Commentary

Commentary on *CAM and NK Cells* by Kazuyoshi Takeda and Ko Okumura

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History of Natural Killer (NK) cells is inextricably woven into the fabric of modern immunology: cells have it!

Innate and adaptive immunity: is innate immunity the hero?

The purpose of this commentary is to extend the ideas presented in the article by Takeda and Okumura (1) on Complementary and Alternative Medicine (CAM) and Natural Killer (NK) cells. Instances of unintentional omissions due to space and time constraints or possibilities of tracing evolutionary origins or using alternative models will be presented briefly. A second overarching concern is to bring the idea of NK cells into the broadest realm of biology, rendering NK cells, like all other living phenomena, accessible in the conceptual, organismic and universal sense. To arrive at this point historically would have been somewhat slower without the ferment that occurred in the world of immunology during the 1800s. As other aspects of history and culture reveal ideas, creations, writings and material concepts were quite different then from our current understanding. In addition, the interplay of events and various other forces, contrived or accidental, may have contributed to the formation of certain ideas or concepts or destroyed existing ones. With respect to immunity, there has always been the dominant anthropocentric theme starting most vividly at the end of the 18th century with Jenner's attempts at vaccination (2). No one really investigated the universality of mechanisms or the possible existence or at least importance of beings other than humans, not at least with respect to immunology. However, the current status of immunity can be described using two general terms: cellular immunity and humoral immunity. These two great camps are in turn subdivided into innate immunity and adaptive immunity. Innate immunity is characteristically nonspecific, natural non-anticipatory, non-clonal and germline. On the other hand, adaptive immunity is specific, induced anticipatory, clonal and somatic. Each of these terms defines particular attributes and when compared represents distinct underlying mechanisms. Invertebrates are considered to possess cells and molecules that almost exclusively effect only innate responses. Vertebrates retained this innate response but also evolved the adaptive response.

Was the evolution of the vertebrate immune system necessary?

From a more personal observation, it is not clear what caused the evolution of the adaptive system as a survival strategy because the long-lived invertebrates—no doubt, extant relatives of extinct species—with a strong innate system have successfully survived millions of years without the overly bureaucratic adaptive systems of vertebrates, especially mammals. Additionally, vulnerability to cancer, once thought to be a syndrome of vertebrates, probably has relevant precursors in certain invertebrates. (3–5). Caution should be exerted when trying to adhere to these rigid characteristics since there is evidence of sharing of components such as signal and mediator molecules. A biologically unifying view might predict this *a priori* because of the ubiquity of DNA and the shared homologies of certain molecules such as hemoglobin (e.g. earthworms and humans). Therefore, why should the cells, tissues, organs and molecules that they synthesize and secrete not bear striking resemblances when the immune system is the discipline being scrutinized?

The development of modern immunology may owe itself to invertebrates

These two great divisions of the immune system (innate and adaptive) that were pioneered by prescience and perhaps the coalescence of anthropocentric ideas on immunity with the genius of a zoologist, resulted from a fortuitous experiment considered as a great experiment in biology (2). This was first recognized when Metchnikoff successfully demonstrated phagocytosis in the 19th century, a discovery worthy of the Nobel Prize in Physiology and Medicine. This discovery of invertebrate phagocytosis dramatically changed the monolithic world of immunology. His careful and detailed observations of white cell motility toward and engulfment of foreign

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bodies in transparent larvae of starfish and in the water flea, *Daphnia*, provoked a major re-evaluation of the nature of immune systems, admittedly restricted to the human good. Before his prescient observations, immune systems were believed to be wholly humoral and there was little emphasis on the role of leukocytes or white cells. Metchnikoff's discovery, however, added cellular immunity to the known armory of humoral immunodefense mechanisms. Serendipity surely intervened and there was probably the impulse to shout Archimedes' *eureka* when the interpretation of why cells were moving toward a foreign body was easily visualized. Thus, the foundation for invoking the concept of *self non-self* recognition was laid.

