Characterization of Phagocytic Pattern Recognition Receptors in *Drosophila melanogaster*

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Our greatest glory is not in never falling,
but in rising every time we fall.

Confucius

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Abstract

Drosophila melanogaster has emerged as a powerful model system to study innate immunity. Insects employ multilayered innate immune defenses including antimicrobial peptide responses and phagocytosis. In *Drosophila,* phagocytosis is carried out by plasmatocytes, a blood cell type similar to mammalian macrophages and neutrophils. The scavenger receptor Eater is expressed by larval and adult plasmatocytes and mediates recognition of a broad range of bacterial pathogens. Eater is required for fly survival after infection with Gram-positive and Gram-negative bacteria. However, the bacterial ligands of Eater, and the mechanisms by which this receptor recognizes these different types of bacteria, remain poorly understood.

To address this problem, I generated a soluble, Fc-tagged receptor variant of Eater comprising the N-terminal 199 amino acids (including four N-terminal EGF-like repeats) and raised antibodies against Eater. Using these tools, I established (i) that Eater is expressed on the surface of macrophage-like *Drosophila* S2 cells, (ii) that it interacts with broad, yet distinct classes of heat- and ethanol-inactivated microbes and (iii) that it binds peptidoglycan from Gram-negative Proteobacteria (*E. coli*) and Gram-positive *Firmicutes* (*E. faecalis* and *S. aureus*), but not Gram-positive Actinobacteria (*M. luteus)*. In order to identify genes involved in the phagocytosis of *M. luteus,* I screened 39 candidate genes by RNA interference-mediated knock down in S2 cells.

A longstanding question was whether Eater recognizes live, naïve bacteria. I found that Eater-Fc bound equally well to naïve or heat-inactivated *S. aureus* or *E. faecalis*, suggesting that *in vivo* Eater directly targets live Gram-positive bacteria, enabling their phagocytic clearance and destruction. By contrast, Eater-Fc was unable to interact with live Gram-negative bacteria (*E. coli, S. marcescens* and *P. aeruginosa)*. Eater binding required prior membrane-disrupting treatments. Cecropin A, a prototypic cationic, membrane-disrupting antimicrobial peptide could promote Eater-Fc binding to live *E. coli,* even at sublethal concentrations. These results suggest a previously unrecognized mechanism by which antimicrobial peptides cooperate with phagocytic receptors.

Zusammenfassung

Drosophila melanogaster hat sich zu einem nützlichen Modellsystem zur Erforschung angeborener Abwehrmechanismen entwickelt. Insekten besitzen ein vielseitiges Immunsystem welches unter anderem antimikrobielle Peptide und Phagozyten umfasst. In *Drosophila* wird Phagozytose von sog. Plasmatozyten durchgeführt, einem Blutzelltyp, der den Makrophagen und neutrophilen Granulozyten des Menschen ähnelt. Der ‚Scavenger Rezeptor' Eater wird von Plasmatozyten in Larven und adulten Insekten ausgeprägt. Er erkennt ein breites Spektrum bakterieller Pathogene und seine Ausprägung ist erforderlich für das Überleben von Infektionen mit Gram-negativen und Gram-positiven Bakterien. Die bakteriellen Liganden und die Mechanismen, mit denen Eater diese verschiedenen Bakterien erkennt, sind unzureichend verstanden.

Um diese Fragen zu erforschen, habe ich eine lösliche Rezeptorvariante hergestellt, Eater-Fc, welche aus den N-terminalen 199 Aminosäuren von Eater (4 EGF-ähnliche Wiederholungen umfassend) und einem C-terminalen Antikörper-Fc-Teil besteht. Zudem habe ich Antikörper gegen Eater-Fc hergestellt. Mit diesen Reagenzien konnte ich zeigen, dass Eater (i) auf der Oberfläche von Makrophagen-ähnlichen *Drosophila* S2 Zellen ausgeprägt wird, (ii) mit einem breiten, jedoch differenzierten Spektrum inaktivierter Mikroben interagiert, und (iii) an Peptidoglykan von Gram-negativen Proteobakterien (*E. coli*) und Gram-positiven *Firmicutes* (*S. aureus, E. faecalis*), jedoch nicht von Gram-positiven Actinobakterien (*M. luteus*), bindet. Um Gene zu finden, die an der Phagozytose von *M. luteus* beteiligt sind, habe ich S2 Zellen untersucht, in denen 39 Kandidaten-Gene mit Hilfe von RNA-Interferenz ausgeschaltet wurden.

Eine bisher ungeklärte Frage war, ob Eater auch vermag, an lebende, unbehandelte Bakterien zu binden. Einerseits konnte ich zeigen, dass Eater-Fc lebende Gram-positive *Firmicutes*-Bakterien (*S.aureus* oder *E. faecalis)* bindet. Es liegt deshalb nahe zu vermuten, dass Eater diese Gram-positive Bakterien *in vivo* direkt erkennen und ihre Phagozytose und Zerstörung einleiten kann. Andererseits war Eater-Fc nicht in der Lage, mit lebenden Gram-negativen Proteobakterien (*E. coli, S. marcescens und*

P. aeruginosa) zu reagieren. Um eine Bindung zu ermöglichen, mussten die Bakterien zuvor einer membran-schädigenden Behandlung unterzogen werden. Cecropin A, ein kationisches, membran-permeabilisierendes Peptid bewirkte, dass Eater an lebende *E. coli* binden konnte, sogar unter sublethalen Bedingungen. Meine Ergebnisse weisen somit auf einen bisher unbekannten Mechanismus hin, der es Antimikrobielle Peptiden ermöglicht, mit Phagozytose-Rezeptoren zu kooperieren.

Abbreviations

AMP	antimicrobial peptide
BHI	brain heart infusion
bp	base pair
BSA	bovine serum albumin
CFU	colony forming units
Da	Dalton
DIC	differential interference contrast (Nomarski microscopy)
DNA	deoxyribonucleic acid
dsRNA	doublestranded ribonucleic acid
EDTA	ethylenediaminetetraacetate
EGF-like	epidermal growth factor – like
EM	electron microscopy
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
Fc	Fragment, crystallizable region of antibody constant region
FITC	fluorescein-5-isothiocyanate
FPLC	fast protein liquid chromatography
GFP	green fluorescent protein
IMD	immune deficiency
kDa	kiloDalton
LB	lysogeny broth
LBP	LPS binding protein
LDL	low density lipoprotein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
Lys	lysine
mDAP	meso-diaminopimelic acid
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline + 1 % Tween 20
PCR	polymerase chain reaction
PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
PI	propidium iodide
poly-C	polycytidylic acid
poly-I	polyinosinic acid
PRR	pattern recognition receptors
S2	Schneider line 2 (S2 cells)
SDS	sodium dodecyl sulfate
Sf-9	*Spodoptera frugiperda* 9 cells
TEV	Tobacco etch virus
TFA	trifluoroacetic acid
TLR	toll like receptor

Tris	Tris (hydroxymethyl) aminomethane
TE	Tris 10 mM/EDTA 1 mM pH 8
RNA	ribonucleic acid
RNAi	RNA interference
RT	room temperature

1. INTRODUCTION

1.1. Background

1.1.1 Innate immune mechanisms in *Drosophila*

Multicellular animals, whether they are invertebrates or vertebrates, are able to defend themselves against pathogens (Kvell et al. 2007). The defense mechanisms against infectious pathogens, namely the innate and the adaptive immune systems, protect animals against attacks from potentially pathogenic bacteria, fungi and viruses. The innate immune system is the first line of defense mounted in response to various microbial invaders (Hoffmann 2003). The innate immune system appeared early during evolution and while this is the only defense mechanism in invertebrates, it is also a major part of the immune system in vertebrates (Janeway 1989). The insect and mammalian innate immune responses against pathogenic microbes show a great amount of evolutionary conservation (Hoffmann 2003; Kimbrell & Beutler 2001; Aggarwal & Silverman 2008). A great example of this conservation was provided by the discovery of the Toll pathway as an important part of the Drosophila immune response and the following identification of the mammalian Toll-like-receptors (TLRs) (Lemaitre 2004). In addition to an innate immune system, vertebrates however developed a very complex adaptive immune system, which cooperates with the innate immune system in host defense (Medzhitov 2007).

The adaptive immune system has the capacity to specifically recognize and remember attacks by pathogenic microbes (immunological memory). Adaptive immunity seemed to dominate pathogen defense, and it seemed that the innate immune system only played a minor role in the fight against microorganisms that cause pathology (Kvell et al. 2007). The innate- and the adaptive immune system deal with the molecular diversity of pathogens in fundamentally different ways. The main distinction between these are the receptor types used to recognize pathogens (Medzhitov 2007). Adaptive immune recognition is mediated by antigen receptors on T and B cells with unlimited specificities generated by somatic rearrangement of receptor genes (Beutler et al. 2006), whereas the innate immune system in mammals

uses germline encoded receptors which recognize evolutionarily conserved microbial molecules, the so-called pathogen-associated molecular patterns (PAMPs) (Medzhitov & Janeway 2000; Janeway & Medzhitov 2002). Such germline encoded receptors have been termed pattern recognition receptors (PRRs) (Medzhitov & Janeway 2000; Janeway & Medzhitov 2002). PRRs initiate signaling cascades leading to the production of immune effectors, such as antimicrobial peptides, cytokines, inflammatory mediators, and the activation of phagocytic and proteolytic cascades.

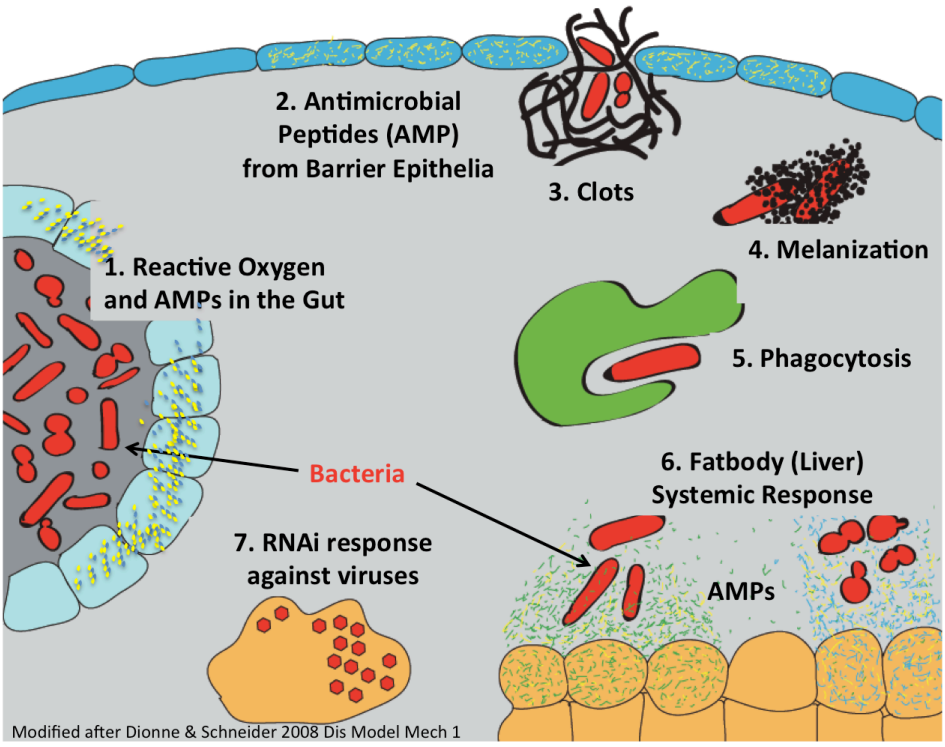


Figure 1. The fruit fly *D. melanogaster* possesses multilayered pathogen defense mechanisms. 1. Regulation of the native microbiota in the gut through AMPs and reactive oxygen species. 2. Barrier epithelial responses produce local AMPs and send signals to the rest of the body. 3. Clotting response seals wounds, prevents bleeding and traps bacteria. 4. Phenoloxidase response deposits melanin at the site of an immunereaction releasing potentially antimicrobial reactive oxygen species. 5. Phagocytic response, through which phagocytes kill microbes directly or indirectly (by releasing systemic signals). 6. Systemic AMP response through NfkB pathways (Toll, Imd) involves massive release of AMPs from the fat body into circulation. 7. Virus infected tissues are defended by RNAi. Modified after Figure 1 in Dionne and Schneider, 2008.

A good model organism to study innate immune functions would be an organism, in which the extra layer of complexity added by the adaptive part of the immune system does not exist. This is the case for *Drosophila melanogaster*. In *Drosophila*, which rely almost entirely on innate immunity to fight microbial infection, a sophisticated multilayered pathogen defense system consisting of at least seven subcategories can be found, including a cellular and a humoral response (Dionne & Schneider 2008).

The humoral defense response relies on the production of antimicrobial peptides (AMPs), which in response to pathogen attack are secreted from the equivalent of the mammalian liver, the fat body of *Drosophila*. Members of the peptidoglycan-recognition protein (PGRP) family act as microbe sensors (receptors) and can be found in the hemolymph, on immune cell surfaces and in the immune cells. These receptors recognize bacterial cell wall components like bacterial peptidoglycans (PGNs) that activate immune signaling pathways, such as the nuclear factor kappa (NFk) B-like Toll and Imd signaling pathways (Hoffmann 2003; Hoffmann & Reichhart 2002).

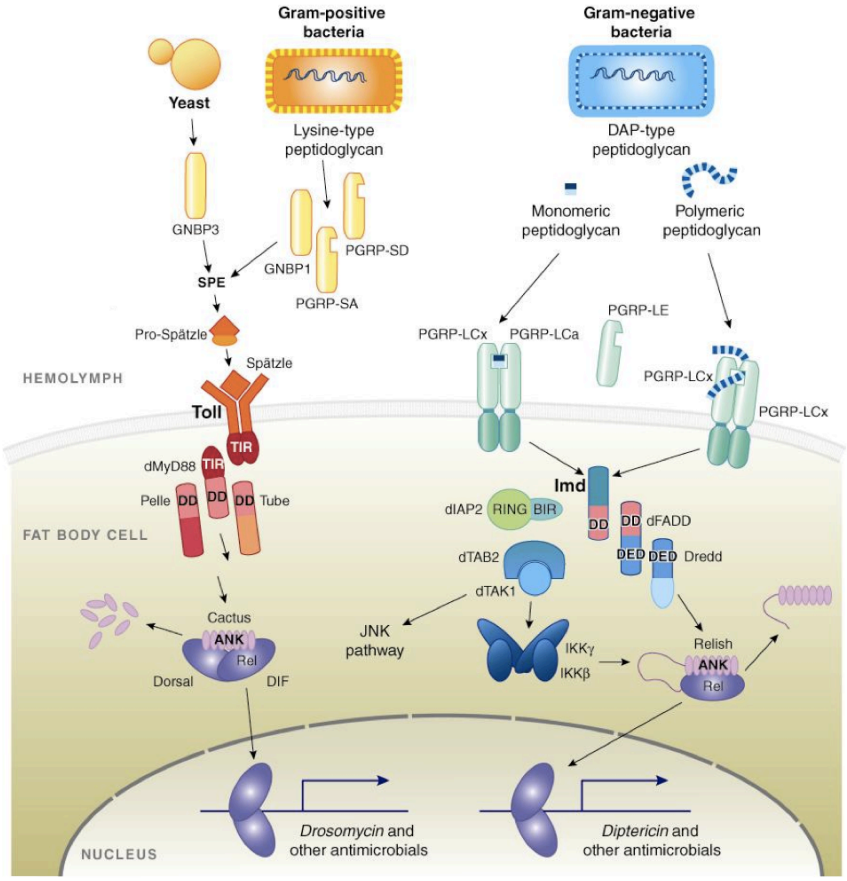
1.1.2. The NFkB-like Toll and Imd pathways

The Toll pathway is mainly activated by fungi and Gram-positive bacteria, whereas the Imd pathway is activated predominantly by Gram-negative bacteria (Fig. 2). The two pathways are triggered by elicitors released from the microbes, such as Lys-type PGN for Gram-positive bacteria and DAP-type PGN for Gram-negative bacteria, that are recognized by recognition proteins which in turn activate proteolytic cascades leading to the production of antimicrobial peptides and other immune effectors (Lemaitre 2004). Depending on the κB sites present in their promotors, antimicrobial peptide genes are under the control of either the Toll or the Imd signaling cascade or can even be coregulated (Lemaitre & Hoffmann 2007).

Drosophila Toll does not function as a pattern recognition receptor as its vertebrate counterparts, the TLRs, but has to be activated by a proteolytically cleaved form of the cytokine Spätzle which then leads to the activation of NFκB transcription factors Dif and Dorsal and the induction of various target genes encoding humoral factors, including antimicrobial peptides (Lemaitre et al. 1996; Weber et al. 2003; Hu et al. 2004).

The Imd pathway was initially defined by the identification of a mutation named *immune deficiency* that impaired the expression of several antibacterial peptide genes (Lemaitre 2004; Lemaitre & Hoffmann 2007). *imd* mutant flies are viable when non-infected however succumb readily to infections by Gram-negative bacteria (Lemaitre & Hoffmann 2007). The recognition of Gram-negative DAP-type PGN activates a

different NFκB transcription factor, Relish, and the induction of genes encoding humoral factors.



Modified after Lemaitre & Hoffmann 2007

Figure 2. Model of Toll and Imd pathway activation. Toll: The Toll pathway is activated by secreted recognition molecules (GNBPs, PGRPs) that sense Gram-positive Lys-type PGN, Glucan and entomopathogenic fungi, which activate the serine-protease SPE which in turn cleaves Spätzle. Mature Spätzle binds to Toll which leads, through a series of phosphorylations, to the release of transcription factors (Dorsal & Dif) into the nucleus and the production of antimicrobial peptides. Imd: Direct binding of peptidoglycan recognition receptors (PGRPs) to monomeric or polymeric PGN and subsequent recruitment of the adaptor Imd leads through proteolytic cascades to the translocation of the transcription factor Rel into the nucleus and the production of AMPs. Modified after Figure 3 in Lemaitre and Hoffmann, 2007.

Certain aspects of both pathways are not yet fully understood, however ultimately both pathways lead to the rapid and massive release of antimicrobial peptides from the fat body into the hemolymph as well as transcriptional upregulation of hundreds of other putative immune effectors whose role remains to be elucidated (Lemaitre 2004; Lemaitre & Hoffmann 2007).

1.1.3. Antimicrobial peptides

Even before the mechanisms of the Toll and Imd pathways were elucidated, Boman and his co-workers were able to characterize the inducible antimicrobial peptides (AMPs) Cecropin and Attacin from the giant silk moth *Hyalophora cecropia* (Steiner et al. 1981; Hultmark et al. 1983). These peptides were rapidly produced by the insect fat body and secreted into the hemolymph after septic injury. Now more than 1200 different AMPs have been either identified or predicted through nucleic acid sequences (Brogden 2005; Bulet & Stöcklin 2005). These include AMPs from many different tissues and cells of a variety of invertebrate, plant and animal species (Ganz 2004; Ganz 2003; Lehrer 2011; Lehrer 2004; Zasloff 2002; Brogden et al. 2003; Vizioli & Salzet 2002). AMPs are a unique and diverse group of molecules which have been divided into subgroups on the basis of their amino acid composition and structure (Brogden 2005). Here, the focus lies on an evolutionarily conserved subgroup that contains linear and amphipathic α-helical AMPs including cecropins (Fig. 3) and cecropin-like molecules conserved from *Diptera*
and *Lepidoptera* even to mammals (pigs) which contain 29-40 amino acid residues (Gazit et al. 1995; Bulet et al. 2004; Bulet et al. 2004). Sequence comparison revealed that cecropins form a homologous group with more than 70 % identity in their amino acid composition (Okada & Natori 1985; Kylsten et al. 1990; Bulet & Stöcklin 2005).

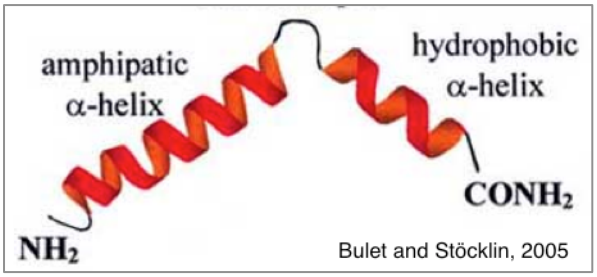


Figure 3. Global fold of *Hyalophora* cecropin A. Peptide has a long N-terminal, basic, amphipatic α-helix and a shorter, more hydrophobic C-terminal helix, linked by a Gly-Pro hinge region. NH_2, N-terminus; $CONH_2$, C-terminus. From Bulet and Stoecklin, 2005.

Despite numerous studies, no definitive consensus explanation has emerged for the mechanism of antimicrobial action of cationic AMPs and their modes of action seem to be pleiotropic comprising direct and indirect antimicrobial functions as well as immunomodulatory activities (Hale & Hancock 2007; Hancock & Scott 2000). However, there is a broad consensus that α-helical AMPs such as cecropins are active with a higher efficacy against Gram-negative than Gram-positive bacteria, are non-toxic for the host and that one site of the antibacterial action is the bacterial plasma membrane (Hancock & Scott 2000). The initial contact between the peptide and the

target organism would be electrostatic, as most bacterial surfaces are anionic, in contrast to animal cells. Their amino acid composition, amphipathicity, cationic charge and size allows cecropins or other cationic AMPs to attach to and insert into membrane bilayers to form pores (Brogden 2005). Several groups showed that various cecropins and cecropin analogues initially form selective voltage dependent ion channels, where the positively charged NH_2-terminal helices bind to negatively charged headgroups on the bacterial membrane and the hydrophobic $CONH_2$-terminal part inserts itself to the membrane core (Christensen et al. 1988; Silvestro et al. 1999; Shai 1995). After application of a positive potential on the side of the peptides, the positively charged NH_2-terminal helices get pushed into the membrane and the channel is formed by the association of multiple transmembrane NH_2-helices, so that the hydrophilic residues form the aqueous pore and the hydrophobic residues are in contact with the aliphatic phase of the membrane (Christensen et al. 1988; Durell et al. 1992). Despite multiple theories and models, the precise mechanism by which cecropin attacks bacteria is still not known. To date, it has also not been shown whether AMPs by themselves are sufficient to combat bacterial infections. While direct bactericidal activities of cationic AMPs have been demonstrated, mostly under rather non-physiological conditions, a mechanistically poorly defined activity that leads to increased phagocytosis by macrophages was noticed long ago (Finlay and Hancock, 2004). Some experiments in my thesis address this latter aspect of AMP function and suggest a molecular mechanism for this phenomenon: permeabilization of bacterial envelopes may lead to 'priming' of AMP exposed bacteria for other innate immune mechanisms, such as phagocytosis.

1.1.4. Phagocytosis in *Drosophila*

Phagocytosis is an evolutionarily ancient mechanism by which cells internalize particles (Metchnikoff 1908; Rabinovitch 1995). It requires cell surface receptors that bind non-self or altered-self molecules displayed on microbes or dying and aberrant cells (Stuart & Ezekowitz 2005). The engulfment of apoptotic particles by macrophages in early stages of embryogenesis for instance has been shown to be essential for development (Tepass et al. 1994; Zhou et al. 1995). Phagocytosis also plays a major role in innate immunity as one of the first lines of defense against

invasive microbes and by mobilizing and instructing adaptive immunity. Phagocytes must constantly monitor their environment to quickly recognize, ingest and destroy foreign intruders or altered cells. Once an invader is recognized, phagocytes start an uptake mechanism that is not yet fully understood (Underhill & Ozinsky 2002). However, multiple studies with a variety of microbes have shown that mammals and *Drosophila* share certain parts of the uptake machinery such as actin and actin-related proteins as critical participants in phagocytosis (Stuart & Ezekowitz 2008; Pearson et al. 2003; Philips et al. 2005; Agaisse et al. 2005; Stroschein-Stevenson et al. 2006).

