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REVIEW

Presence and conservation of the immunoglobulin superfamily in insects: current perspective and future challenges

M Mandrioli[1], M Monti[2], R Tedeschi[2]

[1]*Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy*
[2]Department *of Agricultural, Forest and Food Sciences, University of Turin, Turin, Italy*

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Abstract

Numerous proteins that contain a *bona fide* immunoglobulin domain have been identified in the last decade showing that immunoglobulin-like proteins are quite common in metazoans. In particular, recent surveys identified more than 140 immunoglobulin-like proteins in *Drosophila melanogaster*, *Anopheles gambiae* and *Bombyx mori*. A well-studied example of immunoglobulin-like protein is the *Drosophila* Down syndrome cell adhesion molecule (Dscam) that, accordingly to comparative molecular analyses, showed a high conservation in Diptera, Hymenoptera and Coleoptera, together with a conserved presence of alternative splicing that permitted insects to possess an unsuspected molecular complexity of their innate immune system. At a functional level, immunoglobulin-like proteins seem to be capable of reacting to pathogen challenges and may contribute to the defense against infection so that they are candidates as immune effector molecules in insects. Preliminary findings on insect-borne plant and animal diseases suggest a possible role of the immunoglobulin-like proteins in the vectorial capacity.

Key Words: immunoglobulin-like proteins; insects; innate immunity; non-self recognition

Introduction

The evolution of immunity has been frequently regarded as a sort of tinkering resulting in jawed vertebrates (gnathostomes) in the presence of conserved "building blocks", including immunoglobulins, T-cell receptors (TCRs) and polymorphic MHC class I and class II molecules, as well as a thymus and compartmentalized secondary lymphoid tissues (Zapata and Amemiya, 2000). A relevant role has been also played by the evolution of the recombination-activating gene (RAG)-mediated V(D)J recombination and the somatic hypermutation of immunoglobulin genes that brought to the synthesis of hypervariable immunoglobulins (Igs) that can bind to their nominal antigens with exquisite specificity and neutralize the harmful effects of non-self antigens (Zouali, 2001; Flajnik, 2002).

Urochordates and cephalochordates do not have an adaptive immune system involving the somatic rearrangement of the antigen receptor genes so that the status of their immune system seems to reflect a "primitive" pre-RAG stage. In particular, an immunological "big bang" of the adaptive immunity occurred in an ancestor of jawed vertebrates, where the insertion of a transposon into an immunoglobulin superfamily gene member initiated the antigen receptor gene rearrangement via the RAG recombinase (Bernstein *et al*., 1996; Agrawal *et al*., 1998; Hiom *et al*., 1998; Schatz, 1999; Flajnik, 2002, 2014). Interestingly, numerous proteins that contain *bona fide* immunoglobulin domains have been identified in invertebrates confirming that gnathostome Igs evolved from previously present molecules that gained a primary role in immunity (Pancer *et al*., 1998; Blumbach *et al*., 1999; Azumi *et al*., 2003). Indeed, proteins containing immunoglobulin-like domains are frequently involved in activities other than defense in invertebrates, and just some of them participate to immune responses, as already reported in cephalochordates (Cannon *et al*., 2002; Haire *et al*., 2004) and for the "molluscan defense molecules" identified in the pond snail *Lymnaea stagnalis* (Sun *et al*., 1990; Hoek *et al*., 1996). Even though none of the invertebrate molecules that bear an immunoglobulin-like domain resembles gnathostome antibodies, the fibrinogen-related proteins (FREP) from the freshwater snail *Biomphalaria glabrata* can recognize through its Ig-

Corresponding author:
Mauro Mandrioli
Department of Life Sciences
University of Modena and Reggio Emilia
via Campi 213/D, 41125 Modena, Italy
E-mail: mauro.mandrioli@unimore.it

like domain a wide range of pathogens (from prokaryotes to eukaryotes) and different categories of FREPs seem to exhibit a functional specialization with respect to the encountered pathogens (Zhang *et al*., 2008).

In the last years, the publication of the wholly sequenced genomes of different insect species allowed a deep analysis of the evolution of their immune systems. In particular, proteins containing immunoglobulin-like domains seem to be quite common and, at least part of them, play relevant roles in the immune response making insects valuable models to understand the extent of roles played by the immunoglobulin superfamily (IgSF) in the insect immunity.

