Sequential Immune Responses: The Weapons of Immunity

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Abstract

Sequential immune responses (SIR) is a new model that describes what 'immunity' means in higher animals. Existing models, such as self/nonself discrimination or danger, focus on how immune responses are initiated. However, initiation is not protection. SIR describes the actual immune responses that provide protection. SIR resulted from a comprehensive analysis of the evolution of immune systems that revealed that several very different types of host innate responses occur (and at different tempos) which together provide host protection. SIR1 uses rapidly activated enzymes like the NADPH oxidases and is present in all animal cells. SIR2 is mediated by the first 'immune' cells: macrophage-like cells. SIR3 evolved in animals like invertebrates and provides enhanced protection through advanced macrophage recognition and killing of pathogens and through other innate immune cells such as neutrophils. Finally, in vertebrates, macrophages developed SIR4: the ability to present antigens to T cells. Though much slower than SIR1–3, adaptive responses provide a unique new protection for higher vertebrates. Importantly, newer SIR responses were added on top of older, evo-

lutionarily conserved functions to provide 'layers' of host protection. SIR transcends existing models by elucidating the different weapons of immunity that provide host protection in higher animals.

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Nothing in biology makes sense except in the light of evolution.
Theodosius Dobzhansky (1973)

Introduction

Immunology has long focused on how immune responses are initiated, and it has done so using mostly a 'top-down' approach, considering 'immunity' from a human perspective. This anthropocentric view led to the postulate that self/nonself discrimination is mediated by T cells [1]. More recently, the concept of self/nonself discrimination was modified because of the findings that macrophages/innate leukocytes recognize nonself pathogen-associated molecular patterns [2]. Then, a very different way of looking at how immune responses are initiated was proposed: the 'danger' model [3]. It was hypothesized that disrupted 'self' patterns (e.g. through injury via damage-associated molecular patterns) cause reactions that initiate immune responses.

However, immunity (from the Latin 'immunis') is a broader concept meaning being exempt or having resistance, and resistance does not result from initiation but rather from responses that protect hosts from pathogens or other threats. In this connection, it has recently been recognized that the primary host defense in virtually all animals is carried out by macrophage-like cells/innate immunity [4–6]. This is a fundamental change from the long-held belief that adaptive responses direct macrophage activity. The importance of innate immunity is highlighted by the fact that greater than 95% of the animal kingdom does not have T or B cells and yet displays potent resistance to pathogens. Even when T and B cells appeared in vertebrates, their adaptive responses remained dependent on macrophage responses [6].

When one examines evolution, it can be seen why macrophages (or macrophage-like cells) are central to animal life and defense. Indeed, the first animal seems to be a 'macrophage' [4, 6]. Amoebas are free-living macrophages able to sample their environs, phagocytize foreign objects, repair cellular damage, and kill [7]. The ability of cells to kill (without 'suicide') is central to animal immunity: resistance to pathogens. Killing by amoebas occurs primarily through rapidly triggered effector mechanisms, such as the NADPH oxidases (or Nox), that produce microbicidal compounds from superoxide within minutes [7, 8]. The varied intracellular locations of Nox enzymes also serve an opposite protective role: spatially restricting and protecting cells from overexpression of the destructive molecules [9].

As animals became multicellular, and compartmentalization in cellular functions occurred, macrophages became specialized killers (called M1-type) [10]. Interestingly, macrophage-like cells (called sentinels) may have appeared as early as 'social' amoeba (a multicellular collection of amoebas, such as *Dictyostelium* species) [11] somewhere between 1,000 and 600 million years ago (mya). These earliest 'immune' cells (called hemocytes in insects and some other species) developed additional protective responses, including enhanced phagocytosis and new NADPH oxidase isoforms. Around the time that primitive sponges evolved (550 mya), macrophages developed an enhanced ability to recognize pathogens through Toll, lectin-like, and other receptors [12–14]. At about the same time, macrophages developed the ability to recruit and activate other newly appearing innate killer cells, such as neutrophils and innate lymphoid cells (ILC) [15, 16]. Such innate cellular defenses are found in earthworms and other invertebrates.