In contrast to the nematode *C. elegans, Drosophila* has circulating and sessile blood cells (called hemocytes in *Drosophila*), which play an important role in protecting flies against infection by phagocytosing invading microbes. *Drosophila melanogaster* possesses three major types of blood cells which are derived from the embryonic and larval hematopoietic organs (Meister & Lagueux 2003): 1. plasmatocytes, 2. crystal cells and 3. lamellocytes (Rizki & Rizki 1984, cited after Stuart & Ezekowitz 2008). 95 % of hemocytes, sessile as well as circulating, are plasmatocytes, the counterpart of the mammalian neutrophils and macrophages. These phagocytic cells are long-lived (Stuart & Ezekowitz 2008; Meister & Lagueux 2003) and devoid of neutrophil-like granules (Rizki & Rizki 1984; Lanot et al. 2001). They play essential roles in tissue remodeling during development (Defaye et al. 2009) and in immunity during infection (Defaye et al. 2009; Nehme et al. 2007; Charroux & Royet 2009; Avet-Rochex et al. 2007). Phagocytosis by *Drosophila* hemocytes share many similarities with the process in mammals, but with less anticipated complexity due to *Drosophila's* smaller genome, which makes it a good model system to validate known mechanisms of uptake (Cherry & Silverman 2006; Stuart & Ezekowitz 2008). In all cases, whether in mammals or *Drosophila*, phagocytes are able to discriminate particles and microbes by an array of receptor molecules on the surface of the cells.

1.1.5. Phagocytic receptors in *Drosophila*

As described in a series of recent publications (Philips et al. 2005; Kocks et al. 2005; Rämet et al. 2002; Stroschein-Stevenson et al. 2006), phagocytosis in *Drosophila* is initiated by surface receptors on plasmatocytes which either bind directly to microbes, apoptotic cells or through molecules that opsonize the surface of the phagocytosed

particle (Stuart & Ezekowitz 2008). Genetic screens and other experiments indicate that in *Drosophila* there are four different classes of molecules involved in phagocytic recognition (reviewed by Stuart & Ezekowitz 2005):

(i) Complement-like opsonins in *Drosophila* are thioester-containing proteins (TEPs) which have been found to bind microorganisms and enhance phagocytosis (Stroschein-Stevenson et al. 2006). Functional characterizations of TEPs is derived from RNAi screens in *S2* cells and *in vitro* and *in vivo* analysis in *Drosophila* and the mosquito *Anopheles gambiae* (Moita et al. 2005, Bou Aoun et al. 2011).

(ii) Down syndrome adhesion molecule (DSCAM), a member of an immunoglobulin superfamily, is predicted to have more than 38,000 potential splice variants (Schmucker et al. 2000), possibly 18,000 different extracellular domains of DSCAMs, and also exist in soluble forms (Watson et al. 2005). These may prove to be the innate immune system equivalent of immunoglobulins. This hypothesis is based among other evidence on DSCAM crystal structures (Meijers et al. 2007) and needs further investigation.

(iii) Scavenger receptors in *Drosophila* belong to several classes, which are structurally unrelated and have been shown to bind a wide variety of microbes as well as apoptotic cells: the CD36 homologues croquemort and peste (Franc et al. 1996; Philips et al. 2005) and scavenger receptors of class C. Scavenger receptors have emerged as important pattern recognition receptors (Janeway 1989) also in many other species.

(iv) EGF-like-repeat containing receptors, a newly emerging family of EGF-like-repeat-containing receptors belonging to the scavenger receptor family, recently termed the Nimrod Superfamily (Kurucz et al. 2007; Somogyi et al. 2008) which has homologues in many invertebrates and vertebrates including humans. An example in mammals are the class F scavenger receptors SCARF1 and 2.

Within the Nimrod family, the phagocytic receptor Eater (Kocks et al. 2005) is particularly well characterized (see Chapter 1.1.6 below). Apart from Eater, there have been reports of other *Drosophila* proteins from this family, such as NimrodC1, a transmembrane protein with EGF-like repeats similar to Eater, which seems to act as a phagocytic receptor and a potential adhesion molecule (Kurucz et al. 2007). A more recently discovered member of this family called SIMU, comprising in it's ectodomain 4 EGF-like repeats, is involved in the engulfment of apoptotic neurons by glial cells in the developing nervous system of *Drosophila* (Kurant et al. 2008).

Interestingly there seems to be a connection between SIMU and Draper, another Nimrod family protein containing EGF-like repeats. SIMU is required for the recognition and Draper for the subsequent engulfment of apoptotic neurons (Kurant et al 2008) and programmed axon pruning in the fly central nervous system (Awasaki et al. 2006; Freeman et al. 2003), and has orthologues and homologues in *C. elegans* (CED-1) (Zhou et al. 2001) and in mammals (MEGF10, MEGF11, Jedi, SREC1 and 2) (Hamon et al. 2006).

1.1.6. Eater

Eater was identified as a putative target for Serpent, a *D. melanogaster* GATA transcription factor that had been found to be essential for bacterial phagocytosis by an RNAi screen (Rämet et al. 2002). Silencing of Eater expression in S2 cells led to lower bacterial binding and phagocytic activity. The same result was observed in flies lacking the *eater* gene or after RNAi knock-down of Eater. Such flies show impaired phagocytic activity, with increased bacterial loads and decreased survival rates after bacterial infections (Defaye et al. 2009; Charroux & Royet 2009; Kocks et al. 2005; Nehme et al. 2011). Induction of AMP expression through NFκB-like pathways Toll and Imd however was not affected (Kocks et al. 2005; Nehme et al. 2011), consistent with results obtained with flies in which phagocytes were ablated altogether. (Defaye et al 2009; Charroux & Royet, 2009). These results indicated that Eater is a major receptor for a broad range of pathogens in *D. melanogaster* and that it is critical for immune defense.

Although Eater belongs to a superfamily that comprises mammalian class F scavenger receptors, no clear mammalian orthologue of Eater exists. There are however related scavenger receptors implicated in the removal of apoptotic cells, p120 in the flesh fly (Hori et al. 2000) and CED-1 of *C. elegans* (Zhou et al. 2001) showing overall amino acid identity of 40 % and 25 %, respectively (Kocks et al. 2005).

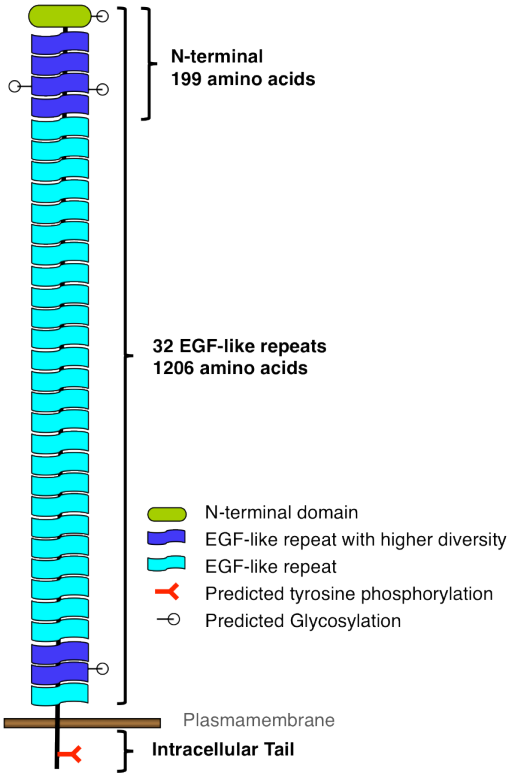


Figure 4. Schematic depiction of the Eater protein as a type-1 transmembrane protein with an extracellular region consisting of 32 EGF-like repeats, a transmembrane region and a short intracellular tail.

mRNA expression analyses revealed that the *eater* gene is a rare example of a gene whose expression is restricted exclusively to adult and larval hemocytes, and their pro-hemocyte precursors in the larval 'lymph gland' (Kocks et al. 2005). This expression pattern indicates that although it is a phagocytic receptor for bacterial particles, Eater does not seem to be involved in the clearance of apoptotic cells during tissue remodeling in embryogenesis and metamorphosis. Transcriptional silencing of *eater* in S2 cells did not affect the uptake of apoptotic cells (Cuttell et al. 2008).

Eater consists of 1206 amino acids and forms a large extracellular domain (Fig. 4). It contains 32 typical, non-calcium binding EGF-like repeats preceded by an N-terminal extension of 40 amino acids that contains a characteristic cysteine-flanked CCXGY-motif (Kocks et al. 2005; Kurucz et al. 2007; Somogyi et al. 2008), with an N-terminal signal sequence, a single membrane spanning domain, and a short C-terminal membrane anchor followed by an intracellular domain of 28 amino acids containing a potential tyrosine phosphorylation motif (Fig. 4) (Kocks et al. 2005). It has been shown that the first four EGF-like repeats which exhibit a high level of amino acid

diversity, repeat length and N-glycosylation participate in direct microbe binding (Kocks et al. 2005) whereas the remainder of the repeats may play a structural role as a 'stalk'. Whether this is the case, or whether the 'stalk' does participate in binding remains to be determined experimentally. However, analysis of the evolution of repeats in the Nimrod gene family (Somogyi et al. 2008), and the haplotype structure of *eater* alleles in wild populations of *D. melanogaster* (Juneja & Lazarro 2010) indicates different modes of evolution of the more 'unique' and 'stalk repeat' regions, and is in good agreement with this concept.

Direct binding of microbes to Eater was shown after purification of a secreted, truncated ectodomain comprising two complete N-terminal tandem repeats (Eater1-199His) from stably transfected *S2* cell supernatants (Kocks et al. 2005). Eater 1-199His bound directly and specificly to heat-inactivated Gram-negative *S. marcescens* as well as Gram-positive *S. aureus* and a yeast associated with termites (*C. silvatica*) (Kocks et al. 2005). Binding experiments aiming at elucidating Eater ligands suggested that Eater is able to recognize multiple polyanionic ligands, a behavior known from scavenger receptors (Greaves & Gordon 2005; Greaves & Gordon 2009; Plüddemann et al. 2007). Eater's affinity to certain polyanionic molecules (such as the typical scavenger receptor ligands oxidized low density lipoprotein (LDL) or acetylated LDL), as well as unpublished data suggesting binding of Eater to bacterial LPS and LTA, supported this view (Kocks et al. 2005; J. Cho & C. Kocks, unpublished). To date, it still remains to be determined whether Eater is also able to recognize other molecules found in the bacterial envelope. Shedding light on this issue would help us understand the mechanism by which Eater is able to bind to bacteria.

1.1.7. *Drosophila* as a model for phagocytosis

In mammals, the innate and adaptive immune system work in synergy making it complex to investigate one part of it without the other part interfering. The fruit fly, however, lacks the adaptive part of the immune system and this makes it inherently useful to study innate immune responses in the absence of antibody-based, acquired immunity (Levitin & Whiteway 2008).

Drosophila has a short generation time (10-12 days at 25°C) and can be maintained at relatively low cost. Furthermore, there are a number of macrophage-like *Drosophila* cell lines derived from mixed embryonic tissues including the widely used *Schneider line 2* (*S2* cells) (Schneider 1972). These hemocyte-derived cells possess properties similar to mammalian macrophages and efficiently phagocytose invading microbes and cell debris in a temperature-dependent manner (Pearson et al 2003; Rämet et al., 2002; Rämet et al., 2001; Stuart & Ezekowitz 2008). Their morphology after phagocytosis is also very reminiscent of that of professional phagocytes (Meister & Lagueux 2003; Pearson et al. 2003; Rabinowitz et al. 1992). S2 cells have been used as a tool to study *Drosophila* immune responses, particularly in regard to phagocytosis since they are readily amenable to genetic manipulation such as knock-down of expression of candidate genes by RNAi (Stuart & Ezekowitz 2008). Therefore, S2 cells have been widely used in high-throughput RNAi screens to identify molecules for their involvement in host pathogen interactions (Rämet et al. 2002; Ramadan et al. 2007; Agaisse et al. 2005; Philips et al. 2005; Boutros et al. 2004; Stroschein-Stevenson et al. 2006; Stuart & Ezekowitz 2008).

With respect to phagocytosis, approximately 600 *D. melanogaster* proteins were identified to be associated with *Drosophila* phagosomes, 70 % of which had mammalian orthologues, validating *Drosophila* as a model system for mammalian phagocytosis (Stuart et al. 2007 reviewed by Stuart & Ezekowitz 2008).

Thus, the powerful genetic tools (Duffy, 2002; Rong et al., 2002) available in *Drosophila* combined with the ease of using RNAi in cellular systems (Clemens et al., 2000) give researchers many options to study the innate immune system in *Drosophila*.

1.2. Aims of this Thesis

Characterization of the *Drosophila* phagocytic pattern recognition receptor Eater

The phagocytic pattern recognition receptor Eater is expressed solely on *Drosophila* blood cells (hemocytes) and their precursors (pro-hemocytes) and was shown to play a critical role in host survival after bacterial infection. Although Eater plays an

important role during bacterial infection, it remains poorly understood how this receptor recognizes different types of bacteria. Therefore one aim of this thesis was to further elucidate mechanisms by which Eater recognizes various microbes. Additionally, it was of interest to determine whether Eater recognizes live, naive bacteria since previous binding studies were carried out only with dead bacterial particles. It also remains unclear what the natural ligands of Eater are, and if and how Eater cooperates with other innate immune mechanisms or effectors to exert its protective effect.

Although Eater was found to recognize multiple ligands and broad classes of bacteria, it nevertheless bound specifically to certain microbes (Gram-negative Proteobacteria and Gram-positive *Firmicutes*) but not to others such as the Actinobacterium *M. luteus* and yeast *C. albicans*. This raises the question how Actinobacteria like *M. luteus* are bound and phagocytosed by *Drosophila* hemocytes. It was another aim of this thesis to identify genes involved in the binding and phagocytosis of *M. luteus* through a screen of 39 candidate genes.

2. MATERIAL & METHODS

2.1. Chemicals and Biologicals

Table 1. Chemicals and Reagents

Product	Catalog-No.	Manufacturer
Acrylamide Solution	BP1410-1	Fisher
Agarose	05066	SIGMA
Ammoniumpersulfat	A3678	SIGMA
Anhydrous Sodium Carbonate BioUltra	71345	SIGMA
β-Mercaptoethanol (β-M)	M7154	SIGMA
Blotting Grade Blocker Non-Fat Dry Milk	170-6404	Bio Rad
Bovine serum albumin (BSA), Fraction V	A3294	SIGMA
Bacto Brain-Heart Infusion Medium	237500	BD Biosciences
CelLytic M	C2978	SIGMA
Complete, Mini protease inhibitor cocktail	11836170001	Roche
Dimethylsulfoxide (DMSO)	D2650	SIGMA
Ethanol, absolute 200 proof	111ACS200	Pharmco
Ethidium bromide	E1510	SIGMA
Fetal Bovine Serum – Heat inactivated (tested on insect cells)	100 82-147	Invitrogen
Fluorescein-5-isothiocyanate (FITC, Isomer 1)	F-1906	Molecular Probes
Full-Range Rainbow Molecular Weight Markers	2892534	GE Healthcare
GelCode Blue Stain Reagent	24590	Thermo Scientific
Gentle Ag/Ab Binding and Elution Buffers	21027	PIERCE
Glacial acetic acid, 99.5 %	124040010	ACROS
Glycerol for Molecular Biology	G5516	SIGMA
HEPES, 99.5 %	H4034	SIGMA
HiTrap Protein A HP	17-0406-01	GE Healthcare

Human Low Density Lipoprotein (LDL)	BT-903	Biomedial Technologies
Isopropanol, 99.9 %	BP2632	Fisher
Laemmli-SDS Sample buffer	161-0737	BioRad
LB Broth Lennox	L1505	USBiologicals
Lithium Cloride Sigma Ultra	L4408	SIGMA
Magnesium chloride	8266	SIGMA
Oxidized Low Density Lipoprotein (oxLDL)	BT-910	Biomedial Technologies
Paraformaldehyde EM Grade 16 %	18814	Polysciences
Phenol	77607	FLUKA
Phenol:Chloroform:Isoamyl Alcohol 25:24:1	77617	FLUKA
Phosphate-buffered saline (PBS)	003000	Invitrogen
Polycytidylic acid (polyC)	P4903	SIGMA
Polyinosinic acid (polyI)	P4154	SIGMA
Potassium Acetate	P1190	SIGMA
Propidium Iodide	P3506	Invitrogen
Protein A Carboxylate Beads	17698	Polysciences
Robb's Drosophila PBS	-	(Robb 1969)
Schneider's Drosophila medium	11720-034	Invitrogen
Sodium Acetate	S825	SIGMA
Sodium Azide	S2002	SIGMA
Sodium chloride	S6191	SIGMA
Sodium dodecyl sulfate (SDS), 20 %	BP1311	Fisher
Sodium Hydrogen Carbonate	S5761	SIGMA
Streptavidin-15 nm colloidal gold conjugate	EM.STP15	Ted Pella Inc.
Sulfo-NHS-LC-Biotin, no weigh	21327	PIERCE
Tetramethylethylenediamine (TEMED)	T-9281	SIGMA
Tris-HCl, 1 M, pH 9	T-1190	TEKNOVA
Tris-HCl, 1.5 M, pH 8.8	T-1588	TEKNOVA

Tris-HCl, 1 M, pH 6.8	T-1068	TEKNOVA
Trypan Blue (0.4 %)	T-8154	SIGMA
Zeba Spin Desalting Columns	89889	PIERCE

Table 2. Enzymes and Enzyme Kits

Product	**Catalog-No.**	**Manufacturer**
All Restriction enzymes		New England Bio labs
Peptide:N-Glycosidase F	P07045	New England Bio labs
'High Fidelity PCR Master' Kit	12140314001	Roche
Megascript High Yield Transcription Kit	AM1334	Ambion
Mutanolysin	M9901	SIGMA
Rapid DNA Ligation Kit	1635379	Roche
RedTaq ReadyMix PCR Reaction Mix	R2523	SIGMA
THROMBIN CleanCleave Kit	RECOM-T	SIGMA

Table 3. Bacterial cell wall components

Supplier	**Cat. No.**	**Compound**	**Origin**
Sigma	L4524	LPS	*E. coli*
	L9143		*Pseudomonas aeruginosa*
	L6136		*Serratia marcescens*
	L3265	LTA	*Bacillus subtilis*
	L2515		*Staphylococcus aureus*
	L4015		*Streptococcus faecalis*
	77140	PGN	*Staphylococcus aureus*
	69554		*Bacillus subtilis*
	53243		*Micrococcus luteus*
InvivoGen	tlrl-pgnek		*E. coli*

2.2. Microbiology

2.2.1. Bacterial strains

Live *E. coli DH10B/TOP10* was purchased from Invitrogen; *E. coli* DH5alpha GFP, *P. aeruginosa* PA14 and *S. aureus* ALC1435 GFP were gifts of Fred Ausubel, *C.*

albicans of Ian Fraser, all at Massachusetts General Hospital, Boston, MA. *S. marcescens* Db11-GFP and LPS mutant 20C2 (Nehme et al. 2007), *E. faecalis* and *M. luteus* CIPA270 were provided by Dominique Ferrandon, IBMC du CNRS, Strasbourg, France. Surface protein A-negative *S. aureus* Wood 46 (ATCC10832) was from ATCC. To obtain non-fluorescent *S. marcescens* Db11, *S. marcescens* Db11-GFP was cured of the GFP plasmid.

2.2.2. Bacterial cultures and inactivation

Bacteria were grown in LB broth Lennox (US Biological) or brain heart infusion medium (BD) (*E. faecalis*) and inactivated by heat (60 minutes at 70˚C, or at 95˚C for 30 minutes (PA14)), Carnoy's fixative (75 % EtOH, 25 % glacial acetic acid for 10 min on ice) or formaldehyde (3 % for 20 min at RT) or used alive. All bacteria were washed in PBS (10 mM sodium phosphate dibasic, 156 mM sodium chloride, 2 mM potassium phosphate monobasic, pH 7.4) before use.

2.3. Molecular Biology

Standard methods of molecular biology were performed according to the respective manufacturer's guidelines or following protocols described by J. Sambrook et al., unless otherwise stated (Sambrook 2001) .

2.3.1. Quantification of nucleic acids

DNA and RNA concentrations were quantified by measuring the sample absorption at 260 nm and 280 nm with a NanoDrop ND-1000 UV-Vis Spectrophotomoter (Thermo Scientific). An optical density of 1 corresponds to approximately 50 µg/ml of double stranded DNA or to 38 µg/ml of RNA. A 260nm/280nm ratio of > 1.8 was used as an indicator of high nucleic acid purity.

Table 4. Primer sequences, all primer sequences are displayed in 5'→ 3' order. All primers were purchased from MGH DNA Core facility, Boston, MA.

Primer	Sequence
pYAC4fwd	CGCGGATCCCGCTCAGATCTGCACTGTTAATGT
pYAC4rev	GGGATAGGCTT ACCTTCGAA

pYAC5fwd	ATAGCTCGGTCCGATGTGGATTTGTAGGATAAC
pYAC5rev	GCTTACCTTCGA AGGGCCCTCTAGA
pYAC2fwd	GTCTAGTCTAGAGTAT ACAACTGATCCCGGTG
pYAC2rev	ACCGCGGGTACCGCGGCCGCTGATATC TCACCTTTGACGA

2.3.2. Extraction of genomic DNA from adult *Drosophila*

Adapted after a Web protocol from Laura Johnston Lab (www.cumc.columbia.edu/dept/genetics/faculties/Johnston/Potocols/DNA%20Prep.pdf); 30 healthy, freshly eclosed flies were collected into 1.5 ml Eppendorf tubes and frozen at –80°C for 5 min. 200 ml Buffer A (RT, 100 mM Tris-HCl, pH 7.5, 100 mM EDTA, 100 mM NaCl, 0.5% SDS) was added and the flies grinded with a tissue grinder until only pieces of cuticle remained. The suspension was incubated at 65°C for 30 min and 800 µl of 1:2.5 [5M]KOAc:[6M]LiCl was added and DNA was precipitated on ice for 10 min. DNA was centrifuged at 14000 rpm for 15 min and the supernatant was transferred to 2 Eppendorf tubes. 7/10 volume of Isopropanol per ml supernatant was added and centrifuged at 14000 rpm for 15 min. The pellet was washed subsequently with 1 ml cold EtOH, 150 ml Phenol (tris-buffered) and aqueous (top) layer was transferred to new eppendorf tube and washed with 150 µl (25:24:1) Phenol : Chloroform : Isoamyl Alcohol. Aqueous (top) layer was transferred to new Eppendorf tube as before and washed with 150 µl (24:1) Chloroform : Isoamyl Alcohol and aqueous (top) layer was transferred to new eppendorf tube. After subsequent addition of 1/10 volume [3M] NaOAc (pH 5.2) and 2x volume 100 % Ethanol, solution was mixed and chilled at -80°C for 15 min then centrifuged at 14000 rpm for 15 min. Ethanol was removed and washed with 1 ml cold 70% Ethanol. The pellet was dried and resuspended in 100 ml TE (Tris 10 mM EDTA 1 mM).

2.4. Cell culture

Drosophila S2 cells were cultivated at 26.5˚C in Schneider's *Drosophila* Medium supplemented with 10 % FBS heat-inactivated. For production of secreted Eater-Fc

protein, *Spodoptera frugiperda 9 (SF-9*) cells (Invitrogen) were grown at 27°C in serum-free HyQ-CCM3 medium (Hyclone, Thermo Scientific).

2.5. SDS PAGE and Western Blots

Samples were separated on SDS discontinuous polyacrylamide gels and blotted onto PVDF membranes (Millipore). Membranes were blocked with blocking buffer (2 % w/v dry milk in PBS-T) for one at RT. Subsequently, primary antibodies (Table 5.) diluted in blocking buffer at indicated concentration were applied and incubated for at least 60 minutes at RT. After 3 washes with PBS-T (PBS + 1 % Tween 20) for 10 minutes each, secondary antibody was applied for one hour at RT. After 3 washes with PBS-T, membranes were incubated for one minute in Pierce ECL Western Substrate (PIERCE) and exposed to chemiluminescence films (Kodak). Films were developed in an automatic developer (Kodak).