From one to thousand: current state of the art of the insect immunoglobulin superfamily

Immunoglobulins have been considered as typical molecules of gnathostomes since the identification of hemolin (previously called P4), an immune protein isolated from the lepidopterans *Hyalophora cecropia* and *Manduca sexta* (Sun *et al*., 1990; Ladendorff and Kanost, 1991; Lindström-Dinnetz *et al*., 1995). Indeed hemolin has been the first Ig-like molecule isolated in invertebrates and it has been assessed that it was also involved in the immune response (Sun *et al*., 1990; Ladendorff and Kanost, 1991; Lindström-Dinnetz *et al*., 1995). Hemolin is present in low, but significant, amounts in the hemolymph of untreated larvae and pupae, and its level increases 18 - 30-fold after injection of live bacteria into the insect body, as a consequence of an increased synthesis at the fat bodies (Rasmuson and Boman, 1979; Faye and Wyatt, 1980; Ladendorff and Kanost, 1991).

Functional analyses clearly assessed that *H. cecropia* hemolin was able to bind to bacterial cell wall components *in vitro*, forming a complex with two other hemolymph proteins (Sun *et al*., 1990), whereas *M. sexta* hemolin was able to bind not only to bacteria (triggering a protective response involving humoral and cellular reactions), but also to hemocytes inhibiting their aggregation *in vitro* (Ladendorff and Kanost, 1991; Eleftherianos *et al*., 2006, 2007). As a whole, hemolin was not only belonging to the immunoglobulin superfamily, but also played a relevant role in immunity, probably as a pattern recognition molecule, which discriminates between self and infectious non-self by the recognition of molecules unique to microorganisms, such as LPS (Schmidt *et al*., 1993).

Since this first identification, the immunoglobulin superfamily has been greatly enriched in insect genes encoding proteins with at least one immunoglobulin domain. The Ig domain is highly conserved and it has a representative sandwich structure of two opposing antiparallel *β*-pleated sheets, stabilized by a disulphide bridge, that make it easily identified by bioinformatics analyses (Bork *et al*., 1994; Halaby e*t al*., 1999). Indeed, searches of the InterPro and Pfam databases showed several types of Ig domains such as I-subtype, I-subtype 2, C2-set_2, V-set, V-type, Ig, I-set and Ig-like (Huang *et al*., 2009). These domains are found in cell adhesion molecules, cell receptors, antigens, cell surface glycoproteins and other proteins (Wang and Springer, 1998; Ossiboff and Parker, 2007; Soroka *et al*., 2010) and they are at the basis of specific adhesion and recognition capability, including cell-cell adhesion, cell-surface recognition and pathogen recognition (Hutter *et al*., 2000; Hynes and Zhao, 2000).

Recent surveys identified more than 140 IgSF proteins in *Drosophila melanogaster* (Vogel *et al.,* 2003), 138 IgSF proteins in *Anopheles gambiae*, with 85 of them increasing their amount after induction with *Plasmodium*, gram-negative or gram-positive bacteria (Garver *et al.,* 2008), and more than 150 IgSF genes containing at least one immunoglobulin domain have been predicted in *Bombyx mori* (He *et al*., 2014).

At a functional level, recent reports showed that insect IgSF proteins are essential for the immune response (*i.e*., Garver *et al*., 2008). For instance, in *A. gambiae*, the IgSF protein Down syndrome cell adhesion molecule (Dscam) is involved in the defense against bacteria and *Plasmodium* (Dong *et al*., 2006) and hemolin-like proteins have been also isolated in the silk glands of *Galleria mellonella* in response to bacterial challenge and might mark apoptotic cells for elimination by hemocytes (Shaik and Sehnal, 2009).

Multiple sequence alignments and phylogenetic analyses indicated that, despite IgSFs evolved rapidly, 58 % of *Anopheles* IgSF genes has orthologues in *D. melanogaster* and *Aedes aegypti* even if they tended to share more sequence similarity with *Aedes* than with *Drosophila* (Garver *et al*., 2008). For instance, 10 genes are homologous between *A. gambiae* and *D. melanogaster*, but not with *A. aegypti*, 28 genes are orthologues between the two mosquito species only, 23 IgSF genes are uniquely present in anopheline and two of these share similarity with *Apis mellifera* only (Garver *et al*., 2008).

Gene ontology annotation indicated that insect IgSF members are involved as cellular components and in different molecular functions and biological processes (Garver *et al*., 2008; He *et al*., 2014). Indeed IgSF proteins were implicated in signal transduction and cell communication and they can be expressed in a wide range of tissue, such as testis, ovary, fat bodies, midgut, integument, hemocytes, malpighian tubules and head, as clearly assessed in *B. mori* (He *et al*., 2014).