The appearance of distinct circulatory systems in various vertebrate fish groups (450–500 mya) brought strong evolutionary pressure to develop yet another layer of immune protection. A separate circulatory system introduced a new risk: pathogens could enter the blood and spread rapidly [17]. In parallel, lymphoid cells with recombination-activating genes (RAG) appeared, allowing enhanced receptor diversity and which circulate more readily in the vasculature than in tissue-resident macrophages [18]. Finally, T and B lymphocytes appeared in fish, with the capacity to produce different and superior types of adaptive responses which were further refined into T cell-mediated cellular (cytotoxic) or antibody-type responses in birds about 300 mya [5]. Highlighting the central role of macrophages in immunity, T cells (in general) do not directly recognize pathogens [19]. They depend on macrophages to present antigens in combination with major histocompatibility class I or II [20, 21]. The newly appearing adaptive immune responses are much slower than earlier appearing innate responses, requiring several days of clonal proliferation to become effective (eukaryotic cell division takes about 16 h). However, like the other earlier appearing protective responses, T and B cells provide unique new protection. Cytolytic T lymphocytes and antibodies can kill targets without the collateral damage of nonspecific innate effector molecules such as H₂O₂ or nitric oxide (NO) [6]. Also, T and B cell responses result in 'memory' (increased frequency of antigen-specific cells) for subsequent rapid same-antigen encounters. Thus, it can be seen that as animal anatomies became more complicated it became necessary to add new responses with unique protective capacities, culminating in adaptive immunity in vertebrates.

A critical feature of sequential immune responses (SIR) is that, as new 'layers' of protection were added, animals retained the evolutionarily older NADPH oxidase and NO-innate-mediated-type protective responses. Also, the different protective responses that occur in vertebrates occur at very different tempos (e.g. from min to days). The evolutionary appearance, the cellular purveyors and the protective molecules of SIR are described below.

Sequential Immune Responses

SIR1: Basic Cellular Defense

As mentioned, even amoebas have an ability to protect themselves. In particular, as shown in figure 1, all cells have SIR1, an ability to rapidly respond to 'stress'.

Fig. 1. SIR. SIR1: all animal cells have basic protective responses. One such type is NADPH oxidases (Nox enzymes) that rapidly self-assemble and produce superoxide. SIR2: separate innate killer cells appeared with additional phagocytic and killing abilities (mainly through the Nox-2 enzyme). SIR3: from about fishes onward in evolution, innate cells acquired enhanced abilities to recognize pathogens, resulting in transcribed enzymes and more

powerful killing molecules like NO. SIR4: macrophages developed the ability to process and present antigens to T cells in combination with major histocompatibility molecules (MHC-I or MHC-II) resulting in clonal proliferation. DAMP = Damage-associated molecular patterns; PAMP = pathogen-associated molecular patterns; iNOS = inducible nitric oxide synthase.

The stress response is primarily mediated by rapidly triggered effector mechanisms, such as the NADPH oxidase (Nox) enzymes that assemble upon stimulation [8]. Such enzymes produce superoxide that can combine with other molecules, generating microbicidal products such as hydrogen peroxide, hypochlorous acid, ozone, and singlet oxygen. Superoxide also maintains cellular homeostasis, with clearly defined roles in development as well as protection against excessive intracellular damage [11, 22–24]. Various ‘ancestral-type’ NADPH oxidases, each with different modes of regulation, are present in primi-

tive cells, though the precise role of individual isoforms in cellular protection is less clear [7, 11, 24, 25]. All cells (whether primitive or human) can protect against stress via Nox enzyme reactions. Important inputs that cause stress include: cellular damage; changes in temperature, pH, isotonicity, or oxygen, and pathogens [8]. There are other protective responses found in many animal cells (e.g. antimicrobial peptides), which will not be discussed separately here [26]. Thus, basic cellular immunity preceded immune cells. As self-contained as the SIR1-type Nox-mediated responses are, they have a major disad-

vantage: the production of self-toxic chemicals. SIR2 helped solve this problem and facilitated the evolution of higher animals.