Table 5. Antibodies and secondary reagents

Product	Catalog-No.	Manufacturer
Anti-human IgG1-Fc, HRPO conjugate	MH1715	Caltag
Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG	111-035-144	Jackson ImmunoResearch
Streptavidin Alexa Fluor 488	S32354	Invitrogen

2.6. Expression and purification of Eater-Fc

2.6.1. Baculovirus Expression Vectors

pYAC4 (encoding Eater^{+}19-199-Fc; with four additional amino acids at the mature N-terminus)

A 594 bp fragment corresponding to amino acids 19 to 199 of the Eater protein was amplified using plasmid pMT/V5His-Eater1-199 (Juhyun Cho) as template with primers pYAC4fwd and pYAC4rev and cloned into pCR2.1-TOPO (TOPO TA Cloning Kit, Invitrogen Cat. No. K4500-01). The 584 bp BamHI-XhoI fragment corresponding to amino acids 19-199 of Eater was cloned into pFastBactevFc

Baculovirus expression vector (Ju et al. 2006 , obtained from Bok-Luel Lee) in order to generate an in-frame fusion between the Baculovirus signal sequence gp67 and amino acids 19-199 of Eater, followed by a TEV cleavage site, thrombin cleavage site and the Fc domain of human IgG1. This vector was called pYAC4.

pYAC5 (encoding Eater'19-199-Fc; corresponding to the mature Eater N-terminus)
A 648 bp fragment corresponding to amino acids 1 to 199 of the Eater protein was amplified by PCR using plasmid pMT/V5His-Eater1-199 (Juhyun Cho) as template with primers pYAC5fwd and pYAV5rev and cloned into pCR2.1-TOPO (TOPO TA Cloning Kit, Invitrogen Cat. No. K4500-01). The 603 bp BamHI-XhoI fragment corresponding to amino acids 1-199 of Eater was cloned into pFastBactevFc Baculovirus expression vector (Ju et al., 2006, obtained from Bok-Luel Lee) in order to generate an in-frame fusion between the first 199 amino acids of Eater, followed by a TEV cleavage site, thrombin cleavage site and the Fc domain of human IgG1. This vector was called pYAC5. All PCR amplifications were done with High Fidelity PCR Master Kit (Roche). The correct sequence of all of the entire insert was confirmed on both strands.

2.6.2. Expression and purification of Eater-Fc

Recombinant bacmids were generated by transformation into *E. coli DH10Bac* (Invitrogen). For production of secreted Eater-Fc protein, *Spodoptera frugiperda 9 (SF-9*) cells (Invitrogen) were grown at 27°C in serum-free HyQ-CCM3 medium (Hyclone, Thermo Scientific). High-titer bacmid stock was used to infect 7 liters of *Sf-9* cells (2 x 10^6/ml) and incubated at 27°C for 42 hrs. Cell culture supernatant was harvested by centrifugation at 5,000 x g for 30 min, filtered (0.22 µm low protein binding) and loaded onto a 5 ml HiTrap Protein A column on an Äkta FPLC (GE Healthcare). Bound protein was washed with 5 column volumes of 20 mM Hepes, 100 mM NaCl, pH 7.0, eluted using Gentle Ag/Ab Elution Buffer at pH 6.6 (Pierce, Thermo Scientific), buffer exchanged into 20 mM Hepes, 150 mM NaCl, pH 7.0 (Zeba Desalt Spin Column; Pierce, Thermo Scientific) and concentrated to 2 mg/ml with Amicon filter devices (Millipore). To assess purity and size, purified protein was analysed by Laemmli SDS-PAGE under non-reducing or reducing conditions in the absence or presence of 710 mM β-mercaptoethanol followed by Coomassie Blue

staining (GelCode Blue; Pierce, Thermo Scientific). For cleavage of Eater-Fc Thrombin CleanCleave KIT (Sigma) was used according to the manufacturer's instructions.

2.7. Deglycosylation of Eater-Fc

Eater-Fc fusion protein was incubated overnight at 4˚C in PBS under non-denaturing conditions with or without (mock control) Peptide:N-Glycosidase F (New England Biolabs) and subsequently used for SDS-gel analysis (uncleaved or after thrombin-cleavage) or in flow cytometry binding assays (uncleaved).

2.8. Eater-Fc binding to bacteria

Eater-Fc fusion protein, or control human IgG_1 or IgG Fc-fragment (Athens Research & Technology), was biotinylated with EZ-Link Sulfo NHS-LC-Biotin reagent (Pierce, Thermo Scientific) according to the manufacturer's instructions. For flow cytometry, 2 x 10^6 bacteria in Robb's *Drosophila*-PBS (Robb 1969) supplemented with 0.5 % BSA and 0.01 % sodium azide were incubated with biotinylated proteins for 30 min at room temperature, sedimented at 9,000 x g for 5 min and washed. For detection of bound biotinylated protein, bacteria were resuspended in the presence of 1 µg/ml streptavidin-Alexa Fluor 488 conjugate (Invitrogen) and incubated for 20 min before analysis on a FACS Calibur (Becton Dickinson). The bacterial population was gated by forward and side scatter and 10,000 events were recorded. For assessment of bacterial viability by propidium iodide (PI) exclusion, 50 µg/ml PI was added on ice immediately before analysis. Fluorescence emissions were detected in the FL-1 channel (Alexa Fluor 488 emission: 519 nm) and, where indicated, in the FL-3 channel (PI emission: 620 nm).

2.9. Generation of anti-Eater-Fc antibodies

Antibodies were generated in two rabbits by Pocono Rabbit Farm & Lab Inc. (Canadensis, Pennsylvania, USA) using the 'Quick Draw' Protocol. The rabbits were

prescreened towards lack of activity against insect proteins and then injected with Eater-Fc fusion protein. After two bleeds (21 and 28 days after initial injection) the sera were tested for antibodies against Eater by flow cytometry. Rabbits were boosted once more and subjected to a final bleed. Sera from bleed 3 showed a significant improvement in the detection of Eater in flow cytometry as well as in Western Blot analysis and was purified over Protein A Sepharose (Data not shown).

2.10. Cecropin A exposure of bacteria

Chemically synthesized cecropin A peptide from the moth *Hyalophora cecropia* (KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK) was purchased from Sigma. Cationic control peptide 2K1 with the sequence $(GK)_6$ AS $(GK)_6$ (Fantner et al. 2010) was synthesized by standard solid phase peptide synthesis at the MGH Peptide/Protein Core Facility. Both peptides were dissolved in PBS at 100 µM and stored frozen in aliquots at -80˚C. Bacteria were grown to mid log phase in LB broth Lennox at 37˚C, centrifuged (3,500 x g, 4˚C), resuspended in PBS, counted and adjusted to 10^8/ml. 50 µl of bacterial suspension was added to 50 µl of PBS containing the indicated concentrations of cecropin A and incubated at 25˚C for the indicated times, placed on ice and analysed immediately by flow cytometry in the presence of 50 µg/ml PI. For assessment of Eater-Fc binding to cecropin A-exposed bacteria, subsequent bacterial Eater-Fc binding assays were carried out in PBS at 4˚C, a temperature non-permissive for AMP activity. TFA (used as counterion to maintain charge balance in solid phase peptide synthesis) (Roux et al. 2008) showed no effect on bacterial viability at 9-fold molar excess over peptide (1 counterion per positive charge), and up to 10 mM. For control CFU counts, samples were split, one half was plated on LB Lennox agar, and one half processed for flow cytometry. Colonies were counted the next day.

2.11. Pre-embedding immunogold labeling of *E. coli*

At 4˚C, 500 µM (25 µg/ml) biotinylated Eater-Fc fusion protein was pre-incubated in PBS for one hour with 2.7 µg/ml Streptavidin-15 nm colloidal gold conjugate (Ted

Pella Inc.) and then rotated in a total volume of 100 µl for 16 hours with 1 x 10^7 *E. coli*, either heat-inactivated, or cecropin A-killed, or live. Labeled bacteria were washed once with PBS and resuspended in 2 % glutaraldehyde and stored at 4˚C. Further processing of the samples was carried out by the Microscopy Core of the Program in Membrane Biology at MGH. Fixed bacteria were pelleted, rinsed once with 0.1 M sodium cacodylate buffer (pH 7.4) and re-pelleted. Bacterial pellets were stabilized with 2.0 % agarose before dehydration and embedding in Eponate resin (Ted Pella Inc.). Thin sections were collected onto formvar-coated slot grids, post-stained with 2.0 % aqueous uranyl acetate and examined in a JEOL 1011 transmission electron microscope at 80 kV. Images were collected using an AMT digital imaging system (Advanced Microscopy Techniques).

2.12. S2 cell surface staining

2.5 x 10^5 S2 cells were stained with anti-Eater-Fc in PBS supplemented with 0.5 % BSA and 0.01 % sodium azide for 20 min on ice followed by 1 µg/ml goat-anti-rabbit-IgG Alexa Fluor 488 conjugate (Invitrogen). For flow cytometry, S2 cells were gated by forward and side scatter and 5,000 events were recorded. For immunofluorescence microscopy, S2 cells were fixed with formaldehyde (3 % for 20 min at room temperature).

2.13. S2 cell binding and phagocytosis of bacteria and RNA interference, for S2 cell binding to live bacteria and for *M. luteus* screen

Flow cytometry based bacterial S2 cell binding assays and RNAi by soaking were performed and data analysed and presented as described (Kocks et al. 2005; Rämet et al. 2001). In short: Double-stranded (ds) RNA directed against Eater or pBR322 (control) was synthesized from a PCR product using T7 MegaScript RNA polymerase (Ambion). 5 x 10^5 S2 cells were incubated first with 7.5 µg dsRNA for approx. 60 hours, and then with bacteria in Schneider's *Drosophila* Medium (Invitrogen) without serum at 4˚C for binding. To assess phagocytosis, cells and bacteria were warmed up

to 26.5˚C for 30 min and then returned to ice until flow cytometry analysis. To distinguish bound from phagocytosed particles, samples were quenched with trypan blue prior to analysis (Rämet et al. 2002). To facilitate direct comparison, GFP expressing bacteria were used in all cases; since heating destroyed GFP, heat-inactivated bacteria were labeled with FITC (Isomer I; Invitrogen).

2.14. Peptidoglycan (PGN) cosedimentation

A suspension of polymeric, insoluble PGN from *E. coli* (InvivoGen), *B. subtilis, S. aureus*, and *M. luteus* (all from Sigma) was made in PBS (5 mg/ml), aliquoted and stored at -20˚C. 60 µg of insoluble PGN was mixed with 1 µg thrombin-cleaved Eater-Fc in 50 µl of PBS supplemented with Protease Inhibitor Cocktail (Complete Mini; Roche) and 0.5 % BSA. At 0 and 15 min, 10 µl was removed from the mixture. After incubation for 15 min at 4˚C, the remaining 30 µl of mixture was centrifuged at 4˚C at 16,000 x g for 15 min (*B. subtilis*, *S. aureus*, *M. luteus* PGN) or 279,000 x g for 1 hour (*E. coli* PGN). The supernatant was removed and the pellet washed two times with 200 µl supplemented PBS, and resuspended in 30 µl. All samples were mixed with Laemmli sample buffer immediately after preparation and incubated at 95˚C for 5 min. Equal amounts of samples (corresponding to 10 µl of starting sample) were analysed by reducing SDS-PAGE and immunoblotting using anti-Eater-Fc antibodies followed by goat-anti-rabbit IgG conjugated to horseradish peroxidase. As control, 60 µg of insoluble PGN was cleaved with 6 µg (55 units) mutanolysin from *S. globisporus* (Sigma) at 37˚C for 16 hours before addition of 1 µg thrombin-cleaved Eater-Fc.

2.15. Plasmid vector for GAL4 reporter fly line

Genomic DNA was prepared from the *D. melanogaster* strain w^1 (Bloomington stock no. 145; obtained by JM Reichart) as described above under 2.3.2. Primers were designed and analysed using the sequence analysis software Vector NTI (Invitrogen). Primers pYAC2fwd and pYAC2rev were used to amplify 2128 bp of the upstream flanking region of the eater gene (eater promoter). PCR amplifications, using genomic

DNA of *D. melanogaster* strain w^1 as the template, were performed with the 'High Fidelity PCR Master' Kit (Roche) according to the manufacturer´s instructions in an Eppendorf master cycler, with a starting step of 2 min at 94°C followed by 10 cycles of (10 sec at 94°C, 70 sec at 60°C, 4:00 min at 72°C) and 20 cycles of (15 sec at 94°C, 30 sec at 60°C, 5:30 min at 72°C) followed by a final elongation step of 7 min at 72°C. The PCR product was ligated into vector pCR2.1-TOPO (TOPO TA Cloning Kit, Invitrogen Cat. No. K4500-01).

The eater promoter (2128 bp) was excised from vector pCR2.1-TOPO with the restriction enzymes XbaI and Acc65I and ligated into vector pJM1398 V (obtained from JM Reichart) using NheI and Acc65I -sites after removal of a 1843bp Acc65I-NheI fragment encoding a different promoter. This resulted in vector pYAC2. pYAC2 was cut with the restriction enzyme NotI and the 5343 bp fragment (containing the *eater* promoter fused to GAL4) was ligated into vector pCaSpeRSXsNN (obtained from JM Reichhart) pre-cut with NotI. The final vector was pYAC3. All transformation steps were carried out using One Shot® Top10 Competent Cells (Invitrogen, Cat. No. C4040-03).

2.16. Fly strains

Fly cultures and crosses were carried out at 25˚C on a standard medium of yeast, cornmeal, agar and molasses, supplemented with propionic acid and tegasept. Transgenic *eater-GAL4* (*pP{eater-GAL4}*) flies were generated in the laboratory of our collaborator, Jean-Marc Reichhart from the University of Strasbourg, using plasmid pYAC3 (see Material and Methods section 2.15). *eater-GAL4*, *UAS-GFP* recombinants were generated by combining *eater-GAL4* (inserted into second chromosome) with *UAS-GFP* (w[*]; P{w[+mC]=UAS-GFP.S65T}T2; Bloomington stock #1521) on the second chromosome. The recombinant chromosome was then made homozygous.

III. Results & Discussion

Passages of the Results & Discussion section were adopted from the following manuscript with no or minor alterations. Those passages are not individually marked.
Chung, Yoon-Suk Alexander and Kocks, Christine. Recognition of Pathogenic microbes by the *Drosophila* phagocytic pattern recognition receptor Eater. Submitted for publication, under revision.

3.1. Expression, Purification and Characterization of a Recombinant Eater-Fc Protein

Aim: The goal of this part of my thesis was to generate a soluble, recombinant Eater receptor variant comprising the putative ligand binding domain of Eater, and to scale up its production sufficiently to allow follow-up studies on Eater's microbial binding specificity.

Rationale: Eater mediates phagocytosis of a broad range of microbes, and its binding behavior is reminiscent of the multi-ligand specificity exhibited by scavenger receptors (Kocks et al. 2005). The putative ligand binding domain of Eater was previously produced in *S2* cells as a soluble, secreted, histidine-tagged fusion protein (Kocks et al., 2005). This construct comprised the first four EGF-like repeats of Eater (amino acids 19-199). While this recombinant protein showed direct binding activity to microbes, protein yields were very low. I therefore decided to fuse the putative ligand binding domain of Eater to an IgG-Fc tag, and to use a Baculovirus expression system in the hope of generating high amounts of 'biologically active' Eater protein. In order to determine the binding characteristics of Eater-Fc protein, I probed binding of Eater-Fc to to various heat- or ethanol-inactivated microbes using a previously established flow cytometry-based, direct microbe binding assay.

3.1.1. Generation of two Baculovirus expression vectors and small scale purification of Eater-Fc fusion proteins

To follow up on previous findings, it was necessary to establish a protein expression system that results in high yields of 'active' protein, which would also enable me to generate better antibodies against Eater, since protein yields from the S2 cell expression system were low (Kocks et al., 2005; 50 to 100 µg/liter). I decided to use a Baculovirus expression system to produce large amounts of protein (Atkinson et al. 1992; Verburg et al. 1993). I chose to fuse the putative ligand binding domain of Eater (amino acids 1-199) to the Fc part of human IgG1, in the hope to stabilize the protein and to allow for a simple one step purification by protein A affinity chromatography.

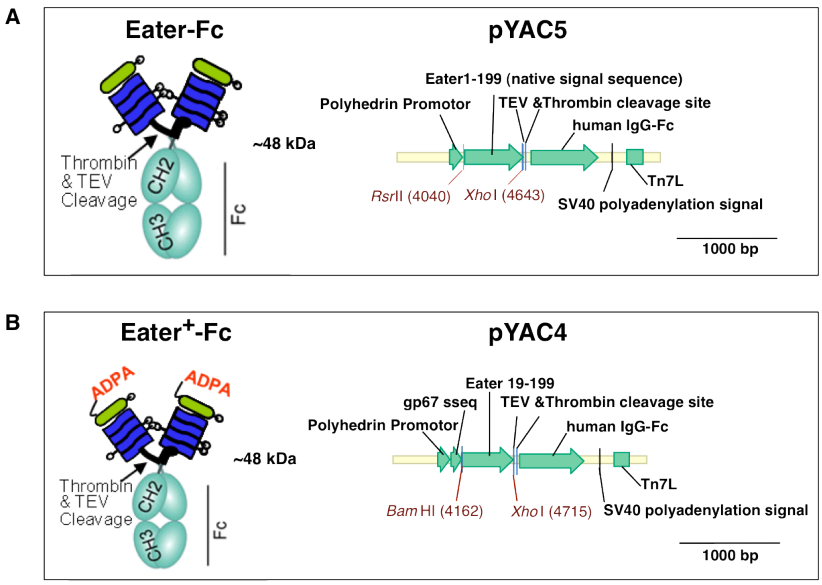


Figure 5. Schematic depiction of Eater-Fc fusion proteins and corresponding Baculovirus expression constructs. (A) Model of Eater-Fc protein. A 611 bp RsrII-XhoI fragment corresponding to amino acids 1-199 of Eater (comprising the signal sequence) was inserted into pFastbactevFc to generate an in-frame fusion of the first 199 amino acids of Eater, with a TEV/thrombin cleavage site and the Fc domain of human IgG1. **(B)** Model of Eater^{+}-Fc protein. A 584 bp BamHI-XhoI fragment corresponding to amino acids 19-199 of Eater was inserted into pFastbactevFc to generate an in-frame fusion between the Baculovirus gp67 signal sequence and amino acids 19-199 of Eater, followed by a TEV/thrombin cleavage site and the Fc domain of human IgG1. Four additional amino acids generated by the cloning site at the N-terminus are shown in red.

I generated two constructs for expression of soluble variants of Eater in the Baculovirus system, plasmids pYAC4 and pYAC5 (Fig. 5), and used them in *Sf-9* cells with the Bac-to-Bac Baculovirus Expression System from Invitrogen. To generate the fusion between antibody Fc and the N-terminal domain of Eater (amino acids 1-199), I based my constructs on a published Baculovirus expression vector, pFastBactevFc (Ju et al. 2006). pFastBactevFc was used successfully to express a soluble EGF-like repeat protein from *Holotrichia diomphalia* larvae, that shares structural similarities to Eater and functions as an opsonin. I used the Baculovirus-derived signal sequence gp67 in addition to the endogenous signal sequence of Eater to determine if the gp67 signal sequence might help to improve yields of Eater-Fc protein (Stewart et al. 1991).

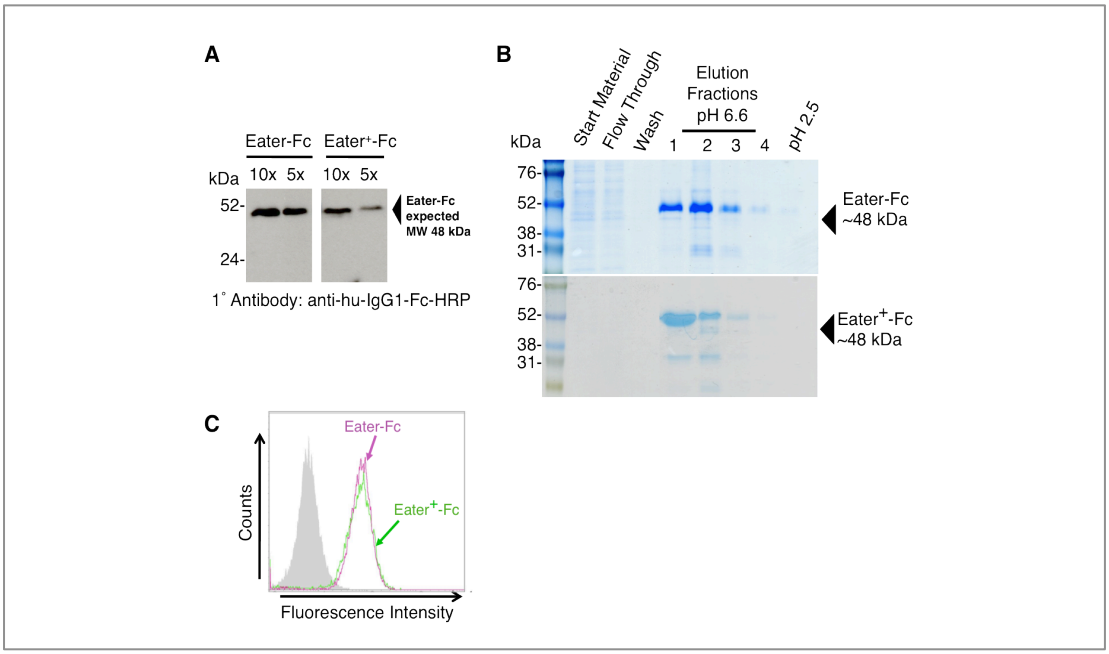


Figure 6. Small scale expression and purification of biologically active Eater-Fc fusion proteins in the Baculovirussystem. (A) Baculovirus-infected *Sf-9* cell culture supernatants (5 or 10 x concentrated) were tested for Eater-Fc expression by Western Blot with anti-human-IgG-Fc-domain specific antibodies. **(B)** Coomassie blue stained 12 % SDS gels showing fractions after affinity purification of Eater-Fc proteins over Protein A sepharose. Elution fractions 1-3 were pooled. **(C)** Flow cytometry analysis of binding of 200 µM biotinylated Eater-Fc (pink curve) or Eater^{+}-Fc (green curve) to heat-inactivated *S. marcescens* compared to control (grey filled curve; secondary reagent only). For a detailed description of the flow cytometry assay see Fig. 7A.

Recombinant N-terminal Eater proteins were overexpressed in *Sf-9* cells and expression was confirmed in concentrated supernatant through western blot analysis against the Fc-domain (Fig. 6 A). I then purified the proteins using a one step Protein A purification protocol (Fig. 6 B). Following pooling, buffer exchange and concentration of Eater-Fc and Eater^{+}-Fc resulted in yields between 350 µg – 1.5 mg

of pure protein per liter of *Sf9* cell culture. Coomassie stained SDS gels confirmed the purity and size of the proteins at approximately 48 kDa (Fig. 6 B). The biological activity of the Eater-Fc proteins was analysed by assessing the binding activity of biotinylated Eater protein to heat-inactivated Gram-negative bacteria (*Serratia marcescens)* (Fig. 6 C).

These results indicated that the Baculovirus system allows the production of larger quantities of Eater-Fc protein compared to the *S2* cell system. Moreover, one step purification resulted in relatively pure protein that showed only few contaminations on a Coomassie blue stained SDS gel (Fig. 6 B).

3.1.2. Large scale production of biologically active Eater-Fc fusion protein

Since the Eater-Fc fusion protein (Fig. 5 A) gave more consistent results in preliminary direct bacterial binding experiments than Eater^{+}-Fc, I decided to scale up the production of this protein. Eater-Fc fusion protein was purified from 7 liters of *Sf-9* cell supernatant by affinity chromatography on a 5 ml HiTrap prepacked Protein A Sepharose column attached to a FPLC system. Eater-Fc fusion protein was eluted under neutral buffer conditions (pH 6.6) with a proprietary buffer (Pierce) (Fig. 7 A). The eluted protein migrated under reducing conditions close to its calculated size of approximately 48 kDa (Fig. 7 B). Following pooling of the relevant fractions (5-9), concentration and buffer exchange, the purification yielded a total of 6.7 mg of pure Eater-Fc fusion protein (corresponding to a yield of roughly 1 mg/liter). Its binding activity showed no decrease after freezing and thawing (data not shown) so that protein could be aliquoted and stored at -70˚C for further use.