A well-studied example of a member of the Ig superfamily is the *Drosophila* Dscam, whose gene comprises clusters of variable exons flanked by constant exons (Schmucker *et al*., 2000) (Fig. 1). Interestingly, fly cells can combine Dscam constant and variable exons by mutually exclusive alternative splicing potentially generating more than 19,000 different extracellular domains (Watson *et al*., 2005; Brites *et al*., 2008, 2013). Despite the absence of a somatic rearrangement similar to that of gnathostome immunoglobulins, Dscam is at the basis of a large protein isoform repertoire with the potential for recognizing diverse ligands and epitopes (Schmucker *et al*., 2000).

Secreted Dscam isoforms have been detected in the hemolymph and hemocyte-specific loss of Dscam impaired the efficiency of phagocytic uptake of bacteria, possibly due to reduced bacterial

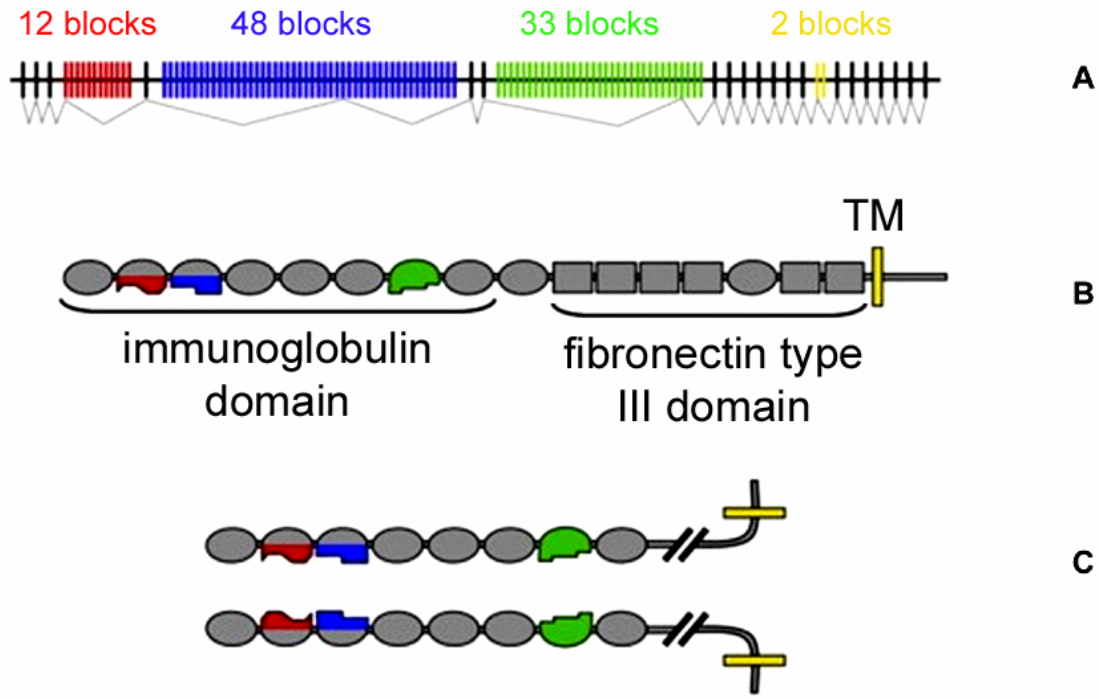

Fig. 1 The Dscam gene contains four variable exon blocks (indicated in red, blue, green and yellow) (A) and multiple isoforms are assembled using single variants from each block through an elaborate process of alternative splicing (B). Each generated protein contain 10 immunoglobulin domain, 6 fibronectin type III repeats, a transmembrane domain (TM) and a cytoplasmic tail. The Dscam proteins show a isoform-specific homophilic binding (C) where the Ig2, Ig3, and Ig7 variable domains match in two opposing isoforms (as represented by the same shape) at all three variable domains is required for protein binding. Modified from Schmucker *et al*. (2000).

binding. Furthermore, Dscam is upregulated after infection in numerous arthropods (Armitage *et al*., 2014). Importantly, comparative analyses of Dscam-like sequences show high conservation of orthologous Dscam genes in Diptera, Hymenoptera and Coleoptera (Graveley *et al*., 2004; Watson *et al*., 2005), together with a conserved expression of diverse Dscam isoforms in immune-competent fat body cells and immunocytes among highly diverged insect species (Watson *et al*., 2005).