SIR2: Separate Killer Macrophages/Innate Cells

As mentioned, separate macrophage-like killer cells became evident in most animal lineages long ago (~800 mya). Segregating protection allowed other cells to specialize for digestion, circulation, and thinking – a brain (though this organ has significant intraspecies variation – humor intended). The separate macrophage developed an enhanced phagocytic ability through additional scavenger receptors, retained the SIR1 stress response, and acquired additional NADPH oxidases, such as Nox-2 (fig. 1). Nox-2 also possesses regulatory subunits that allow for increased superoxide production and the capacity for superoxide to dismutate and enzymatically react with other molecules for more powerful killing: mostly within the confines of a membrane-bound phagosome [9, 27, 28]. Because phagocytosis is involved, SIR2 is somewhat slower than SIR1. There are a few lower animals like *Caenorhabditis elegans* that do not seem to possess macrophage-like cells but do have intestinal cells with functions akin to macrophages (e.g. phagocytosis/killing activity) [29–31]. Such animals do rely on SIR1- and SIR2-type protective responses but represent separate branches of evolution. Neutrophils evolved around 550 mya and developed an even more powerful Nox-based killing ability. Neutrophils may also exhibit neutrophil extracellular trap formation as a means of capturing pathogens [32], though phagocytosis is still finally required for the clearance of pathogens or other debris. Neutrophils seem primarily dedicated to intracellular killing rather than repair processes, like macrophages. In particular, though not the focus of this paper, it is worth noting that, in addition to their M1-type killing ability (mentioned earlier), macrophages are also required to repair and help replace dead or effete cells via what are called M2/heal-type responses [10, 17]. The dual M1/inhibit and M2/heal functions of macrophages are unique and necessary for the survival of all multicellular animals [6]. Another type of killer cell, i.e. ILC, appeared at around the same time [15]. Unlike macrophages (or neutrophils), protection by ILC (e.g. NK cells) involves killing without phagocytosis. Both neutrophils and ILC are recruited or activated by macrophages [33–36]. In addition to the SIR2-type innate protective responses mentioned above, other nonimmune cells (such as gut epithelium) developed enzymes related to Nox called dual oxidases that can produce extracellular H₂O₂ and protect surfaces exposed to the environment [37].

SIR3: Macrophage Advanced Killer Activity

Around the time of the appearance of marine animals with a notochord (~500 mya), macrophages developed additional killing activity to supplement their Nox-based SIR1 and SIR2 responses. SIR3 involves more specific recognition of pathogens through receptors (such as Toll, lectin-like, NLR, and RLR). Though pathogen receptors, such as Toll, were present further back in evolution [38], their dedicated use for pathogen recognition became prominent in macrophages [39–44]. Unlike earlier fast-responding NADPH oxidase enzymes, Toll and related recognition structures induce signals that result in the production of new enzymes. Whereas SIR3 involves the slower DNA-RNA-protein pathway, the production of greater quantities of killing molecules became possible. Also, products like NO appeared with an advanced microbicidal ability [45, 46] (fig. 1) and, unlike Nox-mediated killing, which most often occurs within a phagosome [47], NO (a gas) freely diffuses across membranes, allowing killing at a distance, like a rifle (extracellularly) [45, 46]. NO can also synergize with intraphagosomal superoxide to produce potent killing compounds, such as peroxynitrite [48–50].

Around 475 mya, vertebrates developed with a central pumping organ and a separate circulatory system to distribute oxygen and other nutrients [5, 12]. As will be seen in SIR4: Macrophage Antigen Presentation, this system necessitated the appearance of the newest layer of protection: adaptive immunity.

SIR4: Macrophage Antigen Presentation

The appearance of the complicated anatomies of vertebrates (e.g. a separate circulatory system) introduced a new risk: as mentioned earlier, pathogens could spread rapidly throughout the body [6]. A ‘new’ system was required to protect the vascular system, organs, and isolated locales of vertebrate anatomies.

T and B cells appeared with their vast repertoire of receptors, allowing increased specific recognition of pathogens as well as memory, as mentioned earlier [18]. T cells and antibodies also freely and rapidly circulate, providing systemic protection superior to that afforded by tissue-resident macrophages. However, T cells typically do not respond to antigens directly. Therefore, macrophages (or dendritic cells¹) developed a necessary new capacity to make SIR4 work: the ability to present antigen to T cells

¹ Macrophages and dendritic cells can both present antigens. So, macrophages will be used here to refer to both. Readers are directed elsewhere for discussions on macrophages and dendritic cells [51–54].

(in combination with class I or II histocompatibility molecules) [20, 21]. This newest protective adaptive response, as mentioned earlier, is slow, requiring days to become effective (fig. 1). Therefore, SIR4/adaptive immunity developed to supplement the much faster SIR1–3 responses that are the primary host defense in animals, including humans [6].