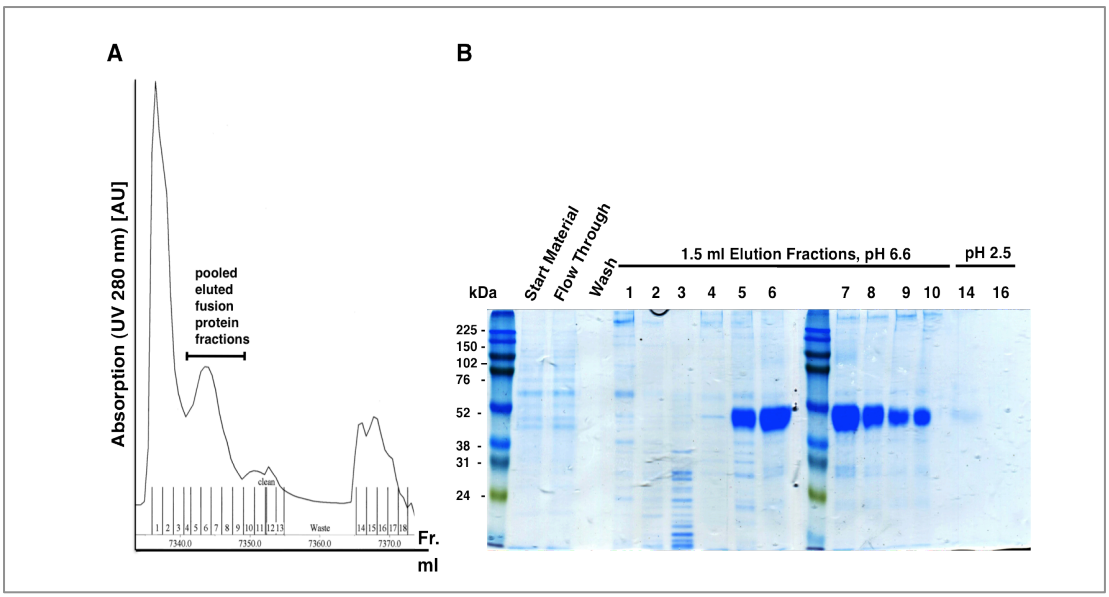


Figure 7. Large scale purification of Eater-Fc fusion protein (A) FPLC Elution profile from 5 ml HiTrap Protein A column. Fusion protein was eluted in 1.5 ml fractions with 2.5 column volumes of neutral elution buffer (pH 6.6; Pierce) followed by a sodium citrate buffer wash (pH 2.5). **(B)** Corresponding Coomassie Blue-stained 10 % SDS gel. Fusion protein eluted in fractions 5-9 was pooled.

In order to assess the biological activity of Eater-Fc, I used a flow cytometry-based direct binding assay. For this assay Eater-Fc is biotinylated and then incubated with a microbe of choice. As depicted in Figure 8 A, Eater-Fc fusion protein bound to the surface of a microbe is detected with Streptavidin Alexa-Fluor 488.

Flow cytometry analysis showed that Eater-Fc binding to heat-inactivated *E. coli* (Fig. 8 B-D) and *S. marcescens* (Fig. 8 E-F) was concentration-dependent (Fig. 8 B & E) and could be competed with unlabeled Eater-Fc (Fig. 8 C & F) confirming that the biotinylation procedure had not changed the binding properties. Biotinylated control proteins IgG_1 and IgG-Fc showed no binding (Fig. 8 D & G), indicating that the observed binding was not due to the Fc tag.

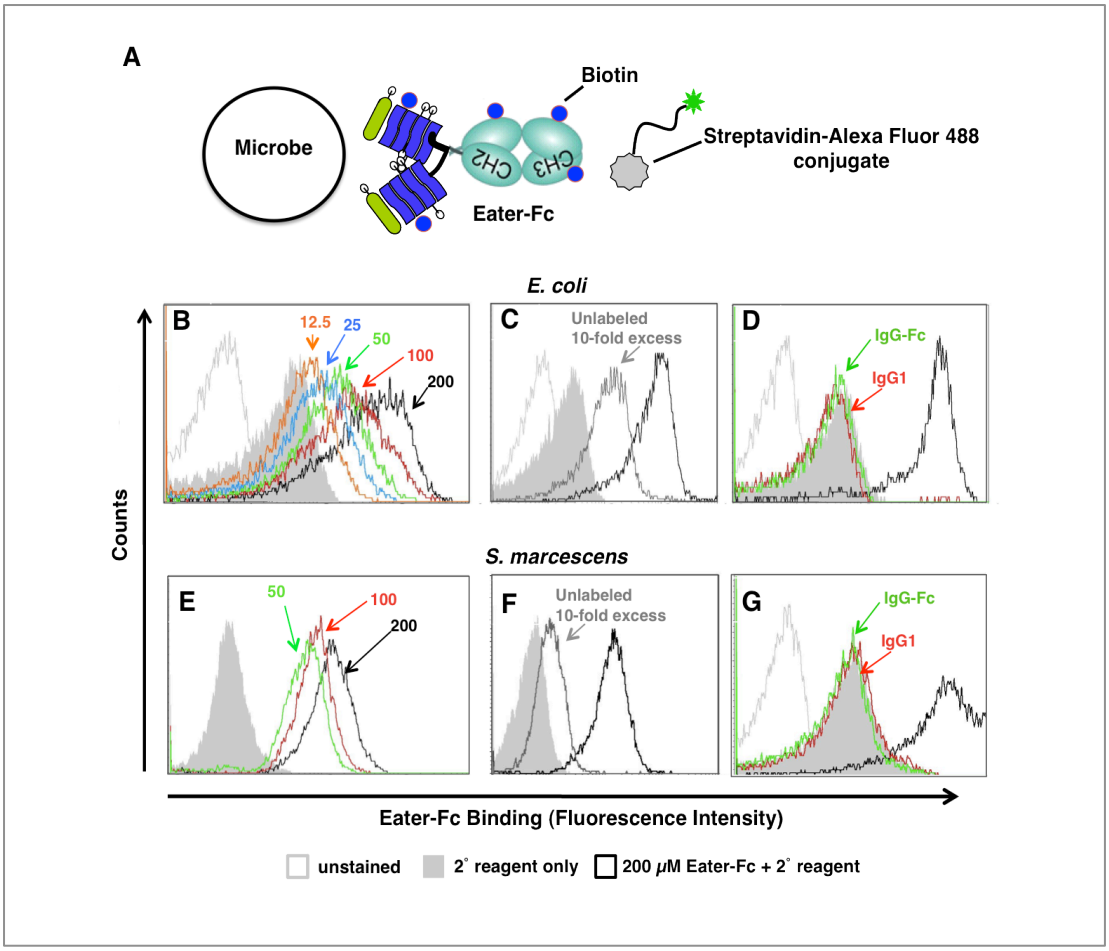


Figure 8. Flow cytometry analysis of Eater-Fc binding to the heat-inactivated Gram-negative bacteria *E. coli* and *S. marcescens*. (A) Schematic depiction of the flow cytometry binding assay. Biotinylated Eater-Fc protein binds to microbes and is subsequently detected by Strepavidin conjugated Alexa Fluor 488 (secondary reagent). Bound Eater Protein is detected by flow cytometry as an increase in fluorescence intensity. **(B-G)** Flow cytometry analysis of direct binding of Eater-Fc to heat-inactivated Gram-negative bacteria **(B-D)** *E. coli*; **(E-G)** *S. marcescens*. **(B & E)** Concentration-dependent binding (red, 100; green 50; blue, 25; orange 12.5 µM). **(C & F)** Inhibition of binding by a 10-fold excess of non-biotinylated Eater-Fc (2 mM; dark grey curve). **(D & G)** Control: No significant binding activity was detected with biotinylated human IgG_1 (200 µM; red line) or IgG-Fc (200 µM; green line).

3.1.3. Eater-Fc binding to distinct classes of non-viable microbes

To further examine the binding specificity of Eater-Fc, I tested binding of biotinylated Eater-Fc fusion protein to various heat-inactivated or ethanol-inactivated bacteria (Fig. 9 A-C), and yeast (Fig. 9 D). Consistent with the previous characterization of Eater as a scavenger receptor with multi-ligand specificity, Eater-Fc protein displayed broad binding activities towards different classes of inactivated, non-viable bacteria such as *S. marcescens* and *P. aeruginosa* (Fig. 9 A), as well as Gram-positive bacteria of the phylum *Firmicutes* (*E. faecalis, S. aureus*) (Fig. 9 B). Control IgG_1 or IgG-Fc

showed no significant binding activity (Fig. 9 A, B). The unrelated Gram-positive bacterium *M. luteus* (phylum *Actinobacteria*), and the fungal pathogen *C. albicans* were not recognized by Eater-Fc (Fig. 9 C, D). This result indicates that microbial binding by Eater-Fc is broad, yet to some extent specific, as would be expected for a pattern recognition receptor (Janeway 1989; Janeway & Medzhitov 2002).

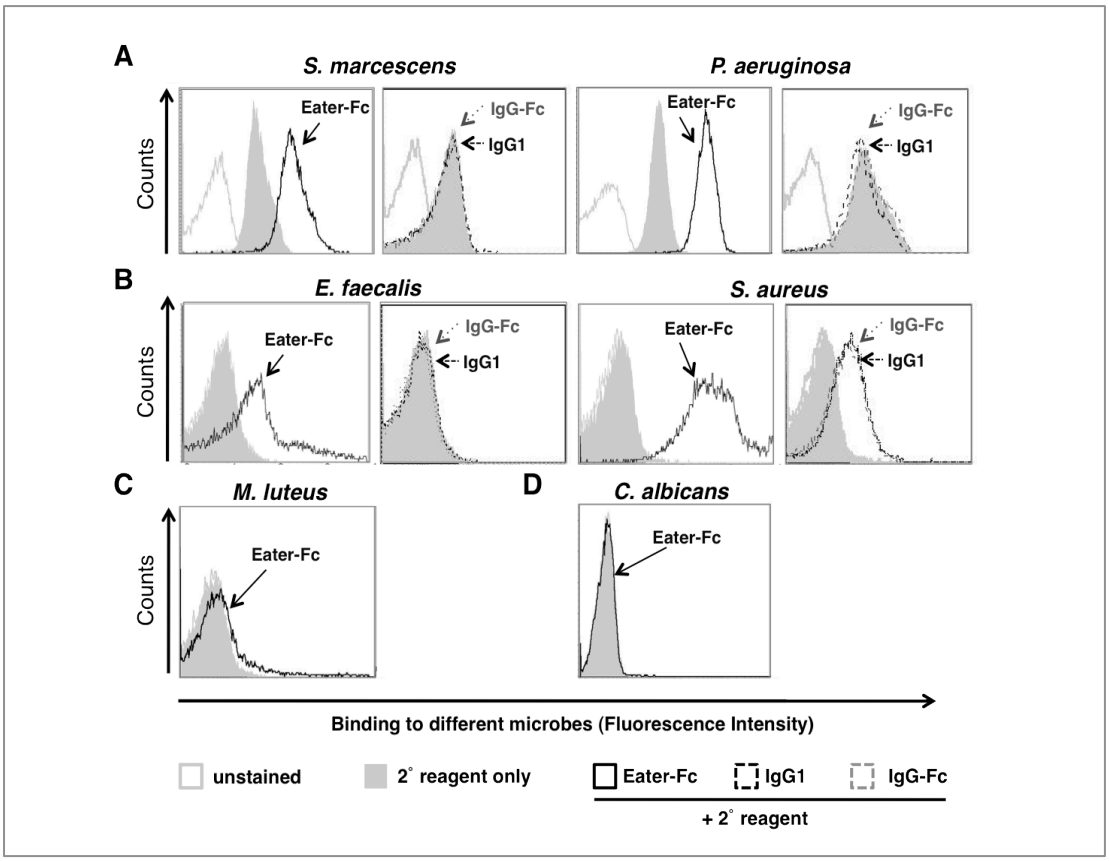


Figure 9. Eater-Fc binds to broad, yet distinct classes of heat- or ethanol-inactivated bacteria. Flow cytometry analysis of binding by 200 µM biotinylated Eater-Fc fusion protein (open black curve) or control biotinylated IgG1 and IgG-Fc (broken black or broken gray curves, respectively) compared to secondary reagent only (gray filled curve) or unstained microbes (open gray curve). **(A)** Binding by Eater-Fc to heat-inactivated *S. marcescens* or ethanol-inactivated *P. aeruginosa* (Gram-negative proteobacteria). **(B)** Binding by Eater-Fc to heat-inactivated *E. faecalis* and *S. aureus* (Gram-positive *Firmicutes*). **(C)** No binding by Eater-Fc to heat-inactivated *M. luteus* (Phylum *Actinobacteria*) or heat-inactivated *C. albicans* yeast **(D)** Experiments were always run in parrallel with positive and negative controls and repeated at least once with similar results.

3.1.4. Characterization of the binding properties Eater-Fc fusion protein

3.1.4.1. Inhibition of Eater-Fc binding by polyanionic molecules

Previous published and unpublished results suggested that the N-terminal four EGF-like repeats of the ectodomain of Eater bind to typical scavenger receptor ligands

(polyanionic molecules such as modified oxidized or acetylated LDL and poly-I), but not to closely related non-scavenger receptor ligands (polyanionic molecules such as LDL and poly-C) (Kocks et al. 2005; J. Cho & C. Kocks, unpublished). Therefore, I tested Eater-Fc binding to these molecules. Poly-I and poly-C are synthetic polyribonucleotides structurally similar to double-stranded RNAs, which are present in some viruses (Plüddemann et al. 2007). As expected, poly-I but not poly-C inhibited the binding of Eater-Fc to heat-inactivated *S. marcenscens* (Fig. 10). I was also able to obtain consistent inhibition profiles using the scavenger receptor ligand oxidized LDL although mostly at higher concentrations (data not shown). However the controls (non-modified LDL) behaved in ways hard to interpret: LDL showed weak inhibition at low concentration, but no inhibition at high concentrations. One possibility is that these results were due to problems with the (commercial) reagent quality, as purified LDL quickly oxidizes upon storage.

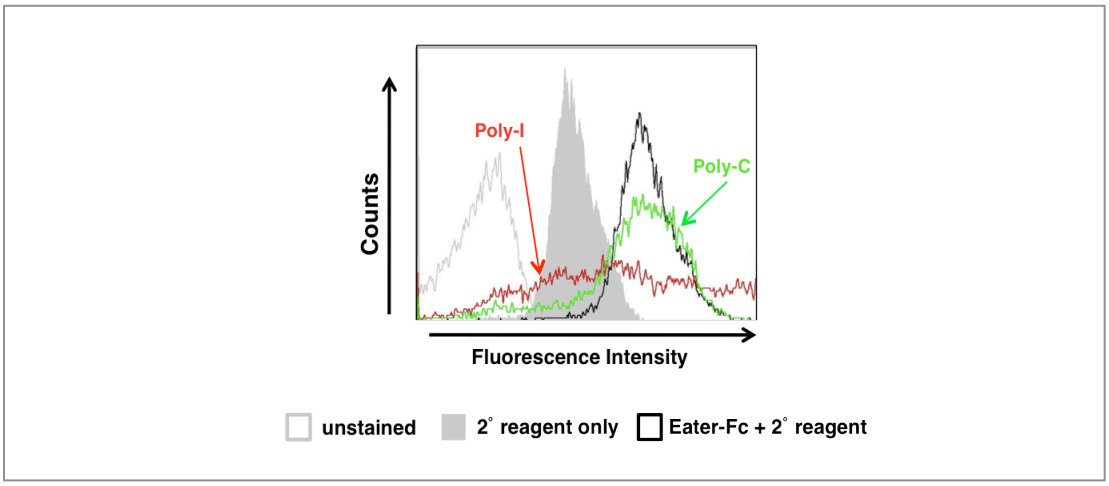


Figure 10. Eater-Fc protein binding to bacteria can be inhibited by poly-I but not by poly-C. Flow cytometry analysis of binding by 200 µM Eater-Fc in the presence of 100 µg/ml inhibitor. Binding by Eater-Fc is inhibited by poly-I (red curve) but not by poly-C (green curve).

3.1.4.2. Dimerization, thrombin-cleavage and heat-denaturation of Eater-Fc protein

Eater-Fc fusion protein migrated in a single band of about 48 or 96 kDa in reducing or non-reducing SDS-PAGE, respectively, in agreement with its expected molecular weight and disulfide bond-mediated dimerization of the C-terminal Fc-Tag (Fig. 11 A).

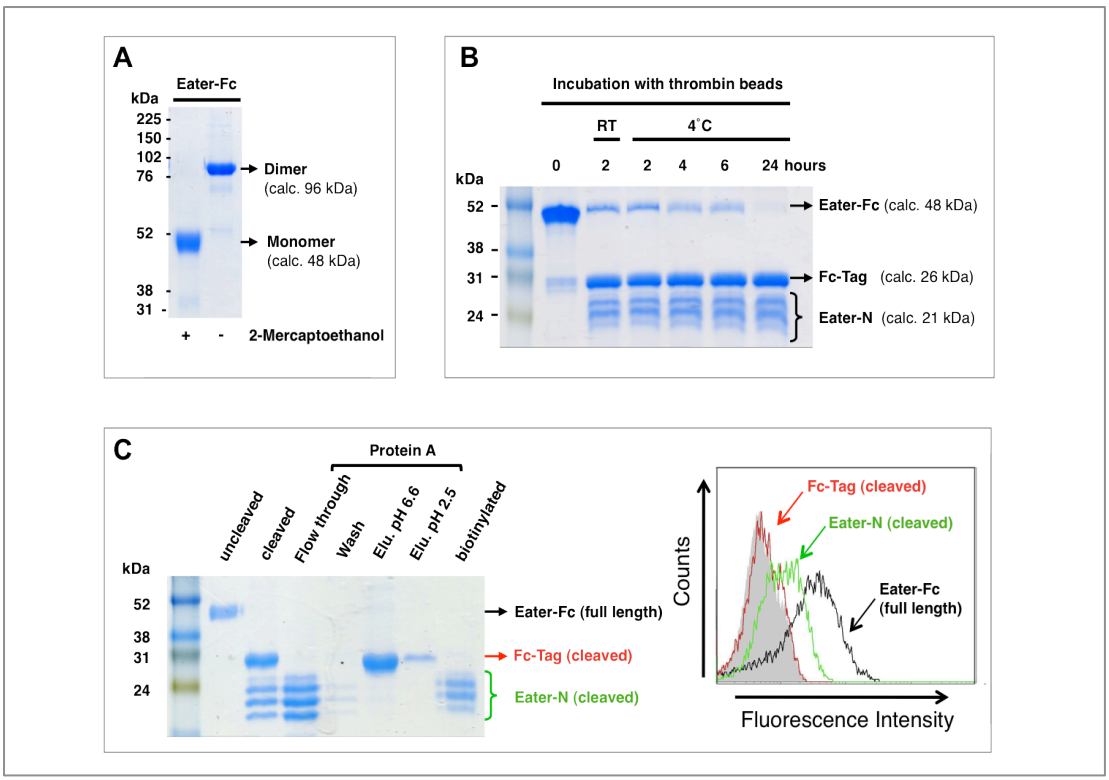


Figure 11. Characterization of intact and thrombin cleaved Eater-Fc. (A-C) Coomassie stained SDS gels. (A) Eater-Fc fusion proteins migrate in non-reducing SDS-PAGE as high molecular weight forms corresponding to dimers. **(B)** Time course of thrombin cleavage of Eater-Fc fusion protein. Note that the cleaved Eater N-terminal fragment migrated in multiple bands consistent with previous published results (Fig. 3 A in Kocks et al., 2005). **(C)** Thrombin cleavage of Eater-Fc and flow cytometry analysis of bacterial binding of thrombin cleavage products: N-terminal fragment (green curve), C-terminal fragment (red curve); full length protein Eater-Fc (black curve), all 200 µM. Heat-inactivated *S. marcescens* was used as bacteria.

Removal of the Fc-Tag after thrombin cleavage yielded an Eater fragment that migrated in multiple bands consistent with, or slightly larger than the calculated MW of 21 kDa (Fig. 11 B). This migration behavior may be due to N-glycosylation and is in agreement with the behavior of histidin-tagged Eater1-199 expressed from S2 cells (Kocks et al. 2005). After separation of the thrombin cleaved N-terminal Eater fragment from the C-terminal Fc domain by Protein A affinity chromatography (Fig. 11 C) and biotinylation of each of the two fragments, I observed either no (Fc-domain) or only weak bacterial binding (Eater-N terminal fragment) (Fig. 11 C) compared to the full-length protein.

Unexpectedly, I found that bacterial binding by Eater-Fc protein was only partially conformation dependent. Eater-Fc was heat-denatured at 80˚C, 90˚C or 100˚C for 5 min in the presence or absence of 0.5 % BSA, and either no or weak loss of bacterial binding was observed (data not shown), possibly due to a stability enhancing effect by the Fc-Tag.

3.1.4.3. Eater-Fc binding activity is dependent on glycosylation

To test whether N-glycosylation is important for the binding activity of Eater-Fc, biotinylated Eater-Fc was deglycosylated under non-denaturing conditions with PNGase F, an enzyme that removes N-glycan chains. Successful deglycosylation was evident as a size-shift of full-length or thrombin cleaved Eater-Fc towards lower molecular weight on reducing SDS gels (Fig. 12 A). To determine if deglycosylation is necessary for Eater-Fc binding to microbes, I performed flow cytometric bacterial binding assays with bioytinylated Eater-Fc fusion protein, either mock treated (black curve) or deglycosylated (red curve). To control that both proteins were successfully biotinylated, I showed binding to protein A-conjugated microspheres (Fig. 12 B). Mock treated Eater-Fc bound to heat-inactivated *E. coli* or *P. aeruginosa*, while deglycosylated Eater-Fc fusion protein did not show any binding to either bacteria (Fig. 12 C). This result indicates that bacterial binding by Eater-Fc is dependent on N-glycosylation. A similar dependency on N-glycosylation was observed with histidin-tagged Eater1-199 (J. Cho and C. Kocks, unpublished data).

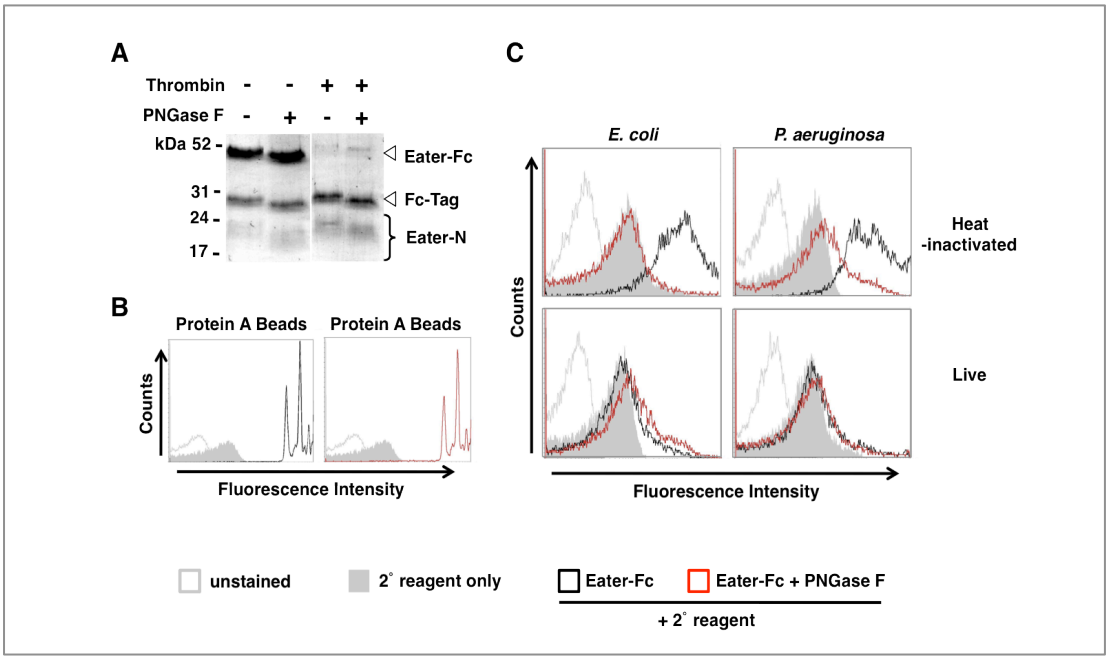


Figure 12. Eater-Fc binding to bacteria is dependent on glycosylation. (A) Coomassie Blue stained SDS gel (15 %). Biotinylated Eater-Fc was deglycosylated with PNGase F under non-denaturing conditions or mock-treated, and thrombin-cleaved where indicated. Deglycosylation is evident as a size shift of full length or thrombin-cleaved Eater-Fc towards lower molecular weights (compared to mock-treated control). **(B & C)** Flow cytometry analysis of binding by 200 µM biotinylated Eater-Fc fusion protein, either mock-treated (black curve) or deglycosylated with PNGase F (red curve). Deglycosylated, PNGase F-treated Eater-Fc showed undiminished binding to Protein A-conjugated microspheres **(B)** In contrast to mock-treated Eater-Fc, deglycosylated, PNGase F-treated Eater-Fc had lost its binding activity to the heat-inactivated Gram-negative bacteria *E. coli* or *P. aeruginosa*. No significant binding was detected to live bacteria Gram-negative bacteria **(C)**.