Roughly a century later, this cellular component, and the animal models from which it was derived are once again at the center of immunology. Moreover, there is a much greater willingness to accept that invertebrate model systems have much more to contribute than was thought, even in the early 1960s when modern immunology was beginning to develop (6,7). Metchnikoff would have relished this turn of events because of which immunology is infinitely richer and even biology in general has reaped substantial benefits, including the harnessing of invertebrate molecules as complementary and alternative approaches to biomedicine (8,9). Of course, we cannot forget the influence of Darwin that surely left its mark on the 19th century and beyond. In fact, broadly interpreted, Darwin led us into the field and Metchnikoff into the laboratory at least with respect to comparative immunology (10,11). Evolutionary immunology reaped the benefits of Metchnikoff and modern immunology advanced conceptually when the clonal selection theory of Burnet was advanced—in essence a Darwinian corollary (12,13). Then came the network theory and the opening up of immunologists to the pervasive extension of the immune system throughout the organism. Additional components and functions existed. Indeed the immune system was broader than plaque forming cells in the spleen, one of the first assay systems that led us to further breakthroughs in immunology.

Organismic approaches are inclusive

This organismic approach involving the cells, tissues, organs and the molecules that they synthesize and secrete has fostered and indeed uncovered an incredible systemic amalgam, discovering almost daily an infinite array of new connections and interconnections, revealing ever more minute complexities almost to the point of incomprehension—as vast as the universe! We know that the maintenance of a balanced milieu as first advanced by Claude Bernard is now known to be due to a finely tuned network whose circuitry is hard wired within the three great regulatory systems—immune, nervous and endocrine—accompanied by a measure of flexibility to allow for changes provoked within and between these systems and those that might be initiated in the internal and external environments. Viewed separately, the immune system has been assumed to have evolved according to the theory of immunologic surveillance, to ensure the capacity to recognize *self* from *non-self*, a concept adhered to by most immunologists. In essence, this view assumes that the immune system evolved to evict internal threats such as cancer. It is canonical—except for the controversial view, the danger hypothesis that assumes that immune responses are set into motion as a result of perceiving danger. The danger hypothesis offers an alternative to the self non-self mechanism associated with surveillance (14).

Tumor immunosurveillance by NK cells in animal models and humans

Early views on immunologic surveillance

According to Burnet (15), 'the concept of immunological surveillance is something which has evolved rather inconspicuously in the last ten years. In my mind, it takes the form of a broad hypothesis, which may soon have the status of a valid generalization that an important and possibly primary function of immunological mechanisms is to eliminate cells which as a result of somatic mutation or some other inheritable change represent potential danger to life. The only fully recognized example of such danger is the initiation of malignant disease—cancer. From human and medical view points, the essence of the hypothesis is that, without immunological surveillance, cancer would be more frequent and occur at younger ages than it presently does. There may also be other lethal conditions related less directly to weakness of the surveillance function, and the theme must be highly relevant to any discussion of the ageing process that ascribes importance to somatic mutation as a factor in senescence. An optimist might hint that a full understanding of the surveillance function might lead in one way or another to a reduction in the incidence of malignant disease and significant prolongation of life span. As yet, there is no real justification for such dreams. [His book was] being written not as a part of the search for an elixir of youth or the cure for cancer but out of the fascination for the theme by a speculative biologist. For at least ten years, my chief intellectual interest has been in immunological and pathological aspects of the interplay between somatic cells within the mammalian body. Over that period there has gradually emerged a conviction that such interactions can be usefully considered from a Darwinian viewpoint. The mobile cells of the body, including red cells, granulocytes and lymphocytes, are constantly being produced and destroyed in large numbers. At least in relation to lymphocytes it is known that there are wide functional differences within the population and, in all somatic cells, mutation and probably other inheritable changes in the genome can occur. Under such circumstances, it is inevitable that something equivalent to Darwinian selection and evolution is going on within those populations (15).' Since this period 30 years ago, there has been an infinitesimal growth in immunology, the most important of which is the elucidation of the cells involved in cancer cell destruction at least *in vitro*.

Current views on cellular mediators against tumors

NK cells are lymphocytes that were first identified for their ability to kill tumor cells without deliberate immunization or activation. Subsequently, they were also found to be able to kill cells that are infected with certain viruses and to preferentially attack cells that lack expression of major histocompatibility complex (MHC) class I antigens. The recent discovery of novel NK receptors and their ligands has uncovered the molecular mechanisms that regulate NK activation and function. Several activating NK cell receptors and costimulatory molecules have been identified that permit these cells to recognize tumors and virus-infected cells. These are modulated by inhibitory receptors that sense the levels of MHC class I on prospective target cells to prevent unwanted destruction of healthy tissues. *In vitro* and *in vivo*, their cytotoxic ability can be enhanced by cytokines such as interleukin (IL)-2, IL-12, IL-15 and interferon α/β (IFN-α/β). In animal studies, they have been shown to play a critical role in the control of tumor growth and metastasis and provide innate immunity against infection with certain viruses. After activation, NK cells release cytokines and chemokines that induce inflammatory responses; modulate monocyte, dendritic cells and granulocyte growth and differentiation; and influence subsequent adaptive immune responses. The underlying mechanism by which NK cells discriminate between normal and tumor cells has provided new insights into tumor immunosurveillance and has suggested new strategies for the treatment of human cancer (16).