The evolutionary pressure for new responses to protect vertebrate anatomies must have been strong because the lymph system and adaptive immunity created a much more complicated immune system. For example, the more involved 2-step system of macrophage antigen presentation to T cells seems to have evolved to ‘fine-tune’ the recognition of antigens. Specifically, it allowed T cells to recognize subtle alterations in self (e.g. intercellular viral infections) in addition to overtly foreign pathogens.

In addition to antigen presentation, macrophages direct T and B cell responses in different ways. Specifically, many pathogens stimulate M1-type responses in macrophages (mentioned earlier), which then also causes them to stimulate T cells to produce Th1-like cellular responses (including cytolytic CD8 T cells) and to further elevate M1-type killer macrophages [6, 55]. Some pathogens do not stimulate M1-type responses; macrophages remain in their day-to-day role of repairing and replacing host tissues (e.g. M2-type) [10, 56]. In this circumstance, M2-type macrophages can stimulate T cells to make Th2-type cytokines and induce antibody production. The ability to direct T cells in these different ways allows macrophages to ‘outwit’ pathogens by stimulating different adaptive responses that are optimally effective for a particular pathogen. Although some earlier innate lymphocytes may display some memory [57], the memory capacity of T and B cells is much more diverse and allows faster responses to the same pathogen encounters. SIR4 also came with a cost to vertebrate hosts. Like any more complicated ‘machine’, the genetic mutations, recombinations, and rearrangements that allow millions of different receptors on T and B cells, also ‘breaks down’. Lymphocyte receptors that recognize self are necessarily produced, which can create ‘autoimmune’ responses [58–60].

Together, it can be seen that SIR1, 2, 3, and 4 each has unique advantages (and disadvantages) such as speed and specificity that together provide layers of protection necessary for complex vertebrate anatomies. SIR is meant to provide an overall framework for understanding immunity in higher animals under which other protective responses can coexist.

Advantages of SIR over Existing Models

Unlike existing models that describe how immune reactions are initiated (be it through self/nonself or danger recognition), SIR is functional: it describes the different protective responses that together make up immunity in higher animals. The advantages of SIR over existing models are discussed below.

Self/Nonself Discrimination

The self/nonself model became popular mainly because of the spectacular ability of the immune system to eradicate important diseases like smallpox and polio through vaccination [1, 17]. Most of us learned that, ‘specificity is the hallmark of immunity’. This is correct in that humans perish without T cells (unless protected). However, self/nonself discrimination is not the sole province of T or B cells or for only recognizing pathogens. For example, as mentioned, simple animals use self/nonself recognition to locate food, find mating partners, or build colonies of like cells [7].

Thus, T or B cell-mediated self/nonself recognition is only the ‘tip of the iceberg’ in immune responses. As mentioned, immunity in greater than 95% of animal species occurs without T cells, even though lower animals (e.g. earthworms) continuously consume contaminated material [6]. The finding that macrophages can specifically recognize pathogens through pattern recognition receptors resulted in modernization of the self/nonself model [39, 41]. Macrophages were shown to add a ‘second signal’ [61, 62], which aids in the recognition of altered self (e.g. virally infected cells). However, the concept that nonself or altered self initiates immune responses incompletely accounts for the fact that there are specific receptors on T and B cells that recognize self antigens and which result in autoimmunity [63]. Though the recently recognized role of innate immunity in nonself recognition importantly advanced our understanding, the self/nonself model still only describes how immune responses are initiated [64].

The Danger Model

That hosts respond to danger because of alterations of self resulting from cellular dysfunctions, injury, or death added an important new dimension about how immune responses are initiated [3]. In this connection, these self-driven reactions resemble SIR1, the rapid initial stress responses present in all animal cells. However, a problem with the concept of danger has always been that immune reactions can be initiated by pathogen-specific recogni-

tion. In particular, specialized immune cells appeared with increasingly specific receptors for recognition of nonself (SIR2–4). Proponents of the danger model argue that specific recognition of pathogens (or altered self) is not sufficient to initiate immune responses [65, 66]. However, robust T (and B) cell responses to allogeneic lymphocytes (GvH) and isolated allergens (allergy) occur in vitro: a circumstance where there is no apparent danger.

Conclusion

Self/nonself and danger models usefully describe different ways in which immune responses can be initiated. However, initiation is not protection. SIR describes the main branch of evolution from lower animals to humans that resulted in an amalgam of evolutionarily preserved and newer protective responses that are very different, occur at different tempos, and work in concert to provide layers of immunity necessary for the survival of higher animals.

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