Taken together, these results indicate that Eater-Fc has some unique properties when compared to histidin-tagged Eater1-199 (dimerization, partial loss of binding activity in thrombin-cleaved N-terminal fragment, partial refractoriness to heat-denaturation), but shares other characteristics such as N-glycosylation in the N-terminal part.

3.1.5. Discussion and Conclusions

Taken together, these data show that Eater-Fc protein is biologically active and recapitulates the known binding behavior of the native Eater receptor, and of a previously described histidin-tagged N-terminally truncated ectodomain (Eater1-199His) expressed from S2 cells (Kocks et al. 2005). Since the latter could only be purified in small amounts (tens of μg), Baculovirus-expressed Eater-Fc represents a significant advance and a useful tool to study the interaction of Eater with pathogenic microbes. Eater binding covered a broad range of killed bacteria including Gram-negative Proteobacteria as well as Gram-positive *Firmicutes*, but did not extend to the Gram-positive Actinobacterium *M. luteus* and the fungal pathogen *C. albicans.* These results are also consistent with the recent finding that Eater mediates phagocytosis of *E. faecalis* and *S. aureus* but not *M. luteus* by fly hemocytes and S2 cells (Nehme et al., 2007). In addition, I confirmed previous results from our lab that the glycosylation of Eater is necessary for the binding activity of the protein (J. Cho & C. Kocks, unpublished).

Binding inhibition experiments with poly-I and poly-C confirmed previous results (J. Cho & C. Kocks, unpublished) and are consistent with the idea that Eater binds to certain polyanionic molecules but not others – reminiscent of the multiligand specificity of scavenger receptors (Greaves & Gordon 2009). My results with oxidized and non-modified LDL were harder to interpret, perhaps due to poor reagent quality. More experiments are needed to draw any conclusions.

3.2. Accessibilty of Eater Ligands on Different Classes of Live, Naïve Bacteria

Aim: The aim of this project part was to investigate whether Eater recognizes live bacteria.

Rationale: To date, most experiments that assessed Eater binding to microbes were carried out with bacteria that had been heat-inactivated or killed by membrane disrupting chemical treatment. To investigate Eater-Fc binding to live bacteria, I used a direct bacterial binding assay in conjunction with naïve, untreated, live bacteria. My results suggest that Eater-Fc can bind to live Gram-positive but not to live Gram-negative bacteria. These results were confirmed using S2 cells which express native membrane-bound Eater receptor on their surface, indicating that recognition of live bacteria by Eater is more complex than anticipated previously based on binding assays with dead bacterial particles. In case of Gram-negative bacteria, my results suggest that Eater ligands are not accessible on the surface of live bacteria.

3.2.1. Eater-Fc binding to live Gram-positive *Firmicutes (E. faecalis, S. aureus)*

Eater is critical for *Drosophila* survival of infections with several Gram-positive bacteria (Defaye et al 2009; Charroux and Royet, 2009; Nehme et al. 2011). To address the longstanding question whether Eater can bind to the surface of live, naïve bacteria, I probed Eater-Fc binding to live bacteria. Heat-, ethanol- or formaldehyde-inactivated bacteria served as controls. Figure 13 shows that Eater-Fc bound well to live or formaldehyde-inactivated *E. faecalis* and *S. aureus* (Fig. 13, first and second rows). Control IgG_1 and IgG-Fc did not show significant binding, although elevated background staining was observed with *S. aureus*. In contrast to *S. aureus* and *E. faecalis*, *M. luteus* was not recognized by Eater-Fc in any condition (Fig. 13, third row). These results suggest that Eater ligands on the surface of Gram-positive *Firmicutes* are always exposed and accessible for Eater binding, irrespective of the pre-treatment of the bacteria. By contrast, it appears that Actinobacteria such as *M. luteus* do not harbor any ligands for Eater.

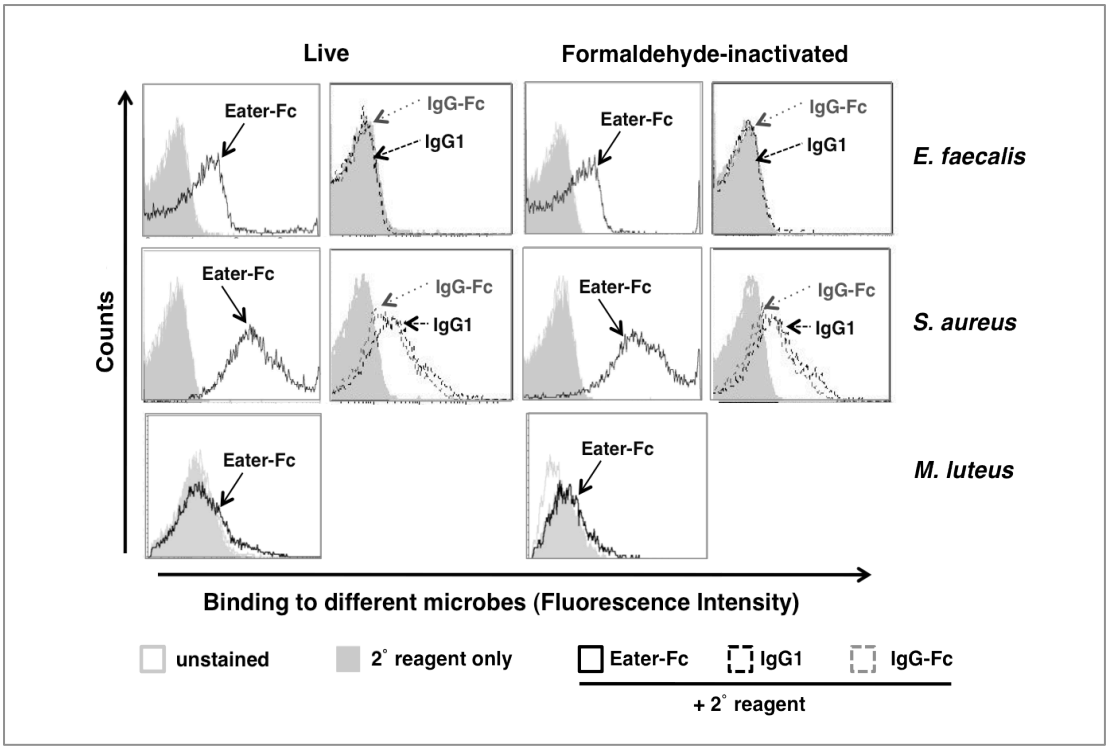


Figure 13. Eater-Fc binds to live, naive Gram-positive *Firmicutes*. Flow cytometry analysis of binding by 200 µM biotinylated Eater-Fc fusion protein (open black curve) or control biotinylated IgG1 and IgG-Fc (broken black or broken gray curves, respectively) compared to secondary reagent only (gray filled curve) or unstained microbes (open gray curve). Upper two rows: Eater-Fc bound to live, as well as to formaldehyde-inactivated, *E. faecalis* and *S. aureus* (Phylum *Firmicutes*). Third row: Eater-Fc did not bind to *M. luteus* in any condition (Phylum Actinobacteria). These experiments were repeated two times with similar results.

3.2.2. Absence of Eater-Fc binding to live Gram-negative Proteobacteria *(E. coli, S. marcescens, P. aeruginosa)*

Eater also plays a critical role in the host defense against invasive Gram-negative pathogens such as *S. marcescens* (Kocks et al. 2005) and *P. aeruginosa* (Limmer et al., submitted). Surprisingly, I could not detect any Eater-Fc binding to naïve or formaldehyde-inactivated *E. coli*, *S. marcescens*, or *P. aeruginosa* (Fig. 14; left and middle panels). By contrast, the controls, heat- or ethanol-inactivated bacteria, bound well to Eater-Fc (Fig. 14; right panels). These results suggest Eater ligands on the surface of intact, naïve, live Gram-negative bacteria are buried or otherwise masked.

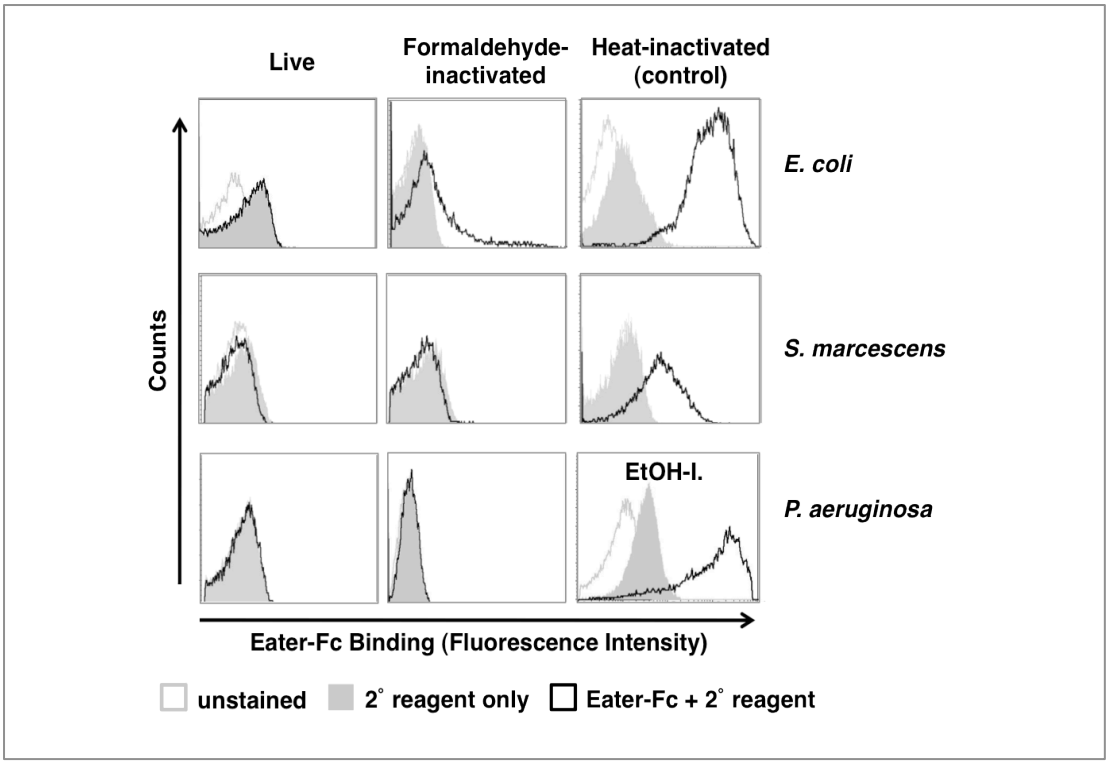


Figure 14. Eater-Fc does not bind to live and formaldehyde-inactivated Gram-negative bacteria. Flow cytometry analysis of binding by 200 µM biotinylated Eater-Fc fusion protein (open black curve) compared to secondary reagent only (gray filled curve) or unstained microbes (open gray curve). Eater-Fc did not bind to live or formaldehyde-inactivated Proteobacteria (left and middle panels). Right panels: Control binding to heat- or ethanol-inactivated proteobacteria. These experiments were repeated two times with similar results. EtOH-I., ethanol-inactivated.

3.2.3. Membrane-bound native Eater receptor on the surface of S2 cells behaves similarly to Eater-Fc

In order to find out whether the native, full length membrane-bound Eater receptor behaves similarly to Eater-Fc, I used *Drosophila* S2 cells which express high levels of Eater (Kocks et al. 2005).

Although there is considerable indirect evidence that Eater is expressed on the surface of S2 cells, this was never shown directly owing to the lack of a suitable antibody against Eater. I raised rabbit antibodies against Eater-Fc and used them for surface staining of S2 cells. Figure 15 A (left and middle panels) shows that rabbit anti-Eater antibodies detect an antigen on S2 cells that was strongly diminshed after RNAi knock-down of Eater. In western blots of S2 cell extracts, the antibodies detected a band corresponding roughly to the predicted MW of native Eater (128 kDa). This band disappeared after RNAi-knockdown of Eater (Fig. 15 A left panel).

Together these results suggest that the native Eater receptor is indeed expressed on the cell surface, as would be expected for a phagocytic scavenger receptor.

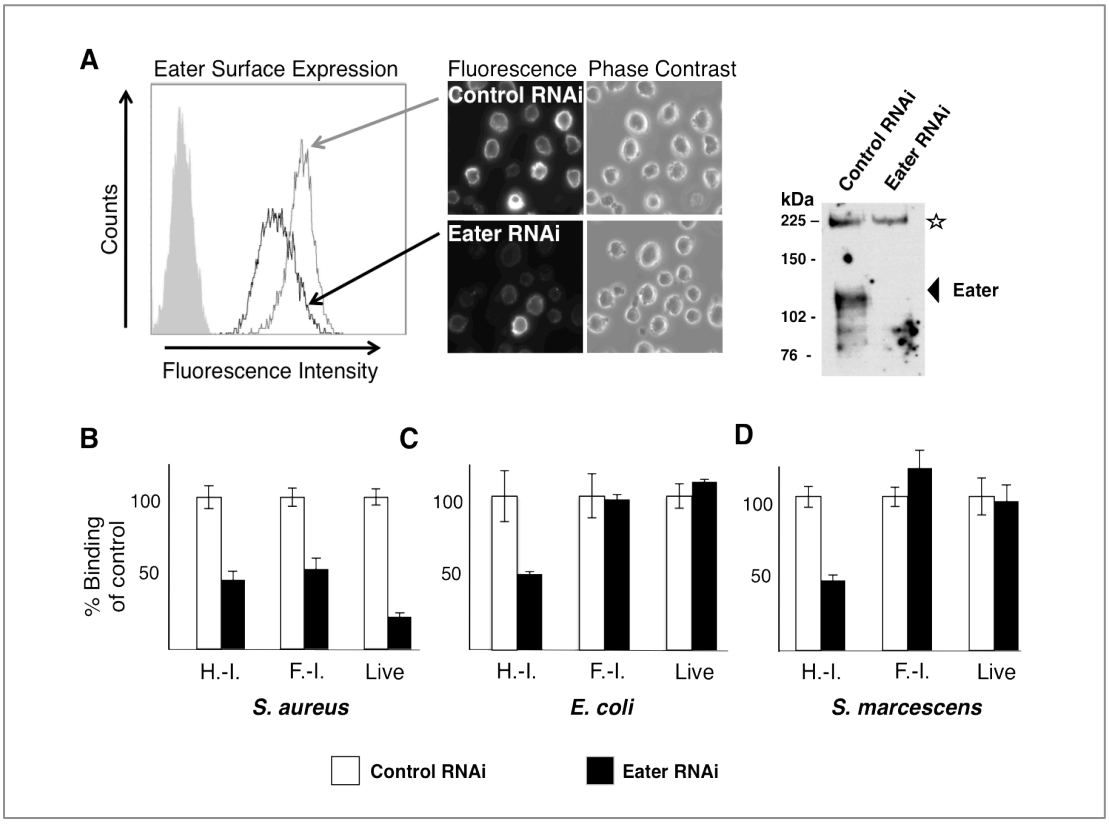


Figure 15. Membrane-bound, native Eater receptor behaves similarly to Eater-Fc. (A) Specific cell surface staining of Eater on S2 cells. Polyclonal rabbit anti-Eater-Fc antibodies reacted with S2 cells by cell surface staining of live S2 cells (left panel: histogram of flow cytometry analysis; filled gray curve: secondary reagent only) or formaldehyde-fixed (non-permeabilized) S2 cells (central panels: microscopy, 40x magnification). Cell surface staining strongly diminished after Eater RNAi knockdown. In a Western blot of S2 cell lysate, anti-Eater-Fc antibodies recognized a specific band consistent with Eater's predicted molecular mass (128 kDa; arrow) that disappeared after Eater RNAi knock-down (upper right panel). Star marks a non-specific band serving as loading control. **(B-D)** Binding of bacteria to S2 cells, normalized to dsRNA-treated controls. **(B)** Binding of S2 cells to heat- or formaldehyde-inactivated and live *S. aureus* is partially Eater-dependent, since the signal decreased after RNAi knock-down of Eater. **(C, D)** Binding of S2 cells to heat-inactivated Proteobacteria (*E. coli, S. marcescens*) was partially Eater-dependent (signal decrease after Eater-specific RNAi), while binding to live proteobacteria was not Eater-dependent (no change in signal after Eater-specific RNAi). H.-I., heat-inactivated; F.-I., formaldehyde-inactivated. All experiments were repeated with similar results.

I made use of S2 cells in conjunction with Eater-specific RNAi to probe the binding behavior of the native Eater receptor towards different classes of live and killed bacteria. *S. aureus* was used as a representative of the Gram-positive *Firmicutes*. *eater* RNAi lead to a significant decrease of *S. aureus* binding by S2 cells irrespective of whether the bacteria were heat-, or formaldehyde-inactivated or alive (Fig. 15 B). The remaining S2 cell binding activity to *S. aureus* is likely due to a combination of

incomplete *eater* knock-down and binding by other phagocytosis receptors (see also Kocks et al. 2005). These results confirmed that Eater is a major phagocytosis receptor for *S. aureus* on S2 cells (Kocks et al. 2005), and that Eater ligands seem to be always accessible, irrespective of the pre-treatment of the bacteria, thus validating the observations obtained earlier with Eater-Fc fusion protein.

Analysis of Eater-dependent S2 cell binding to Gram-negative bacteria was also in good agreement with the binding behavior of Eater-Fc: As shown previously (Kocks et al. 2005), binding of heat-inactivated Gram-negative bacteria was to a large extent Eater-dependent (Fig. 15 C, D). In contrast to this, binding of live and formaldehyde-inactivated Gram-negative bacteria to S2 cells was not dependent on native Eater (Fig. 15 C, D). Taken together, these results suggest that the binding behavior of Eater-Fc towards live and killed bacteria reflects the binding behavior of the native, full length, membrane-bound Eater receptor.

3.2.4. Discussion and Conclusions

The generation of anti-Eater antibodies enabled the direct demonstration of the native Eater receptor on the surface of the hemocyte-derived cell line S2 - as would be predicted for a phagocytic receptor. This newly generated anti-Eater antibody may be a useful tool for the identification of Eater interacting proteins to address the question whether the Eater molecule is part of a larger, multi protein receptor complex.

Membrane-bound native Eater receptor on the surface of S2 cells and Eater-Fc, both failed to bind to live Gram-negative bacteria, whereas they could react with live Gram-positive bacteria. Thus, bacterial binding assays with S2 cells confirmed that the binding behavior of Eater-Fc closely mimics the binding properties of the native, full length, membrane-bound receptor.

Previous findings implicated Eater in the direct binding and clearance of Gram-positive *Firmicutes in vivo* (Defaye et al. 2009; Charroux & Royet 2009; Nehme et al. 2011). Consistent with this, the finding that Eater-Fc is able to bind to live *S. aureus* and *E. faecalis* provides a simple, straightforward explanation for the *in vivo* protective role of Eater against *S. aureus* and *E. faecalis* infections (Defaye et al. 2009; Charroux & Royet 2009; Nehme et al. 2011). It suggests that Eater may directly

target naïve Gram-positive *Firmicutes* in the host leading to their phagocytic clearance and destruction, and to the effective control of bacterial loads.

By contrast, Eater-Fc showed no binding to live or formaldehyde-inactivated Gram-negative bacteria suggesting that Eater ligands are buried beneath the surface of live and formaldehyde-fixed Gram-negative bacteria, or in other ways masked. Membrane-disrupting treatments such as heat- or ethanol-inactivation lead to unmasking of normally inaccessible ligands. These results raise the intriguing question of how Eater ligands may become accessible *in vivo* in the host during an infection.

3.3. Unmasking of Eater Ligands on Gram-negative Bacteria by Cationic AMPs

Aim: The aim of this project part was to test whether antimicrobial peptides can promote Eater binding to live Gram-negative bacteria.

Rationale: I found that Eater was unable to interact with the surface of naïve, live or formaldehyde-fixed Gram-negative bacteria, suggesting that Eater ligands are somehow masked or buried in the intact bacteria. How could exposure of Eater ligands happen *in vivo*? A strong antibacterial peptide response, systemically as well as locally in various epithelia, is a hallmark of *Drosophila* immunity (Dionne & Schneider 2008). This raises the possibility that AMPs might play a role in exposing Eater ligands, leading to recognition and destruction of Gram-negative bacteria by Eater dependent phagocytosis.

3.3.1. Eater-Fc binds to *E. coli* killed by exposure to cationic AMP

Since AMPs are well known to destabilize bacterial membranes (Hancock 1984; Boman & Hultmark 1987; Bulet et al. 2004), I was interested to determine whether AMPs might be able to unmask Eater ligands.

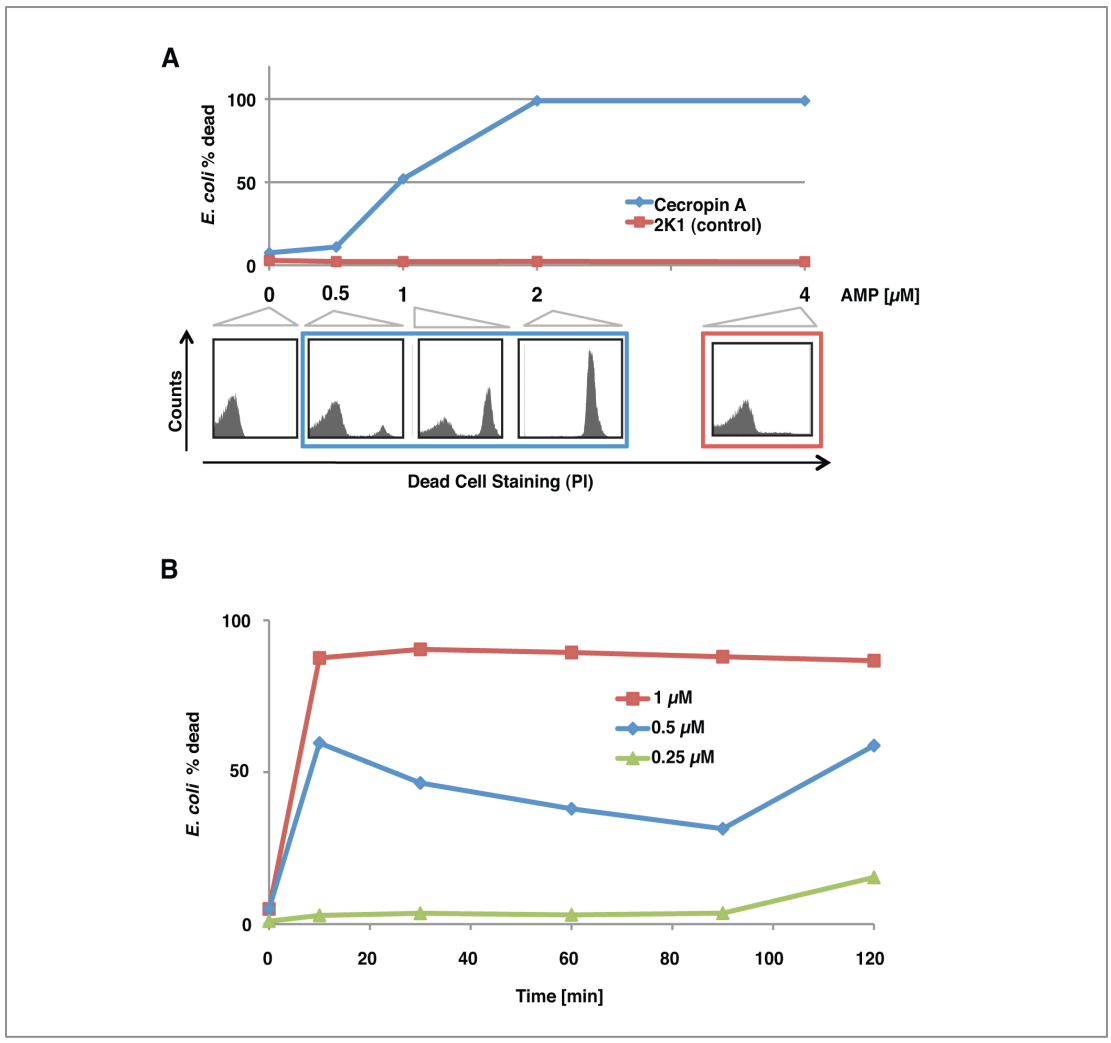


Figure 16. Concentration and time-dependent killing of *E. coli* by cationic AMP. Live *E. coli* were incubated in the presence of cecropin A at 25˚C, transferred briefly to ice and analysed immediately by flow cytometry in the presence of the viability stain PI. **(A)** Dose dependent killing of *E. coli* by cecropin A (0 to 4 µM; blue curve) compared to cationic control peptide 2K1 (red curve). Histograms corresponding to increasing amounts of cecropin A (0.5 to 2 µM; blue boxes) or 4 µM cationic control peptide 2K1 (boxed in red) are shown below the line graph. These experiments were repeated three times with similar results. **(B)** Time and concentration-dependency of *E. coli* killing by cecropin A. Time dependent killing kinetics for three different concentrations of cecropin A: 1 µM (red), 0.5 µM (blue) and 0.25 µM (green). At 1 and 0.5 µM of cecropin A bacterial killing was rapid. Slower killing kinetics were observed with lower amounts of cecropin A (0.25 µM). The apparent viability decrease and increase in the later time points in the blue curve likely reflects experimental variability.