NK cell receptors for tumor recognition

NK cell receptor NKG2D

NK cells function through a diverse array of cell-surface natural killer receptors (NCRs). NCRs specific for classical and non-classical MHC class I proteins, expressed in complex patterns of inhibitory and activating isoforms on overlapping, but distinct, subsets of NK cells, play an important role in immunosurveillance against cells that have reduced MHC class I expression as a result of infection or transformation. NKG2D is an activating NCR, which was first identified on NK cells but subsequently found on macrophages and a variety of T cell types. NKG2D ligands in rodents include the MHC class I-like proteins RAE-1 and H60 whereas in humans they include ULBPs and the cell stress-inducible proteins MICA and MICB. Expression of either NKG2D ligand by target cells triggers NK cell cytotoxicity and IFN-γ secretion by NK cells as well as nitric oxide release and tumor necrosis factor α transcription by macrophages. Thus, through their interaction with NKG2D, H-60 and RAE-1 β are newly identified potent stimulators of innate immunity (17). NKG2D-MIC and -RAE-1 recognition events have been implicated in anti-viral and antitumor immune responses. Crystallographic analyses of NKG2D-MICA and -RAE-1 complexes reveal an unusual mode of recognition that apparently tolerates a surprising degree of ligand plasticity while generating affinities that are among the strongest TCR- or NCR-ligand affinities described so far (18).

Crystal structure of NKG2D and two adapters

NKG2D, a homodimeric lectin-like receptor, is a unique stimulatory molecule that is found on NK cells, T cells and activated macrophages. The natural ligands for murine NKG2D are distant major histocompatibility complex homologs, retinoic acid early transcript (RAE-1) and H-60 minor histocompatibility antigen. The crystal structure of the extracellular region of murine NKG2D reveals close homology with other C-type lectin receptors such as CD94, Ly49A, rat MBP-A and CD69. However, the precise mode of dimeric assembly, surface topography and electrostatic properties varies among these natural killer receptors. The NKG2D structure provides the first structural insight into the role and ligand specificity of this stimulatory receptor in the innate and adaptive immune system (19). *In vitro* studies indicate that NKG2D provides costimulation through an associated adapter, DAP10, which recruits phosphatidylinositol-3 kinase. In DAP10-deficient mice, CD8+ T cells lack NKG2D expression and are incapable of mounting tumor-specific responses. However, DAP10-deficient NK cells express a functional NKG2D receptor due to the association of NKG2D with another adapter molecule, DAP12 (also known as KARAP), which recruits protein tyrosine kinases. Thus, NKG2D is a versatile receptor that, depending on the availability of adapter partners, mediates costimulation in T cells and/or activation in NK cells (20).

NKG2D ligand receptor activates NK cells and macrophages inducing tumor immunity

NK cells employ various modes of immune recognition, 'induced self recognition' exemplified by the NKG2D receptor-ligand system. The NKG2D immunoreceptor, expressed by NK cells, and by activated CD8+ T cells and macrophages, recognizes one of several cell surface ligands that are distantly related to MHC class I molecules (i.e. H60 and RAE1 proteins in mice, and MHC class I chain-related proteins and UL-16-binding proteins in humans). These ligands are not expressed abundantly by most normal cells but are upregulated on cells exposed to various forms of cellular insults. Transcripts of this ligand are found in many different tissues and in various tumor cells. Cross-linking of NKG2D with the novel ligand potently activates NK cells and macrophages. Tumor cells ectopically expressing the molecule are efficiently rejected by naive mice and induced strong protective immunity to the parental, ligand-negative tumor cells (21).