Lastly, the molecular diversity of Dscam transcripts generated through alternative splicing is highly conserved across the major insect orders suggesting a common (and unsuspected) molecular complexity of the innate immune system of insects. Interestingly, the availability of numerous genomes and transcriptomes belonging to different insects could allow to deeply explore Dscam presence, expression and alternative splicing in insects (Palmer and Jiggins, 2015).

As a whole, insect IgSF members seem to be capable of reacting to pathogen challenges and to control events that contribute to the defence against infection so that they are intriguing candidates as immune effector molecules in insects.

Immunoglobulin-like proteins and the insect vectorial capacity: from theory to the field

Several insects are involved in the transmission of pathogens to both animal and plant species. Some of these insect-borne pathogens may circulate within the vector body after acquisition and eventually be inoculated into new hosts during insect salivation event (circulative pathogens), while others may bind to various sections of the foregut of vectors without host internalization (Killiny *et al*., 2012). Biological and molecular interactions in both of these models are very complex leading to a high specificity between a pathogen and insect vectors and determining transmission success and different efficiency. For instance, it has been demonstrated that the gut microbiota is able to decrease viral and parasitic infections in mosquito and tsetse fly vectors by activating their immune responses or directly inhibiting pathogen development (Cirimotich *et al*., 2011a). In particular, Cirimotich *et al*. (2011b) identified an enterobacterium that confers total refractoriness to *P. falciparum* through the production of reactive oxygen molecules. However, the mechanisms responsible for bacteria-mediated reduction in vectorial capacity can also be indirect. In this case, the bacteria seem to impede the development of malaria by priming the mosquito immune system (Lefèvre *et al*., 2013).

Intrinsic factors of the vectors such as age, reproductive status, and body size can have important consequences on vectorial capacity, as well. For example, the host immune system may weaken with age, resulting in an increased susceptibility to pathogens (Lefèvre *et al*., 2013).

Moreover, genetic variability of both vectors and pathogen strains can differently act on these interactions, as reported for phytoplasmas (Seemüller and Schneider, 2007; Suzuki *et al.,* 2006). This could be translated in the insect capacity to only acquire the pathogen or transmit it with low or high efficiency (Galetto *et al*., 2009). Recent gene expression analyses of infected and non-infected insect vectors of plant and animal pathogens offer novel opportunities to exploit new knowledge about host-parasite interactions in the different pathosystems. In particular, this approach pointed out the activation of particular genes encoding antimicrobial peptides, such as defensin and cecropin. However, genes involved in pathogen recognition, such as those encoding lectins, and those encoding receptors that activate the innate immune response, such as Toll-3 have been studied, together with genes encoding for members of the signal transduction pathways activated by Toll-like receptors, such as JNK kinase (Medeiros *et al*., 2004; Fisher *et al*., 2014; Vyas *et al*., 2014; Padrón *et al*., 2014; Colpitts *et al*., 2011; Shigihara *et al*., 2008).

Concerning immunoglobulin-like proteins, so far little is known about their direct correlation with the vectorial capacity. It is reported for examples in *Anopheles gambiae* an interplay between AgDscam and the resistance to *Plasmodium* infection. In the specific, this study demonstrated that a RNAi-mediated AgDscam depletion in mosquitoes, which fed on mice infected with GFP-expressing *P. bergei* parasites, resulted in a statistically significant increase of oocystis numbers on the midgut as well as at 13d of feeding (Dong *et al*., 2006). As well, in *A. gambiae* it has been reported that the immunoglobulin domain 6 gene (IRID 6) seems to be involved in limiting *Plasmodium* infection, and so in the resistance to the pathogen invasion, since it was strongly down-regulated in the gut during *P. berghei* ookinete invasion (Garver *et al*., 2008). On the other hand, the plant virus Tomato Spotted Wilt Tospovirus (TSWV) activates the immune system of its main insect vector, *Frankliniella occidentalis* and homologues of gene members of the immunoglobulin superfamily of proteins, such as hemolin are among the genes activated after TSWV infection (Medeiros *et al*., 2004).

In the light of these findings, it could be interesting to verify if the large protein isoform repertoire related to the Ig superfamily is involved in the establishment of the different insect vectorial capacity (Fig. 2). This role could be particularly intriguing since it could allow us to disrupt some pathogen-vector interactions leading to the blockage of transmission.