I first killed *E. coli* with cecropin A, a prototypic membrane-perturbing cationic peptide conserved from invertebrates to humans (Boman & Hultmark 1987; Bulet et al. 2004). Bacterial killing by cecropin A was concentration-dependent and rapid, while the cationic control peptide 2K1 (Fantner et al. 2010) had no effect (Fig. 16 A, B).

I then used cecropin A-killed *E. coli* to measure Eater binding by two-color flow cytometry analysis to simultaneously monitor Eater-binding and bacterial viability.

Figure 17 shows that Eater-Fc bound well to cecropin A-killed bacteria, whereas control IgG-Fc did not. Moreover, the control peptide 2K1 did not increase Eater-Fc binding beyond background. These results suggest that cationic AMPs might play a role *in vivo* in unmasking Eater ligands on the surface of Gram-negative bacteria.

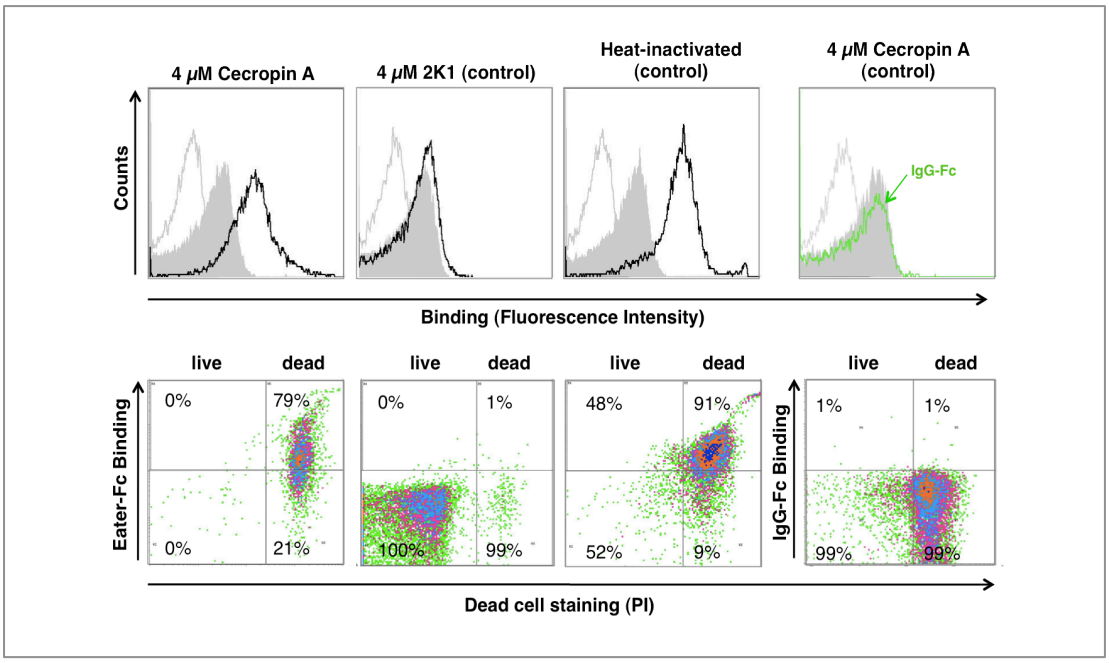


Figure 17. Eater-Fc binds to *E. coli* killed by a cationic AMP. (Upper panels) Histograms of flow cytometry analysis of binding of 200 µM Eater-Fc (open black curve) or IgG-Fc (green curve) to *E. coli* compared to secondary reagent only (gray filled curve) or unstained microbes (open gray curve). Eater-Fc bound to *E. coli* killed by exposure to 4 µM Cecropin A for 10 minutes at 25˚C, but not to *E. coli* treated with 4 µM control cationic peptide 2K1. Eater-Fc binding to control heat-inactivated *E. coli* are shown for comparison. (Lower panels) Dot plots of two-color analysis of the same samples as in the upper row, allowing assessment of Eater binding simultaneously with bacterial viability. % of Eater-Fc-binding to live or dead bacteria respectively are indicated. Experiments were repeated three times with similar results.

In order to visualize and confirm Eater-Fc binding to heat-inactivated and cecropin A exposed *E.* coli at the subcellular level, I performed pre-embedding immunogold labeling electron microscopy. Heat-inactivated, cecropin A-killed and live *E. coli* were incubated with pre-formed Eater-Fc:Streptavidin Alexa Fluor 488 conjugates, postfixed with glutaraldehyde and embedded. Fig. 18 A shows thin sections of heat-killed *E. coli* that reveal gold labeling at the bacterial cell envelope. The number of bacteria carrying gold labeling was 5-fold elevated as compared to secondary reagent alone. Furthermore, live, intact, naïve *E. coli* showed almost no Eater-Fc labeling (Fig. 18 B), while cecropin A-killed bacteria showed a 25-fold higher incidence of

Eater-Fc labeling at the cell envelope. These results support the conclusion that AMPs are able to expose Eater ligands, presumably by disrupting the bacterial cell envelope.

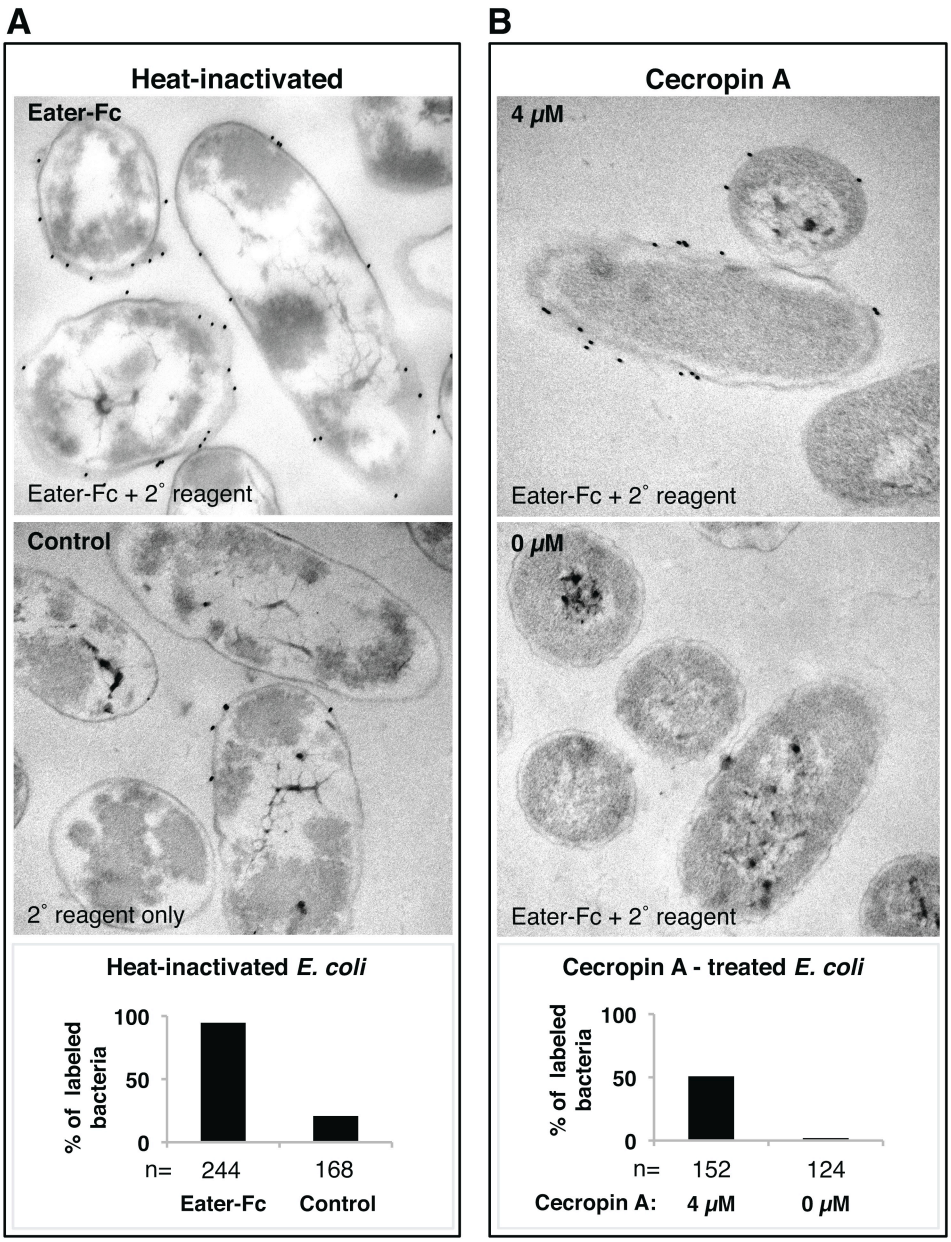


Figure 18. Pre-embedding gold labeling EM shows Eater-Fc binding to cell envelopes of *E. coli* after membrane disruption. *E. coli* was either heat-inactivated, killed with cecropin A or live, and then labeled with 500 µM pre-formed, biotinylated Eater-Fc-Streptavidin-gold conjugate before processing and thin sectioning for EM. **(A)** Heat-inactivated *E. coli* labeled with Eater-Fc (upper panel) or secondary reagent only (middle panel). **(B)** *E. coli* killed with cecropin A (4 µM) for 10 minutes at 25˚C (upper panel) or used live (0 µM cecropin A) (middle panel). **(A, B)** Magnification 60,000x. (Lower panels) Quantification of gold label associated with bacteria. "n" indicates number of bacteria analyzed.

3.3.2. Eater-Fc binds to live, AMP exposed *E. coli*

The finding that cecropin A-treatment was sufficient to expose Eater ligands on killed bacteria raised the possibility that it might also be sufficient under sublethal concentrations, which may more closely mimic the *in vivo* situation and presumably is predominant in the tissues of a living host (Bomann and Hultmark 1987; Bulet et al. 2004). I tested this hypothesis by incubating *E. coli* with lower concentrations of cationic AMP. Figure 19 A shows a subpopulation of live *E. coli* that became accessible to Eater-Fc after exposure to increasing concentrations of cecropin A (1 to 4 µM).

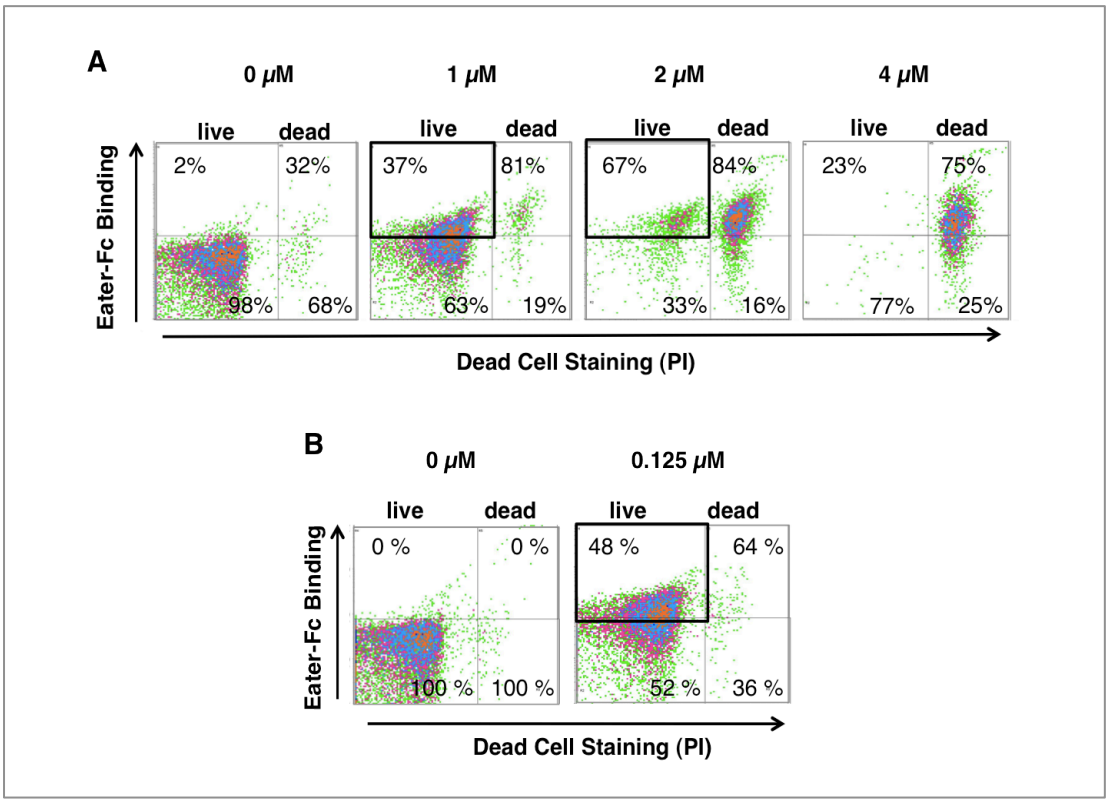


Figure 19. Eater-Fc binds to live, AMP-exposed *E. coli*. Dot plots of two color flow cytometry analysis of live *E. coli* exposed at 25˚C (A) to increasing concentrations of cecropin A (0 to 4 µM) for 10 minutes, or **(B)** to 0.125 µM cecropin A for 2 hours. % of Eater-Fc-binding to live or dead bacteria respectively are indicated. Bold rectangles highlight Eater-Fc binding to bacteria that were exposed to cecropin A at sublethal concentrations. The boxed bacteria were alive because they excluded PI; this was confirmed by bacterial CFU counts in one repeat experiment. Experiments were repeated twice with similar results.

Since bacterial killing by cecropin A under these conditions was very rapid (less than 10 minutes), I confirmed this result by performing a prolonged incubation course (over 2 hours) at a lower concentration of cecropin A (0.125 µM; Fig. 19 B). CFU counts of the AMP-treated sample were indistinguishable from control, and

independently confirmed the viability of the bacteria (8 x 10^4 CFUs in 25 µl; one experiment).

3.3.3. Discussion and Conclusions

Taken together, my results suggest a novel, previously unrecognized role for AMPs. They indicate that cationic AMPs may be able to alter and 'prime' the surface of Gram-negative bacteria in a way that leads to unmasking of previously inaccessible phagocytic receptor ligands.

The bacterial cell envelope is a highly dynamic organelle that undergoes extensive changes *in vivo* in response to its host environment (Li et al. 2007; Nehme et al., 2007b; Peschel & Sahl 2006; Silhavy et al. 2010; Weidenmaier & Peschel 2008; West et al. 2005). Therefore, and because of the natural ionic composition of tissue fluids, the exact conditions by which innate immune molecules interact with their targets are hard to reproduce in the laboratory (Boman & Hultmark 1987; Finlay & Hancock 2004). In my hands it was evident that in the presence of physiological concentrations of divalent cations (1.2 mM $MgSO_4$, 1 mM $MgCl_2$, 1 mM $CaCl_2$), cecropin A lost it's bactericidal activity towards *E. coli* (data not shown). Even at concentrations of up to 10 µM cecropin A, *E. coli* remained alive showing that *in vivo* the AMP's may not be as potent as under artificial conditions *in vitro*.

Even so, I was able to demonstrate that pre-treatment of live *E. coli* with the cationic antimicrobial peptide cecropin A was a way to unmask and expose hidden Eater ligands (Fig. 17-19). By making Eater ligands available for phagocytosis receptors AMPs may 'prime' bacteria for uptake. It is therefore reasonable to speculate that 'priming' of the bacterial envelope by AMPs for subsequent recognition by Eater may be an important mechanism *in vivo*. AMP concentrations *in vivo* may in many circumstances not be sufficient to efficiently kill bacteria, not least in light of the fact that most Gram-negative bacteria would be expected to be more resistant to AMPs than the laboratory *E. coli* strain that I used in my experiments. It is unlikely that cecropin A acts as an opsonin that bridges the bacterial surface and Eater, since a cationic control peptide that is expected to bind to the bacterial surface via its positive

charges (Boman & Hultmark 1987; Fantner et al. 2010) did not have any effect (Fig. 16 & 17).

Atomic force microscopy has emerged as a powerful tool for direct, non-invasive imaging of the living bacterial surface (Dupres et al. 2010). A recent study measured cationic AMP activity on individual, live, naïve *E. coli* cells (Fantner et al. 2010). Surface corrugation caused by AMP activity correlated with killing kinetics in a two-stage process exhibiting a long lag phase followed by a short "execution" phase. These findings are compatible with a previously unrecognized role for cationic AMPs in non-opsonic phagocytosis: making inaccessible ligands available for phagocytic receptors.

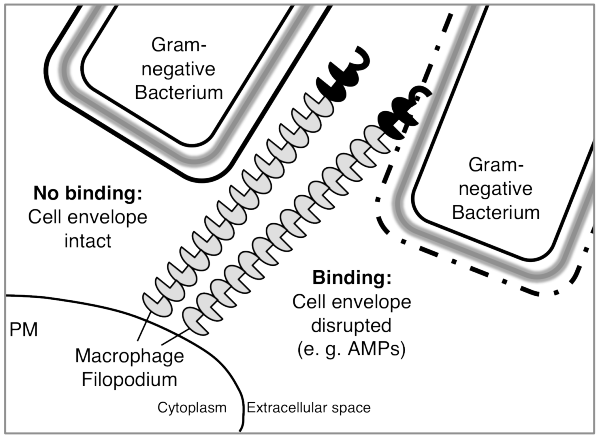


Figure 20. Proposed model for non-opsonic phagocytosis of Gram-negative bacteria by the pattern recognition receptor Eater. (Left) Eater ligands on cell walls of naïve (live) Gram-negative bacteria are masked. (Right) Cationic AMPs destabilize the bacterial outer membrane, disrupt the cell envelope and lead to exposure of Eater ligands which renders the bacteria accessible for Eater binding and non-opsonic phagocytosis. Note that bacteria are not drawn to scale here and in reality are larger relative to the size of the Eater protein.

I propose a model by which AMPs under sub lethal conditions might contribute to the clearance of live Gram-negative bacteria *in vivo* by perturbing the bacterial surface and making previously inaccessible ligands available for non-opsonic phagocytosis (Fig. 20) leading to more efficient clearance and destruction of invasive bacteria. This scenario is supported by an oral-intestinal infection model in which local overexpression of the AMP Diptericin in *Drosophila* midgut epithelium conferred increased protection to invasive *S. marcescens* (Nehme et al. 2007). One interpretation of these data is that local AMP responses contribute to increased host

resistance by 'priming' bacteria for subsequent Eater-mediated phagocytosis when bacteria manage to cross the gut epithelium.

It remains unclear at present what the mechanistic basis for the opening up of the Gram-negative cell wall by cationic AMPs may be (Hale & Hancock 2007; Hancock & Scott 2000). For *P. aeruginosa* it was shown that cationic AMPs can displace divalent cations from non-covalent LPS cross-bridges leading to destabilization and permeabilization of the outer membrane, allowing access of hydrophobic probes or lysozyme (Hale & Hancock 2007; Hancock 1984; Sawyer et al. 1988). This modification of the bacterial surface manifests in membrane blebs observable by electron microscopy (Sawyer et al. 1988). The periplasm (the space between outer and inner membranes of *E. coli*) is a potentially harmful and highly regulated environment akin to the lysosomes of eukaryotic cells (Silhavy et al. 2010). One might speculate that the destructive power of bacterial cell wall remodeling enzymes or lipases could be unleashed upon disruption of outer membrane homeostasis somehow leading to exposure of normally hidden PGN or PGN-bound molecules (Peschel & Sahl 2006). It is noteworthy that the outer membrane modifications induced by cationic AMP did enhance the non-opsonic phagocytosis of *P. aeruginosa* by mammalian macrophages (Sawyer et al. 1988), as would be predicted by a scenario such as proposed in the model in figure 20.

The finding that an AMP can promote the exposure of ligands for a phagocytic receptor may have some broader implications: It may point to a more general mechanism by which AMPs could cooperate with phagocytic pattern recognition receptors and thereby enlarge the spectrum of microbes that can be recognized by a single germ-line-encoded receptor. This may be important *in vivo*, since the efficiency of non-opsonic phagocytosis, especially locally in uninflamed tissues such as lung, is an important determinant for prevention of infection through early clearance of bacteria (Rabinovitch 1995; Speert 1993) .

AMPs may not be unique in their ability to make previously hidden bacterial ligands accessible, and may act synergistically with other defense molecules (Boman & Hultmark 1987; Hale & Hancock 2007; Ganz 2003). For an innate immune system, the advantages of extending the microbial ligand repertoire are clear, given 'the need

for thrifty use of a limited set of germ-line encoded receptors' (Beutler 2003). My findings add a further dimension to this theme: compartmentalization and accessibility of microbial ligands – an emerging topic of increasing importance in the cell biology of innate immune processes in general (Barton & Kagan 2009).

3.4. Eater-Fc Binding to Bacterial Cell Wall Components

Aim: The goal of this project part was to measure the interaction of Eater-Fc with microbial cell wall components.

Rationale: It remains unclear what the bacterial ligands are that Eater recognizes. To address this issue, I used various commercially available bacterial cell wall components in binding inhibition and co-sedimentation assays with Eater-Fc. My results suggest that certain peptidoglycans are ligands for Eater.

3.4.1. Differential Eater-N binding to different types of polymeric PGN

A nearly ubiquitous cell wall component of bacteria is the murein sacculus, made of PGN, a heteropolymer composed of long glycan chains cross-linked by short peptides (Fig. 21). It forms a mesh-like exoskeleton outside the plasma membrane of bacteria. On the surface of live Gram-positive bacteria PGN seems to be at least partially accessible (Steen 2003). However, in live Gram-negative bacteria PGN is much less abundant and hidden, since it is embedded in the cell envelope under an outer membrane containing LPS (Mengin-Lecreulx & Lemaitre 2005; Silhavy et al. 2010). Although PGN is strongly conserved among all bacteria, a major source of variation in PGNs are the peptide stems and crosslinking bridges between the glycan strands (Fig. 21 A) (Silhavy et al. 2010; Mengin-Lecreulx & Lemaitre 2005; Dziarski & Gupta 2005).

