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Toward a Modern Synthesis of Immunity: Charles A. Janeway Jr. and the Immunologist’s Dirty Little Secret

Peter M. Gayed

MD and PhD candidate, Immunobiology Department, Yale School of Medicine, New Haven, Connecticut

This essay chronicles the major theoretical and experimental contributions made by Charles A. Janeway, Jr. (1943-2003), Howard Hughes Medical Institute investigator and Yale Professor of Immunobiology, who established the fundamental role of the innate immune system in the induction of the adaptive arm.

Charles Alderson Janeway, Jr., (1943-2003) did not like secrets. Indeed, as a Yale professor and scientist, it was his goal to shed light on the “immunologist’s dirty little secret” [1], that is, that foreign antigen alone was insufficient to elicit the adaptive immune response and that scientists instead had to routinely pepper their experiments with crude extracts like mineral oil, mycobacteria, and aluminum hydroxide in order to get T and B cells to do their bidding. If lymphocytes were the discerning finger that identified self from non-self, why was it that non-self antigens alone failed to be recognized? What role did these crude substances, euphemistically termed “adjuvants,” play in the initiation of the body’s immune response? And why did they appear to be required for the production of activated T and B cells? Janeway decided to find out.

JANEWAY’S EARLY YEARS AND TRAINING

Janeway’s loyalty to what we now call the adaptive immune system might have begun when he developed measles as a young boy. Janeway’s father was an eminent Harvard pediatrician, researcher, and...
department head at the Children's Hospital Boston. At the time, Janeway Sr. was administering pooled immunoglobulins for the treatment of agammaglobulinemia, a condition of antibody deficiency, as well as for the prevention of measles. As Janeway describes it, his father “lined up all the kids in the neighborhood, injected them with gamma globulin, and then systematically exposed each of them to me” [2]. As the young Janeway would learn (and as his father reported in a 1944 issue of the Journal of Clinical Investigation), this measure would reduce the likelihood of measles transmission shortly after treatment but not provide long-lasting immunity [3].

As a Harvard medical student, Janeway would be reacquainted with the particulars of antibodies in the laboratory of J.H. Humphrey at the National Institute for Medical Research, London, England, where he spent two years. There, he studied the antibody response to injections of two enantiomers of a synthetic tripeptide known as TGA. Janeway found that antibodies produced in response to D-TGA did not cross-react with L-TGA and vice-versa, supporting the idea that an antibody binds to its antigen with specificity. Later, he would devise a red blood cell-marking technique to show that antibodies against blood type A antigens would exclusively crosslink type A red blood cells in a mixture of type A and B blood. Such novelty afforded him another English sabbatical, this time at the University of Cambridge with Robin Coombs, a noted immunologist and inventor of the clinical test that bears his name.

In 1977, after a postdoctoral fellowship at the NIH Laboratory of Immunology and further training at the Uppsala Biomedical Center, Sweden, Janeway began his professorship at Yale in the Department of Pathology’s Division of Immunology, which would later become one of the first freestanding immunology sections in the United States, in large part due to efforts led by Janeway himself.

At the helm of his own lab, Janeway began steering toward unaddressed questions concerning the adaptive system he had come to know so well. At the time, there were examples of immune responses that could occur in the partial or complete absence of an adaptive system. The activation of macrophages without T cell help, the initiation of the complement system independent of antibody (the so-called “alternative pathway”), the ability to generate an antibody response in the absence of what was thought to be a required T cell population — all this was evidence against a conventional view that T and B cells were necessary and sufficient to launch a response against antigen. This, along with the “immunologist’s dirty little secret,” goaded Janeway into a line of inquiry that would bring to the fore of immunological research an often ignored corner: the innate immune system.

THEORETICAL INSIGHTS AND THE TWO-SIGNAL HYPOTHESIS

Previous to 1989, immunologists believed that the immune system, effectively defined by T and B cells, could recognize, attack, and clear whatever stimulus had activated it. Researchers could drive the expansion of T cells or antibody-secreting B cells with any antigen — provided they followed the immunologist’s cookbook. What fixed Janeway was the ubiquitous call for adjuvant in each recipe, an almost alchemic mixture of mycobacterial components or alum with killed Bordetella.