In particular, it would be the starting point to guide strategies in order to block the transmission of vector-borne pathogens to plants or animals altering gene expression. However, deep analyses of insect vector transcripts under infected and non-infected conditions prove to be a pivotal tool for identification of genes involved in the host immunity response to pathogen infection and transmission.

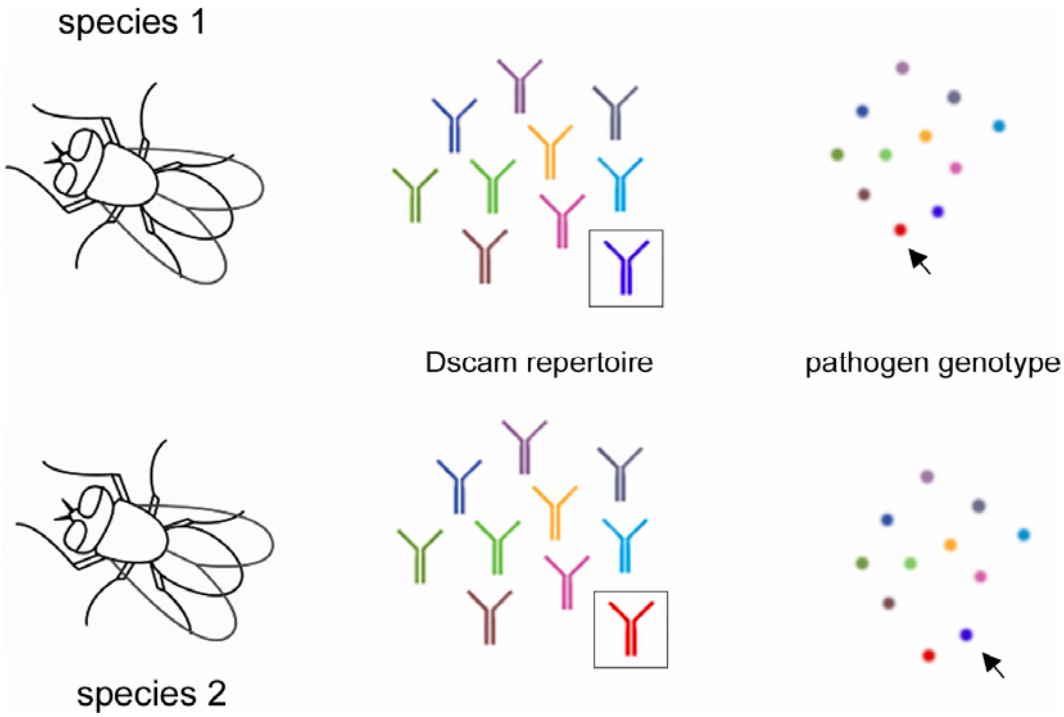

Fig. 2 Despite the conservation of the Dscam genes, the repertoire of proteins that may be assembled is different among diverse species so that two phylogenetically related species may have very similar (but not identical) Dscam repertoire. As a consequence, some pathogen genotypes could be detected in one species, but

undetected in the other (indicated by the arrow in each species) explaining the different vectorial capacity of insect for similar pathogenic species.

Conclusions

Most animals, including insects, have not acquired adaptive immunity, but present a large receptor diversity that increases the effectiveness of immune responses of individual animals. In view of the absence of the adaptive immunity, the insect immune system has been described as simple, term that has been often misinterpreted as evolutionarily inferior to the complex vertebrate immune system. This is a viewpoint that falsely equates the phylogenetic position with a sort of functional inferiority. Insects are the most successful class of organism on the planet (*e.g.*, Gullan and Cranston, 2000) and their simple immune system plays an important role in that success. At this regard, the insect open hemocel provides some advantages in terms of the function of the immune system in contrast to the closed circulatory networks of higher vertebrates, since mediators, effector systems and immunocytes can be more rapidly disseminated and organised. However, the open architecture also presents a problem when the insect is faced with systemic immune insult. Indeed, an open body cavity facilitates rapid movement of infective agents through the host so that selection may have favoured the evolution of effector systems that rapidly and efficiently localise and neutralize invaders. As a consequence, any acquired, or acquired-like, immune response could be not effectives, since such responses are also characterised by a relatively slow response time.

A better knowledge of genes involved in insect immune system by means of the study of vector transcriptomes can therefore lead to a deeper understanding of the mechanisms at the base of the insect-mediated pathogen transmission.

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