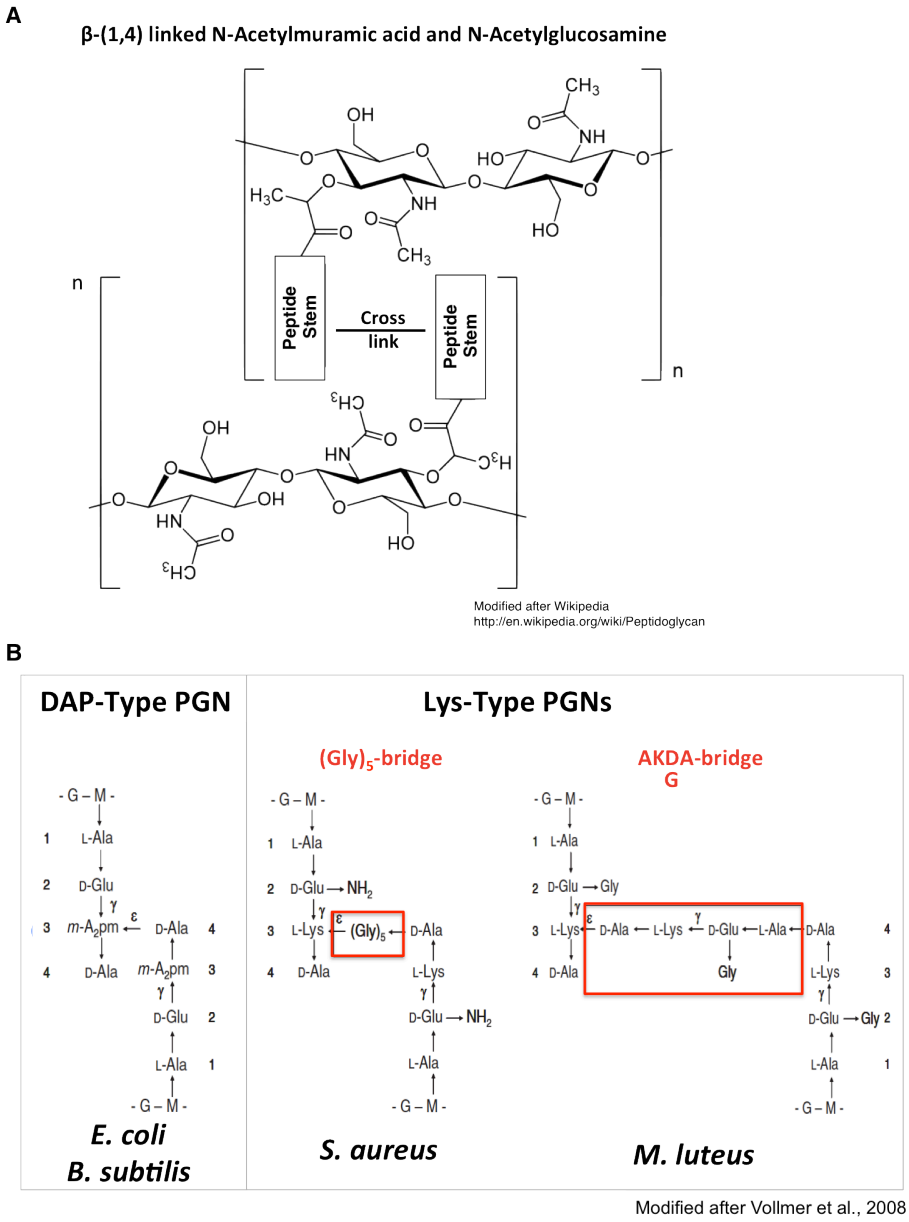


Figure 21. Schematic Depiction of Peptidoglycans. (A) Peptidoglycan (PGN) is formed from linear chains of alternating amino sugars, N-acetylglucosamine and N-acetylmuramic acid which are connected by a β-(1,4)-glycosidic bond. N-acetylmuramic acid is also attached to a short amino acid chain comprising of 3-5 amino acids, also called peptide stem. Crosslinking between peptide stems results in a strong and rigid 3 dimensional structure. **(B)** Structural differences between DAP-type (left panel) and Gram-positive Lys-Type PGNs (right panel). Gram-negative bacteria and Gram-positive bacteria such as *B. subtilis* use meso-DAP in the third position of the stem-peptide which directly crosslinks to D-Ala in position 4 of the opposing peptide stem (no peptide bridge). Lys-type Gram-positive bacteria have L-Lys in the third position of the peptide-stem and use a peptide bridge for crosslinking. The amino acids in the peptide bridge vary between phyla (highlighted in red boxes).

Bacilli and Gram-negative bacteria synthesize meso-diaminopimelic acid (DAP)-type PGN (with identical stem peptides and crosslinks), while *S. aureus* and *M. luteus* contain lysin (Lys)-type PGNs with (identical peptide stems and) different peptide bridges (Fig. 21 B) (Schleifer & Kandler 1972).

I tested whether polymeric PGN could be an Eater ligand that becomes accessible in *E. coli* after membrane perturbation. I cleaved Eater-Fc with thrombin to separate Eater N-terminal fragment from the Fc-tag and incubated the mixture with PGN. Co-sedimentation assays revealed that Eater N-terminal fragment bound to *E.coli, B. subtilis* and *S. aureus* PGN, but not to *M. luteus* PGN (Fig. 22, upper panel). The Fc-tag displayed partial binding to *S. aureus* PGN (possibly due to contamination with surface protein A), but no significant binding to the other PGNs.

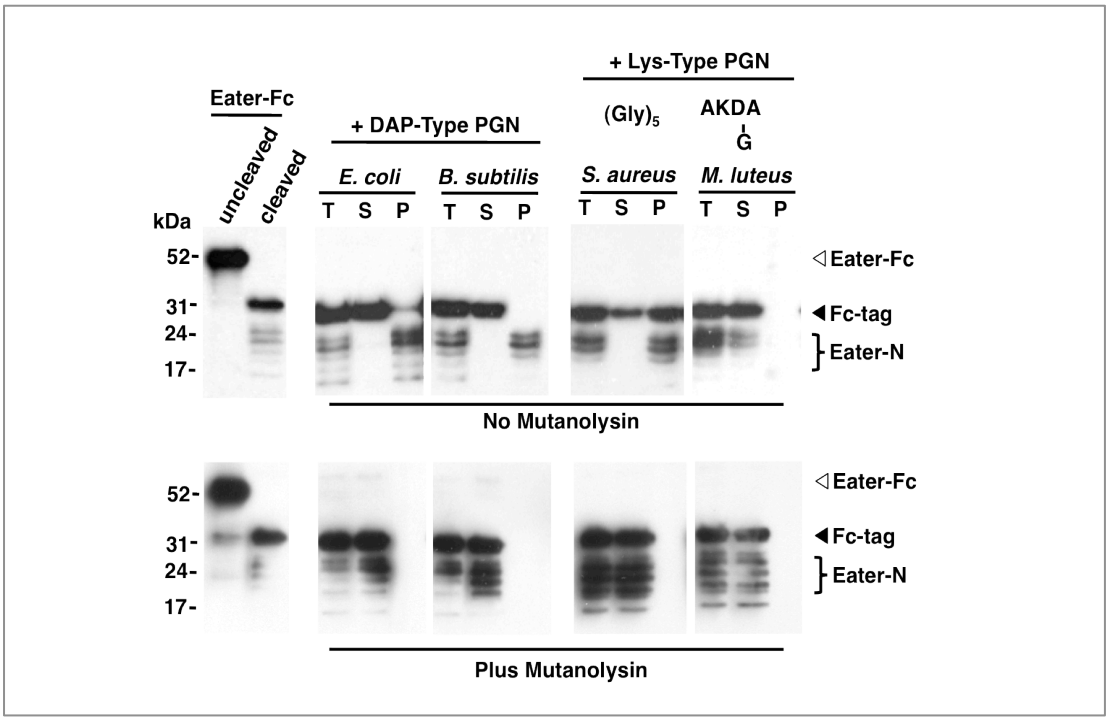


Figure 22. Eater-N displays differential binding to different types of peptidoglycan (PGN). PGN co-sedimentation assay. Thrombin-cleaved Eater-Fc was incubated with different types of insoluble, polymeric PGNs, sedimented by centrifugation and analysed by SDS-PAGE followed by immunoblot analysis using anti-Eater-Fc antibodies. T indicates total protein, S supernatant (unbound) and P pelleted (bound) protein fractions. Bands corresponding to intact Eater-Fc or thrombin-cleaved Fc-tag and N-terminal fragments are indicated. (Upper panel) Eater-N bound to *E. coli*, *B. subtilis* and *S. aureus* PGN, but much less to *M. luteus* PGN. Results are representative of 3 independent experiments. (Lower panel; control) Eater-N could not be detected in the pellet fractions after cleavage of PGNs with mutanolysin, a muramidase that specifically cleaves the glycan backbone of polymeric PGN.

The binding profile of Eater-N-terminal fragment towards PGN correlated well with Eater-Fc binding to the corresponding classes of heat-inactivated bacteria (Fig. 8 B, 9 A-C), and suggested that PGN might be a ligand of Eater. No co-sedimentation could be observed with Eater-Fc alone (Fig. 22, first 2 lanes) or when PGNs were digested with mutanolysin (Fig. 22, lower panel).

3.4.2. No significant binding of Eater-Fc to LPS and LTA

To assess whether Eater-Fc might be able to recognize other cell wall components, I performed binding inhibition studies with LPS and LTA two conserved macromolecules prevalent in Gram-negative, or Gram-positive bacteria, respectively.

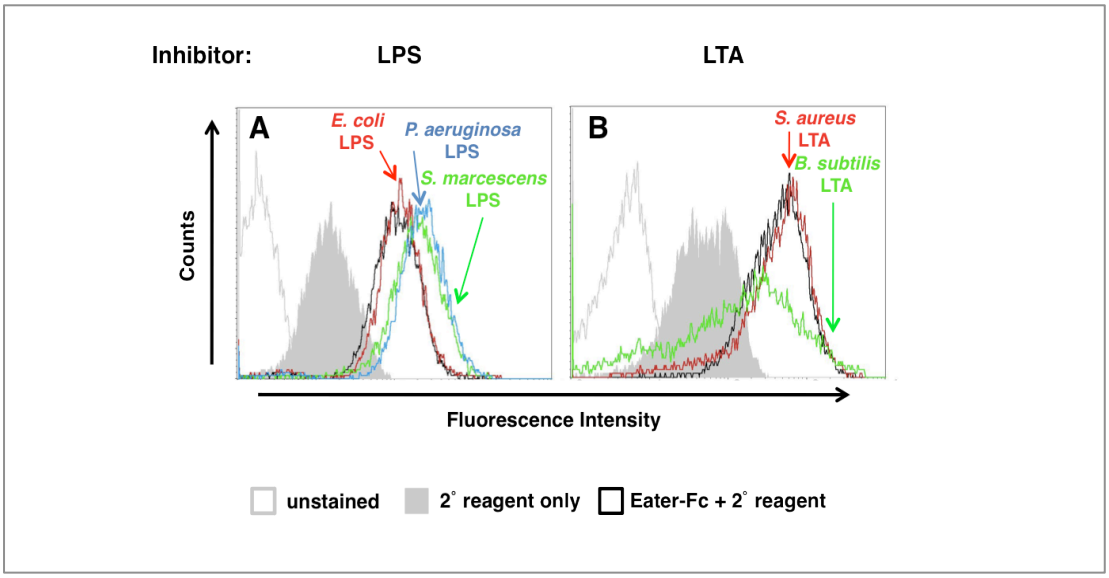


Figure 23. Lipopolysaccharide (LPS) and Lipoteichoic acid (LTA) show no significant inhibition of Eater-Fc binding to bacteria (heat-inactivated *S. marcescens*). (A & B) Flow cytometry analysis of binding by 200 µM Eater-Fc in the presence of 100 µg/ml inhibitor. (A) Binding by Eater-Fc is not inhibited by *E. coli* LPS (red curve), *S. marcescens* LPS (green curve) or *P. aeruginosa* LPS (blue curve). (B) Eater-Fc binding is weakly inhibited by B. subtilis LTA (green curve) but not by *S. aureus* LTA (red curve).

LPS or LTA from various bacteria were incubated with Eater-Fc prior to adding Eater-Fc to heat-inactivated *S. marcescens* and analysed in the flow cytometry-based Eater-Fc binding assay described above (see Fig. 8 A). As shown in Figure 23 A, at a concentration of 100 µg/ml of LPS showed no inhibitory effect on the binding of Eater-Fc to bacteria. At a very high concentration of 900 µg/ml or higher, inhibition could be observed, although the data were variable and ultimately inconclusive (data not shown). At mg/ml concentrations of the commercial preparations used, impurities are likely to confound data interpretation. Slight binding inhibition was observed with LTA from *B. subtilis* but not with LTA from *S. aureus* (Fig. 23 B).

These data suggest that LPS and LTA are not major ligands for Eater-Fc. In support of this interpretation, I found that Eater-Fc bound equally well to heat-inactivated

S. marcescens wild type (strain Db11) and the "rough" transposon insertion mutant 20C2 of *S. marcescens* which lacks LPS O-antigen (Fig. 24) (Kurz et al. 2003).

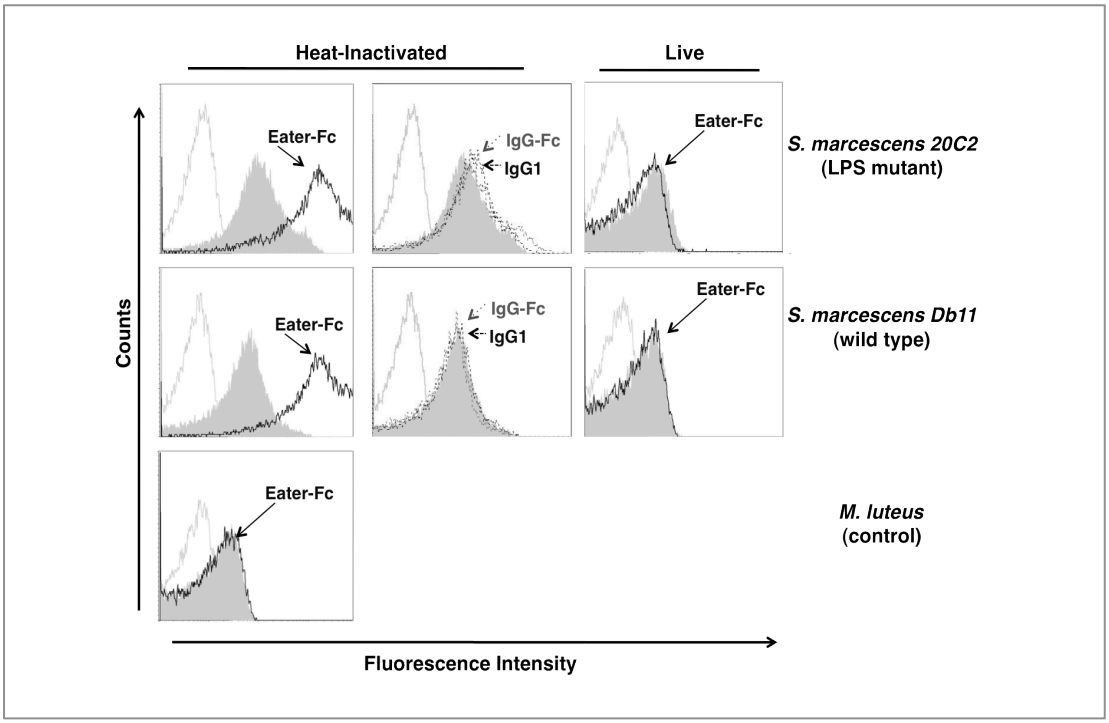


Figure 24. Eater-Fc binds to heat-inactivated, rough LPS mutant *S. marcescens.* Flow cytometry analysis of binding by 200 µM biotinylated Eater-Fc fusion protein (open black curve) or control biotinylated IgG1 and IgG-Fc (broken black or broken gray curves, respectively) compared to secondary reagent only (gray filled curve) or unstained microbes (open gray curve). Eater-Fc bound well to heat-inactivated, rough LPS mutant and wildtype *S. marcescens* , but not to heat-inactivated *M. luteus* (control). No binding was detected to live rough LPS mutant or wildtype *S. marcescens.*

3.4.5. Discussion and Conclusions

Previous, unpublished data with histidin-tagged Eater1-199 (expressed in S2 cells) suggested that Eater might recognize LPS and LTA, two lipid-anchored polyanionic molecules prevalent in the cell envelopes of Gram-negative and Gram-positive bacteria (clear inhibition by 100 µg/ml LPS; J. Cho and C. Kocks, unpublished data). However, this finding could not be confirmed with my Baculovirus expressed Eater-Fc fusion protein. By contrast, I found that Eater-Fc showed differential binding to polymeric PGN, a universal bacterial cell wall component.

The finding that Eater-Fc shows binding avidity to polymeric PGNs is consistent with

earlier characterization of Eater, as displaying a binding preference for polyanionic ligands (Kocks et al. 2005), reminiscent of scavenger receptors (Greaves & Gordon 2005) and LPS-binding protein (LBP) (Weber et al. 2003). It seems that, similar to Eater, several mammalian pattern recognition molecules can bind cell wall components of Gram-negative and Gram-positive bacteria: CD14, Toll-like receptor 2 (TLR2), peptidoglycan recognition proteins (PGRPs) and LBP can bind to LPS, lipoteichoic acid and polymeric PGN, in some cases with overlapping binding sites (Dziarski & Gupta 2005; Weber et al. 2003; Dziarski et al. 1998; Wang et al. 2005). A pattern of multiply iterated anionic charges was suggested to be the common denominator for all these ligands (Weber et al. 2003).

Eater's much higher avidity to DAP-type and *S. aureus* PGN compared to *M. luteus* PGN offers a tentative explanation for its inability to bind to the actinobacterium *M. luteus*, and suggests that recognition may be mediated in part by the nature of the petide stems and crosslinks in PGN. However, PGN preparations are often contaminated with other cell wall molecules, some of which are co-valently linked with PGN (Silhavy et al 2010) (Dziarski & Gupta 2005; Rosenthal & Dziarski 1994). It therefore remains possible that Eater binds to other microbial cell envelope molecules instead of, or in addition to, PGN. The molecular nature of these may be different for different classes of bacteria; moreover, a group of Eater molecules might use a combination of multiple targets. For example, Gram-positive cell envelopes contain cell wall glycopolymers (CWGs) such as LTAs that are attached to the peptidoglycan or to membrane lipids, which are known to be highly diverse and often species- and strain-specific (Weidenmaier and Peschel, 2008). This diversity in CWGs may explain the differences in the binding of Eater to *S. aureus* on the one hand and *M. luteus* on the other. *S. aureus* incorporates lipoteichoic acid as a membrane anchored CWG into its cell wall, whereas *M. luteus* uses lipomannan and teichuronic acid.

Several ligands have been identified for EGF-like repeat containing molecules that are related to Eater: LPS for the *Holotrichia* LPS recognition protein LRP (Ju et al. 2006), lipoteichoic acid for the *Drosophila/C. elegans* phagocytosis receptor Draper/CED-1 (Hashimoto et al. 2009), beta-glucan for the mammalian scavenger receptor of class F SCARF1/*C. elegans* CED-1 (Means et al. 2009), and bacterial

outer membrane protein OmpA for SCARF1 (Jeannin et al. 2005). Since Eater-Fc did not bind to naïve Gram-negative bacteria (Fig. 10 and Fig. 20), it seems less likely that Eater recognizes LPS O-antigen, or outer membrane proteins like the mammalian scavenger receptors SR-A and SCARF1, and the phagosomal microbial sensor SLAM (Jeannin et al. 2005; Areschoug & Gordon 2009; Berger et al. 2010; Peiser et al. 2006). Recent atomic force microscopy measurements even suggest that teichoic acids may obscure the access to PGN on the surface of naïve Gram-positive bacteria (Andre et al. 2008).

Sonicated PGN (i.e. non-polymeric, soluble) from *S. aureus, B. subtilis* and *M. luteus* was also used to test whether they would inhibit Eater-Fc binding to heat-inactivated *E. coli* (data not shown). These experiments remained inconclusive however, since I observed that the commercial PGN preparations used contained a proteolytic activity (likely residual trypsin used to remove proteins from crude cell walls in the PGN purification process (Rosenthal & Dziarski 1994)) that cleave and degrade Eater-Fc (data not shown). Further investigation and different approaches are clearly required to identify biologically relevant Eater ligands.

3.5. A Limited RNAi Screen to Search for Molecules Involved in Binding and Phagocytosis of the Gram-positive Actinobacterium *M. luteus*

Aim: The aim of this part was to carry out a limited RNAi screen of 39 candidate molecules to test their involvement in the binding and phagocytosis of *M. luteus*, a G+C-rich bacterium which belongs to the phylum Actinobacteria, a dominant and evolutionarily ancient group of bacteria comprising the soil bacteria *Streptomyces* and pathogens such as *Mycobacteria* and *Corynebacteria*.

Rationale: Multiple lines of evidence indicate that the Actinobacterium *M. luteus* gets phagocytosed by *Drosophila* hemocytes, but that Eater is not involved in this activity*:* 1) Macrophage-like *Drosophila* S2 cells display a *M. luteus* binding and phagocytosis activity that is not dependent on Eater (Fig. 25 B; Nehme et al. 2011). 2)

Phagocytosis of *M. luteus* is important in adult *Drosophila in vivo*, but is not mediated by Eater (Nehme et al. 2011). 3) My results consistently show that *M. luteus* is not recognized by Eater-Fc (Fig. 9, 13, 25), and that this may be due to an inability of Eater to react with PGN or PGN associated cell wall glycopolymers from Actinobacteria (Fig. 23). Taken together, these results point to other molecules or receptors that mediate phagocytosis of Actinobacteria in *Drosophila*. I set out to identify such molecules.

Figure 25 A illustrates the lack of Eater-Fc binding to *M. luteus*. In agreement with this finding, native Eater on the surface of S2 cells did not mediate binding of FITC-labeled heat-inactivated *M. luteus* to S2 cells, since RNAi knock down of Eater did not have any effect (Fig. 25 B). These data suggest that S2 cells have the ability to bind and phagocytose *M. luteus*, but that the binding and phagocytosis is not dependent on Eater. Therefore, other molecules must be involved.