Toll-like receptors

Innate sensing

According to Medzhitov and Janeway (22), the survival of multicellular organisms is dependent on their ability to recognize invading microbial pathogens and to induce a variety of defense reactions. Recent evidence suggests that an evolutionally ancient family of Toll-like receptors plays a crucial role in the detection of microbial infection and the induction of immune and inflammatory responses. According to Beutler *et al.* (23), in humans, innate immune sensing usually proceeds through the activation of 10 Toll-like receptors (TLRs), which in turn leads to the production of cytokine mediators that create the inflammatory milieu and abet the development of an adaptive immune response. Each TLR senses a different molecular component of microbes that have invaded the host. TLR4 senses bacterial endotoxins (lipopolysaccharide), TLR9 senses unmethylated DNA, and TLR3 senses double-stranded RNA. Each receptor has a conserved signaling element called the TIR (Toll/IL-1 receptor/resistance) motif that transduces a signal through five cytoplasmic adapter proteins, each of which has a homologous motif. The integration of signals that the receptors emit is a key mechanism that needs to be resolved with respect to TLRs. By creating random germline mutations in mice and screening for individual animals with differences in signaling potential, the complex biochemical circuitry of the innate immune response can be unraveled. Till date, more than 35 000 germline mutants have been produced, and approximately 20 000 have been screened to predict innate immunodeficiency states (23).

Toll in a protostome invertebrate: mosquito

In their study on mosquitoes, Christophides *et al.* (24) have identified 242 *Anopheles gambiae* genes from 18 gene families implicated in innate immunity and have detected marked diversification relative to *Drosophila melanogaster*. Immune-related gene families involved in recognition, signal modulation and effector systems show a marked deficit of orthologs and excessive gene expansions, possibly reflecting selection pressures from different pathogens encountered in these insects' very different lifestyles. In contrast, the multifunctional Toll signal transduction pathway is substantially conserved, presumably because of counter selection for developmental stability. Representative expression profiles confirm that sequence diversification is accompanied by specific responses to different immune challenges. Alternative RNA splicing may also contribute to the expansion of the immune repertoire.

Vertebrate ancestors: the tunicates typical deuterostomes

According to a multi-authored effort (25), the first chordates appear in the fossil records at the time of the Cambrian explosion, nearly 550 million years ago. The modern ascidian tadpole represents a plausible approximation to these ancestral chordates. Therefore, to explain the origins of chordates and vertebrates, Paramvir *et al.* (25) generated a draft of the protein-coding portion of the genome of the common ascidian, *Ciona intestinalis*. The *Ciona* genome contains ~16 000 protein-coding genes, similar to that of other invertebrates, but only half that found in vertebrates. Vertebrate gene families are typically found in simplified form in *Ciona*, suggesting that ascidians contain the basic ancestral complement of genes involved in cell signaling and development. The ascidian genome has also acquired a number of lineage-specific innovations, including a group of genes engaged in cellulose metabolism that is related to those in bacteria and fungi. All metazoa possess a variety of innate mechanisms to resist infection by pathogens. In contrast, the lymphocyte-based adaptive immune system seems to have suddenly emerged in the jawed vertebrate lineage (26,27). It is still not clear, however, how this highly sophisticated system involving hundreds of specific genes has evolved. The genome-wide identification of immunity-related genes in non-vertebrate chordates is expected to help elucidate the evolution of both the innate and adaptive immune systems in vertebrates.

For this analysis, systematic search of the *C. intestinalis* genome failed to identify any of the pivotal genes implicated in adaptive immunity, such as immunoglobulin, T cell receptor and major histocompatibility complex (MHC) class I and II genes, although we cannot exclude the possibility that *Ciona* has highly divergent orthologs of one or more of these genes. A more convincing 'negative' result was obtained by analyzing the genes that encode the 20*S* proteasome, which destroys misfolded proteins (28). Eukaryotic 20*S* proteasomes are composed of 14 different gene products; three possess catalytic activity. Mammals contain a second copy of each of the genes that encode these three catalytic subunits. These duplicated genes encode components of an immunoproteasome that is essential for the presentation of antigen to T cells. The *Ciona* genome contains orthologs for each of the 14 vertebrate proteasome genes, but none for the immunoproteasome-specific genes. These observations strongly suggest that *Ciona* lacks the antigen-presenting system for T cells. Putative *Ciona* homologs of the vertebrate MHC-encoded genes neither exhibit an extensive linkage among them, nor syntenic conservation with the vertebrate MHC.

