express vector by *in vivo* excision using the ExAssist helper phage (Amersham Pharmacia) with the XLOR strain and sequenced on both strands. Database searches and sequence comparisons were performed with the BLAST servers at the Berkeley *Drosophila* Genome Project and the National Center for Biotechnology Information.

6.14. Bioassay for lamellocyte induction

One-week-old females of the parasitic wasp, *Leptopilina boulardi*, strain G486 were used to infest second-instar larvae. Fifty larvae were exposed to five *Leptopilina boulardi* females overnight at 18°C and then kept at 25^0C. Two days after infestation, the hemocytes isolated from the infested larvae were stained with 4B8 antibody. The hemocytes were counted and the ratio of lamellocytes was determined after staining with the lamellocyte-specific antibody L1.

6.15. Immune induction

For sterile wounding larvae, second stage larvae were collected, washed in water and placed in 75% ethanol for 10 minutes, air-dried on sterile microscopic slides. They were punctured at the posterior end with sterilized glass needles, which approximate the size of the wasp's ovipositor and removed into sterile wet chambers on sterile filter paper. Three hours later the larvae were transferred into vials with cornmeal-yeast food. Controls were age-matched, unwounded larvae. For injection of live *E.coli* bacteria similar needles we used. Tenfold diluted overnight culture was injected into the posterior end of the larvae. Hemocytes were isolated from individual larvae 48, 72, 96 and 120 hours after wounding or following infestation.

7. List of publications

The thesis was prepared on the basis of the following publications and presentations:

Publications

Rus Florentina, Kurucz Éva, Márkus Róbert, Sinenko Sergey A., Laurinyecz Barbara, Pataki Csilla, Gausz János, Hegedűs Zoltán, Udvardy Andor, Hultmark Dan, Andó István, **Expression pattern of Filamin-240 in *Drosophila* blood cells**, *Gene Expression Patterns*, doi:10.1016/j.modgep.2006.03.005

Márkus Róbert, Kurucz Éva, **Rus Florentina**, Andó István, **Sterile wounding is a minimal and sufficient trigger for a cellular immune response in *Drosophila melanogaster***, *Immunology Letters* 101,2005, 108-111.

Andó István, Laurinyecz Barbara, Márkus Róbert, **Rus Florentina**, Váczi Balázs, Zsámboki János, Kurucz Éva**: Our ancient heritage: innate immunity; the celular immunity of the *Drosophila,*** *Magyar Tudomány 2004/10*

Andó István, Laurinyecz Barbara, Nagy István, Márkus Róbert, **Rus Florentina,** Váczi Balázs, Zsámboki János, Fehér Lászlo, Gateff Elisabeth, Hultmark Dan, Kurucz Éva, **Our archaic heritage: the innate immunity. The cellular immunity of *Drosophila*,** 2003, *Magy. Immunol*.2(4):39-45.

Presentations

Rus Florentina, Kurucz Éva, Gausz János, Hegedűs Zoltán, Udvardy Andor, Andó István, **Expression and a novel function of Filamin-240 in lamellocyte development in *Drosophila melanogaster*,** A Magyar Immunológiai Társaság XXXIII. Kongresszusa, Győr, 2003, October 15-16.

Laurinyecz Barbara, Kurucz Éva, Nagy István, **Rus Florentina**, Andó István, **Az antigén-indukálta sejtes immunválasz folyamata Drosophilában**, A Magyar Immunológiai Társaság XXXII. Kongresszusa, Kaposvár, 2002, October 30-02.

Rus Florentina, Kurucz Éva, Andó István, **Identification of hemocyte-antigens in *Drosophila melanogaster***, ITC 200/2001, Proceeding of the Closing Seminar, BRC, Hungarian Academy of Sciences, 99-110.

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