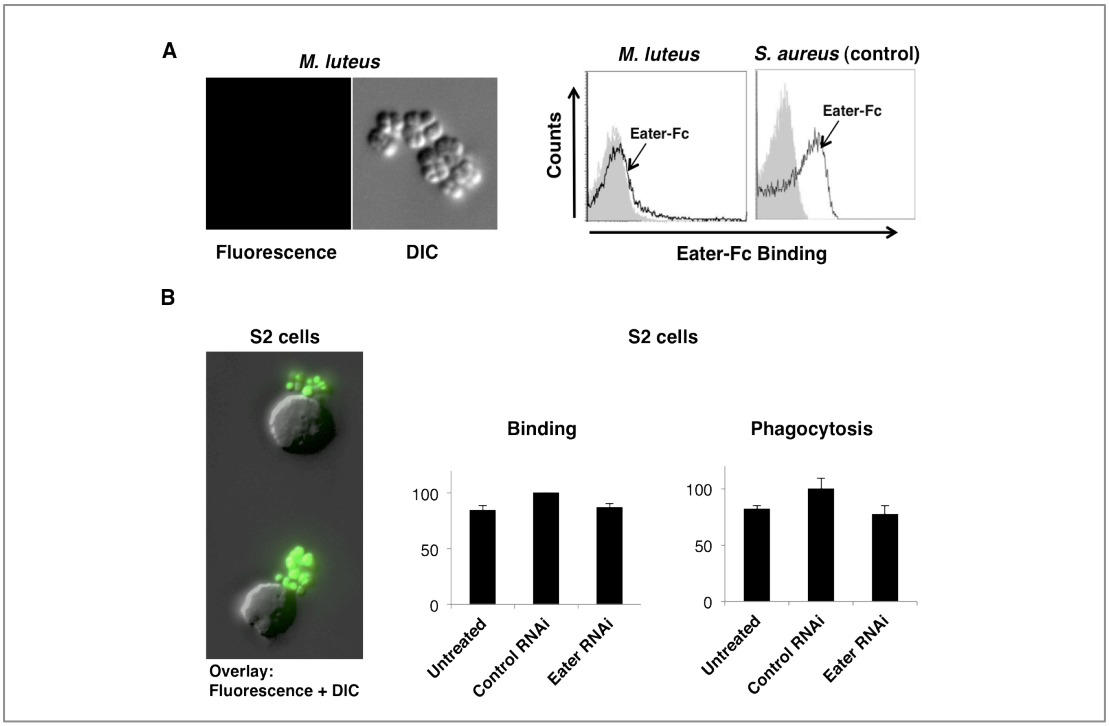
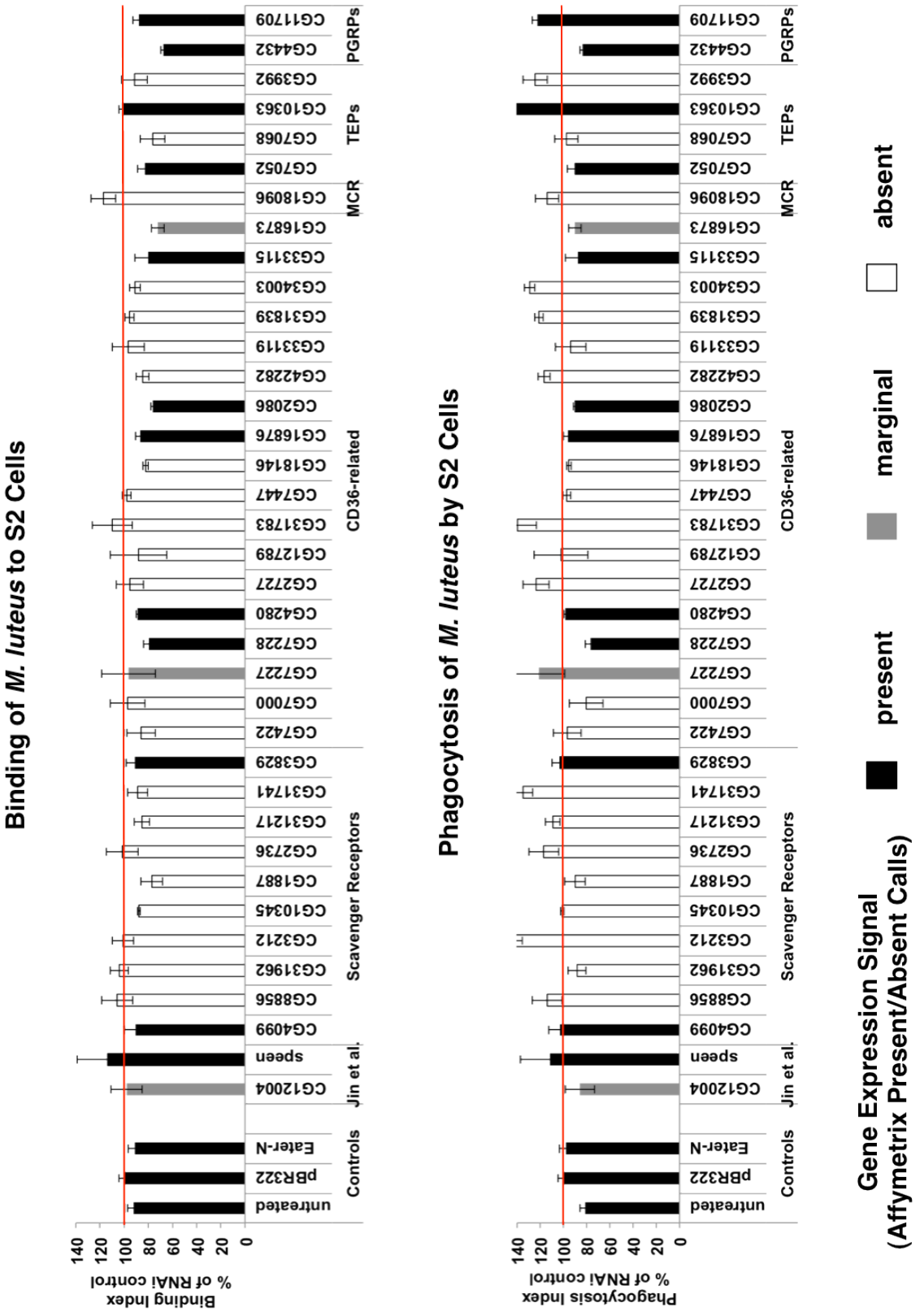


Figure 25. Both, Eater-Fc and native Eater on the surface of S2 cells do not bind to *M. luteus*. **(A)** Immunofluorescence shows that Eater-Fc does not bind to heat-inactivated *M. luteus* (left panel). Flow cytometry analysis of binding by 200 µM biotinylated Eater-Fc fusion protein (open black curve) to *S. aureus* but not to *M. luteus* (right panel). **(B)** RNAi treated S2 cells. (left panel) Immunfluorescence of RNAi treated S2 cells binding heat-inactivated *M. luteus* showing that binding of *M. luteus* is not dependent of Eater. (right panel) Flow cytometry binding and phagocytosis assay of *M. luteus* to S2 cells, normalized to dsRNA-treated controls. S2 cell binding and phagocytosis to heat-inactivated *M. luteus* was not Eater-dependent (no signal decrease after Eater-specific RNAi).

To identify molecules involved in Actinobacteria uptake, in the first instance, I focused on one particular candidate receptor, a gene designated CG12004 which was recently implicated in host defense against *M. luteus* and the fungal pathogen *B. bassiana* (Jin, Shim, et al. 2008b; Jin, Choi, et al. 2008a). I performed computational analysis which revealed that CG12004 is predicted to encode for a protein consisting of 7 transmembrane domains (Kurusu et al. 2008). Microarray gene expression data confirmed that CG12004 is expressed in S2 cells (C. Kocks, unpublished data). I used RNAi against CG12004 and our flow cytometric binding and phagocytosis assay (Rämet et al. 2002) with fluorescently labeled heat-killed *M. luteus*. The results depicted in Figure 26 revealed that RNAi knockdown of CG12004 had no effect on binding or phagocytosis of *M. luteus* by S2 cells. A parallel control sample (knock down of *eater*) - as expected - had no effect on binding and phagocytosis of *M. luteus* but showed a decrease in binding and phagocytosis of the control heat-inactivated *S. aureus* (data not shown).

Since CG12004 was not involved in the binding or phagocytosis of *M. luteus* by S2 cells, I decided to carry out a limited candidate screen of a set of 37 genes. These genes represent the *Drosophila* scavenger receptors with members of class C type I to IV (Rämet et al. 2001), the Nimrod receptor family (Kurucz et al. 2007), the CD36 family (Saleh et al. 2006), the TEP family (Stroschein-Stevenson et al. 2006) and PGRP-LC (Rämet et al. 2002) and PGRP-SA (Garner et al. 2006) previously implicated in phagocytosis.

Figure 26. (see below) Candidate screen for *M. luteus* receptor on S2 cells did not reveal a putative receptor. Results show the level of binding (upper panel) and phagocytosis (lower panel) normalized to pBR322 control RNAi-treated S2 cells (indicated by red line) (A binding/phagocytic index was obtained by multiplying the percentage of binding or phagocytosing cells with the mean number of bound or internalized bacteria as measured by flow cytometry). Genes were divided into seven categories according to predicted classification of the genes based on sequence homologies. Gene expression levels as determined by Affymetrix Arrays are indicated (C. Kocks, unpublished).


Binding of M. luteus to S2 Cells
Binding Index
% of RNAi control
140
120
100
80
60
40
20
0
untreated
pBR322
Eater-N
Controls
CG12004
spaen
Jin et al.
CG4099
CG8856
CG31962
CG3212
CG10345
CG1887
CG2736
CG31217
CG31741
CG3829
Scavenger Receptors
CG7422
CG7000
CG7227
CG7228
CG4280
CG2727
CG12789
CG31783
CG7447
CG18146
CG16876
CG2086
CG42282
CG33119
CG31839
CG34003
CG33115
CG16873
CG18096
MCR
CG7052
CG7068
CG10363
TEPs
CG3992
CG4432
PGRPs
CG11709
CD36-related
Phagocytosis of M. luteus by S2 Cells
Phagocytosis Index
% of RNAi control
Gene Expression Signal
(Affymetrix Present/Absent Calls)
present
marginal
absent

RNAi knockdown of those 37 genes had no effect on binding and phagocytosis of *M. luteus*. Genes that initially showed a reduction in binding (>20 %) were retested but could not be confirmed as candidates. These data suggest that the recognition of different microbes involves different mechanisms. In the case of *M. luteus*, my data suggest that the recognition of this bacterium is either mediated by a novel receptor or by an uncharacterized set of receptors acting together.

3.5.1. Discussion and Conclusions

Since I was not able to find a candidate with this limited RNAi screen, the next logical step would be to carry out a genome-wide screen. However, currently no RNAi screening center offers a flow-cytometry-based read out. Thus, either a fluorescence-microscope-based assay would have to be developed, or the logistics of carrying out the screen in our laboratory would have to be improved.

The current approach suffered from the limitation that the amount of dsRNA that had to be synthesized was not only time consuming but also relatively costly. One approach to making RNAi knock-down of S2 cells more efficient would be by using S2 cells stably transfected with *C. elegans* SID-1 (Feinberg & Hunter, 2003). SID-1 is a multispan transmembrane protein that enables SID-1 expressing S2 cells to rapidly and sensitively silence gene expression in respone to low dsRNA concentrations (Feinberg & Hunter 2003; Shih et al. 2009). Using a SID-1 stably transfected S2 cell line would make the screening process much more efficient, since only a fraction of the dsRNA (about 1000-fold less; 10 ng compared to 7.5 µg) theoretically would be needed to achieve efficient knock-down. If this would hold true in a pilot experiment, SID-1 transfected S2 cells would also allow me to screen multiple samples at once by combining multiple dsRNAs.

Another limiting issue was the manual readout through flow cytometry which could be much more streamlined towards a high-throughput way by using a 96-well automated sample handler available for most FACS machines. This would allow us to use the specialized and reliable binding and phagocytosis assay in a higher throughput manner (Rämet et al. 2002)

These modifications in combination would enable us to expand screening to a genome wide level using published RNAi libraries (Agaisse et al. 2005; Stroschein-Stevenson

et al. 2006), as has been successfully done before by multiple laboratories resulting in the discovery of phagocytic receptors such as Peste (using microscopy-based read-outs). Peste is a member of the CD36 family of scavenger receptors and has been shown to be required for the uptake of *Mycobacterium fortuitum, Mycobacterium smegmatis* and *Listeria monocytogenes* (Philips et al. 2005). Although *Mycobacteria* and *Micrococcus* belong to same Phylum (Actinobacteria), my screen did not identify *peste* to be involved in the recognition of *M. luteus,* further highlighting that *Drosophila* hemocytes appear to possess multiple receptors and pathways for the phagocytoic recognition of different microbes.

3.6. An eater-Gal4 Fly Line to Assess Eater Expression in Different Stages of Fly Development

Aim: The goal of this part of my thesis was to generate a transgenic *eater*-GAL4 fly line and to use this line to characterize *eater* expression.

Rationale: The *eater* gene is a rare example of a known gene whose expression may be restricted to adult and larval hemocytes and their precursors, the pro-hemocytes in the larval 'lymph gland'. It was therefore of interest to generate transgenic fly lines that express GAL4 from the *eater* promoter, and hence in larval and adult hemocytes. Such a fly line may be a valuable tool for the fly community in general to drive tissue-specific expression of UAS-transgenes in larval and adult hemocytes.

3.6.1. Eater expression in different stages of fly development

Aim: To follow Eater expression during fly development from embryo to adult fly.

The GAL4/UAS transgene system is used in *Drosophila* to achieve targeted gene expression by selective activation of any cloned gene in tissue- and cell-specific patterns (Brand & Perrimon 1993). The yeast transcriptional activator GAL4 is cloned behind an enhancer/promoter and drives a transgene of interest which is cloned behind the GAL4 binding sites (UAS). Combined with an UAS-Eater strain,

this line can be used as a tool to rescue the immunodeficient phenotype of Eater-KO flies. This system can then hopefully be used to dissect *eater* gene function by generating at will mutations, truncations or deletions in eater. I used a 2128 bp fragment upstream of the *eater* gene to be certain that this fragment comprises the putative promoter region of the *eater* gene (Fig. 27).

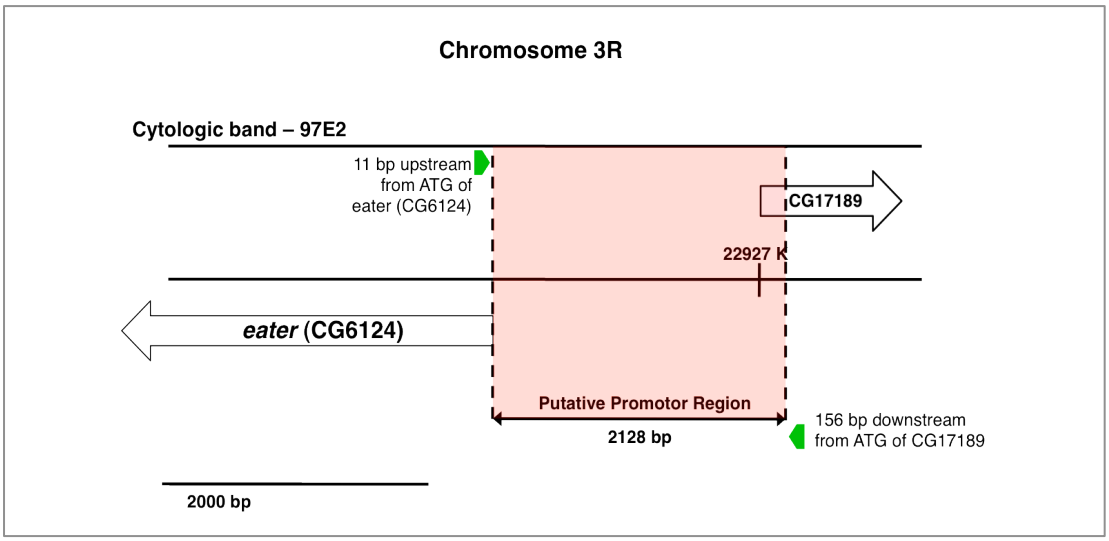


Figure 27. Schematic depiction of the promotor region of the *eater* gene (CG6124). The eater gene is situated on the right arm of chromosome 3 (cytologic band 97ER2). ORF of genes in this region are represented by open arrows. A 2128 fragment (shaded in light red) comprising the putative promoter region of the *eater* gene was amplified by PCR. It was cloned together with the yeast GAL4 gene in fly transformation vector pCaSpeRXsNN (not shown). Primer binding sites are indicated (green arrows).

I crossed *UAS-GFP* transgenic flies to transgenic *eater-GAL4* flies. F1 progeny of such a cross showed tissue specific expression in larval and adult hemocytes (data not shown). In order to study expression of Eater expression in more detail and to obtain a stronger GFP signal I generated a homozygous fly line in which the *UAS-GFP* transgene was recombined onto the same chromosome as the *eater-GAL4* transgene.

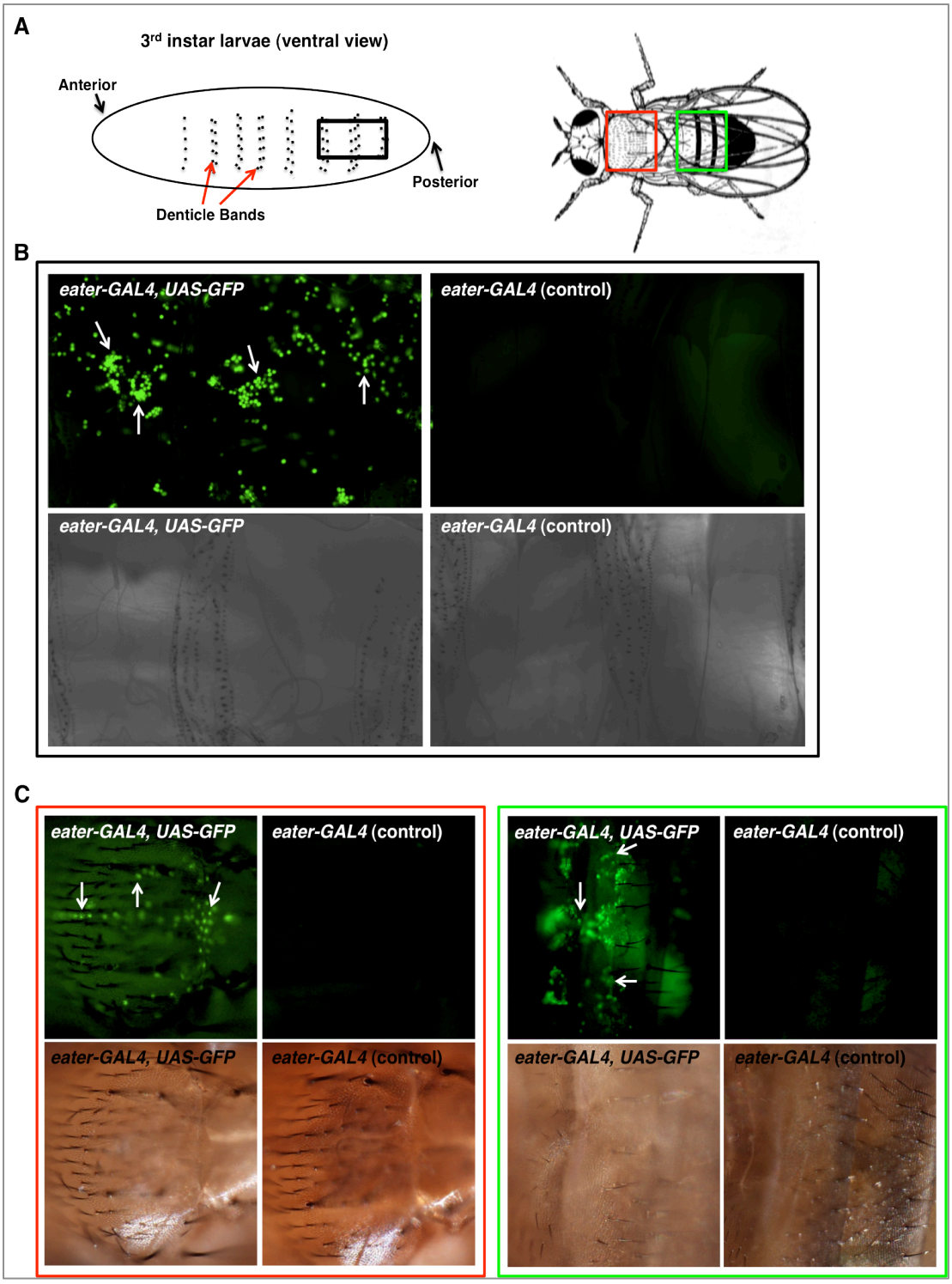


Figure 28. *eater* promotor driven GFP-expression in 3rd instar larval and adult hemocytes. (A) Schematic drawings of larva (left panel) and adult fly (right panel), modifed from: The physical basis of heredity. Thomas Hunt Morgan Philadelphia: J.B. Lippincott Company 1919 (right). **(B & C)** Tissue-specific GFP expression in live larval **(B)** and adult **(C)** plasmatocytes *in vivo*. Fluorescence and corresponding brightfield micrographs of *eater-GAL4, UAS-GFP* (left panels) and *eater-GAL4* control animals (right panels). Imaged areas are indicated on drawings. Arrows point to single or aggregated hemocytes.

Homozygous *eater-GAL4, UAS-GFP* flies carrying both transgenes on the same chromosome were used to monitor Eater expression during fly development. Clearly

detectable eater expression started to occur in late embryos shortly before larval hatching and did not diminish through all larval stages (data not shown). Figure 28 A shows the expression of *eater* promotor driven GFP in hemocytes of 3rd instar larvae and adult flies (Fig. 28 B). Quantitative analysis of hemocytes from 3rd instar larvae revealed that 89 % of hemocytes express GFP (Fig. 29). Preliminary observations also revealed that GFP positive hemocytes seemed visible throughout the pupal stages; however, further analyses are needed to confirm these observations.

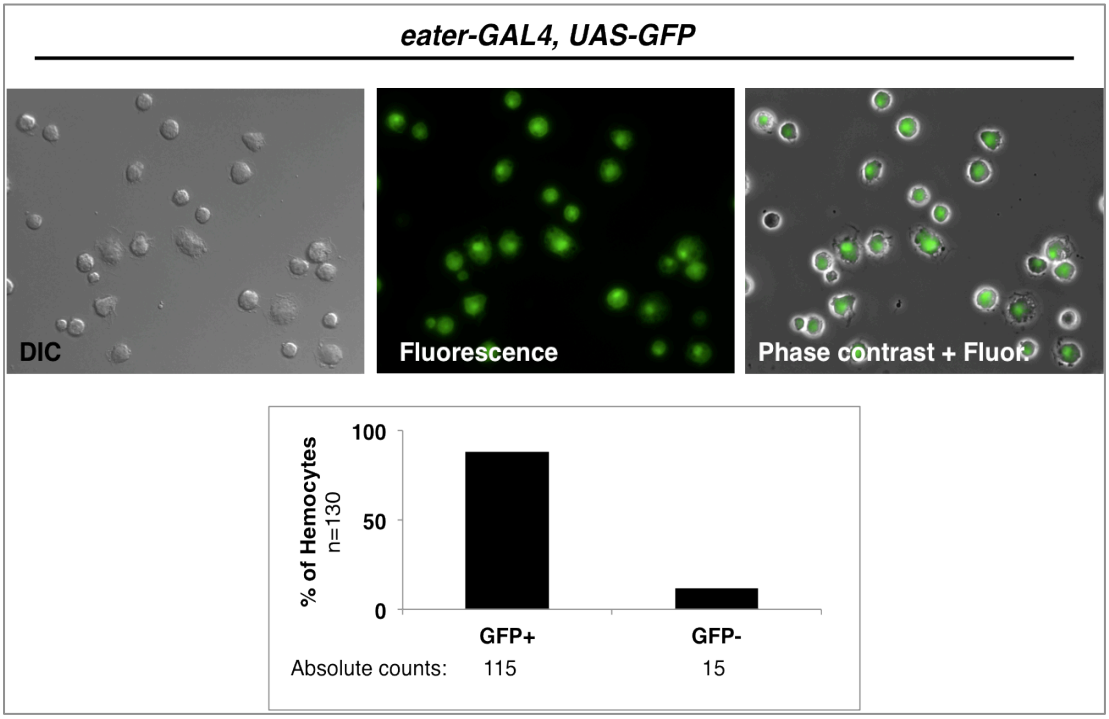


Figure 29. The majority of circulating hemocytes in 3rd instar lavae are GFP-positive. Hemocytes from wandering 3rd instar *eater-GAL4, UAS-GFP* larvae were bled into Schneider medium, allowed to adhere and fixed with formaldehyde. (Upper panels) Micrographs of a representative field taken at 60x magnification. (Lower panel) Quantification revealed that 89 % of hemocytes were GFP-positive (n=130).

These results were in agreement with previous expression analysis (Kocks et al. 2005) which showed that *eater* was not detectable in embryonic macrophages and in pupae, but was expressed in third instar larvae and adults.

3.6.3. Disscussion and Conclusions

The *eater-GAL4* driven gene expression was consistent with previously reported tissue distribution of eater mRNA, namely that Eater expression is absent in

embryonic macrophages and limited to larval and adult hemocytes and their precursors (pro-hemocytes) in the larval 'lymph gland' (Kocks et al. 2005). During the generation of our *eater-GAL4* transgenic flies, Tokusumi et al. reported that larvae expressing *eater-GAL4>UAS-2XEYFP* also show a comparable pattern and timing of expression in circulating hemocytes as compared to the endogenous *eater* gene (Tokusumi et al. 2009). Tokusumi et al. used a 1.7 kb enhancer region upstream of the *eater* start codon, whereas we used a 2.2 kb region (Fig. 27). This suggests that the 1.7 kb Eater promoter DNA sequences contain necessary and sufficient transcription factor binding sites to recapitulate endogenous Eater expression, although none of these *eater-GAL4* constructs has been used yet to rescue *eater* mutant flies.

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