Although there is no evidence of adaptive immunity, a search of the *Ciona* genome reveals a variety of genes that are likely to mediate innate immunity. There are a large number of possible complement genes, including C1q-like and C6-like genes, three Toll-like receptor genes and a variety of lectin genes. No interleukin or interleukin-receptor genes were identified except for an IL-1 receptor and an IL-17 receptor gene. It is possible that *Ciona* has evolved distinctive innate-immunity genes, because a search for the protein domains found in vertebrate innate-immunity genes identified a number of *Ciona* genes that contain these domains in previously unknown combinations. Despite this somewhat negative information, there is evidence of lytic

activity in numerous invertebrates that destroy experimental targets (11).

One year later, Azumi *et al.* (29) proposed that the mammalian genome encodes several TLRs, with each TLR responsible for detecting corresponding pathogen-associated molecular patterns (20). The *Ciona* genome has only three TLR genes, characterized by the extracellular leucine-rich repeat (LRR) motif and the intracellular Toll/IL-1R (TIR) domain. The genes involved in the TLR signaling pathway have been identified, including MyD88, characterized by the TIR and Death domains, IRAK (IL-1 receptor-associated kinase), TRAF (TNF receptor-associated factor), NFκB and IκB. Mouse RP105 protein is an atypical member of the mammalian TLR family as it possesses only multiple LRR motifs and no TIR domain. Ten gene models with domain architecture similar to that of RP105 have been identified. As LRR is a motif that also functions in protein–protein interactions and is involved in cell–cell communication, it is conceivable that some of the LRR-containing *Ciona* genes actually encode cell-adhesion molecules and not pathogen-recognizers.

Lectin receptors

According to Vasta *et al.* (30), 'The modern era of research on animal lectins has seen a vast expansion on these foundations. The number of lectins described and the variety of species in which these are known has increased rapidly. Sequence data allows classification into structurally similar groups with distinct properties, the C-type and S-type (later renamed galectins) lectins. Functional understanding of lectins from vertebrates revealed their participation in innate immune functions, as *non-self* recognition factors binding to LPS or bacterial surfaces, opsonizing bacteria and activating complement. The most well characterized molecule, now recognized as a participant in the innate immune system, is the serum mannose-binding lectin (MBL), a C-type lectin. As a component of the acute phase response, the lectin-dependent complement-activation pathway initiated by MBL may constitute the most ancient *non-self* recognition/defense mechanism. In vertebrates, C-type lectins in another subcategory known as selectins function to facilitate the adaptive immune response through lymphocyte, neutrophil and platelet homing or localization.'

According to King *et al.* (31), the existence in unicellular choanoflagellates of proteins used for cell adhesion, i.e. cadherins, C-type lectins, several tyrosine kinases (TKs) and tyrosine kinase signaling pathway components and signal transduction in animals raises the question of their ancestral function in the progenitor of animals and choanoflagellates. For example, TKs may act in choanoflagellates to detect changes in the extracellular environment, as has been demonstrated through available nutrition. Moreover, animal cell adhesion proteins, i.e. cadherins, may be derived from ancestral proteins that stabilized the interactions between protozoan cells during conjugation or colony formation. C-type lectins might allow choanoflagellates to distinguish between and capture different bacterial species by binding specific sugar groups displayed on bacterial cell walls. This last conclusion suggests the early origins of immune systems.

Perspectives on origins of immune system components

According to King, *et al.* (31), a central question in animal evolution is how multicellular animals evolved from a protozoan ancestor. Of course, we include in this question all the known functions. One approach to origin of animals is to determine which developmental proteins predated the origin of animal and were subsequently co-opted for animal development. Comparative genomics can identify the minimal set of genes in place at the outset of animal evolution by revealing those shared by all animals and their nearest relatives. To resolve the mystery of origin, this group has sampled the diversity of genes expressed by choanoflagellates. These are unicellular and colonial protozoa closely related to metazoa, crucial for providing a possible window into early animal evolution. They found that choanoflagellates express representatives of a surprising number of cell signaling and adhesion protein families that have not previously been isolated from non-metazoans, including cadherins, C-type lectins, several tyrosine kinases and tyrosine kinase signaling pathway components. Choanoflagellates have a complex and dynamic tyrosine phosphoprotein profile, and cell proliferation is selectively affected by tyrosine kinase inhibitors. The expression in choanoflagellates of proteins involved in cell interaction in metazoa demonstrates that these proteins evolved before the origin of animals and were later co-opted for development.

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