In a lucid essay [4] he co-authored with his now most famous postdoctoral fellow, Ruslan Medzhitov, Janeway explains why the adaptive system’s response against antigen would require an additional signal in the form of adjuvant. The conversion of T and B cells into formidable destroyers, he explains, must rely on a separate system of cells and receptors. This is because the binding domain of the T and B cell receptors (TCRs and BCRs†) that recognize antigen are formed randomly during lymphocytic development. This randomness endows our adaptive immune system with the ability to respond to never-before-seen pathogens, but it also means that T and B cells can form receptors against self proteins and thus medi-
ate autoimmunity. More profoundly, Janeway offered this theoretical enigma: How can a receptor with a randomly generated ligand-binding site but a constant signaling domain convey meaningful information? In other words, how is it that a lymphocyte “knows” when to proliferate and attack, and when not to, if antigen alone is the sole conveyor of information? According to Janeway, antigen could not be the sole conveyor. The adaptive system, he reasoned, must rely on other receptors that are not randomly generated — that is, it must rely on interactions that would have been selected over evolutionary time. His first hunch about these interactions, which was to birth a frenzy of discoveries continuing today, was that they had something to do with microbes.

Just prior to Janeway’s start at Yale in 1977, a theoretical paper was published by two Australian immunologists regarding the immune response observed following transplantation. The rejection of transplanted tissue was explained by the presence of “histocompatibility molecules,” which were believed to be any number of non-self antigens carried by the graft. These antigens would be recognized and attacked by the host’s T or B cells. The sequitur of this concept would be that the greater the unrelatedness between donor and recipient, the more abundant the antigens and the greater the immune response. However, it became clear that reactivity was more vigorous “between different strains within a species than between species” [5]. Additional observations led the authors to hypothesize that in addition to antigen, a “species-specific . . . signal passes between the [immune and transplanted] cells,” thus accounting for the more pronounced response observed when donor and recipient were of the same species.

Experimental evidence for the existence of this “second signal” would not come until 1987, when two American immunologists at the NIH showed that antigen presenting cells (APCs) displaying a fragment of pigeon cytochrome c could not drive the expected proliferation of T cells (specific to this fragment) if the APCs were pre-treated with a cross-linking agent known as ECDI [6]. After additional experiments confirmed that ECDI treatment did not modify the antigen itself or the surface molecules displaying the antigen, the authors concluded that ECDI “inactivated an accessory function of the APC.” Such was the first experimental suggestion that antigen alone was necessary but not sufficient to drive the adaptive response. The “accessory function” provided by APCs appeared not to be so accessory. Rather, it appeared this second signal was crucial for activation of T cells.

Similar requirements seemed to exist in B cells, where the addition of antigen plus the Gram-negative membrane component lipopolysaccharide (LPS), but not antigen alone, could induce antibodies [7]. Hence, LPS became known as a “non-antigen” B cell mitogen because it activated B cells and drove their proliferation without triggering an antibody response to itself.

In the same period, there were many groups interested in the microbial defense strategies used by innate cells such as neutrophils and macrophages [8]. Indeed, there was a growing list of effector mechanisms that included phagocytosis, cell degranulation, and lytic peptides, all contributing to efficient clearance of pathogens. As one might expect, these arms could be induced by a variety of microbial products, including LPS.

**EVOLUTION OF IMMUNITY: MORE THAN SELF VS. NON-SELF**

Despite the parallels, no formal theory had satisfactorily reconciled the two-signal hypothesis with the increasingly visible role of microbial components in the activation of both the innate and adaptive arms. In his introduction to the 1989 Cold Spring Harbor Symposium, Janeway would articulate such a theory [1]. In it he explains how early and important studies by Karl Landsteiner, which demonstrated that proteins could be modified chemically to induce antibody formation [9], ingrained in immunologists the misperception that all “foreign macromolecules are equally able to give rise to an im-
mune response.” Though it appears evident today, Janeway proposed what was then an unorthodox belief that the immune system evolved not simply to discriminate self from non-self, but “noninfectious self from infectious non-self.” In other words, that the immune system evolved against, and thus for its induction depended on, the presence of microbes.

He further implicated that it was the innate arm that was responsible for this discrimination and that invariant, germline-encoded receptors on innate cells—as opposed to the randomly generated BCRs and TCRs expressed by B and T cells—would recognize conserved microbial patterns. Support for this idea came from growing evidence that the invertebrate kingdom lacks any sign of an adaptive system and yet is able to mount immune responses against natural pathogens. Thus it appeared likely that a system to identify “self from non-self” had developed early in evolutionary history, far before the arrival of lymphocytes.

By 1990, Janeway knew he had to prove two things experimentally: that microbial components were recognized by innate cells and that this recognition was transmitted as the “second signal” required for lymphocyte activation. In a remarkable series of discoveries over the next decade, Janeway and others would confirm the tenets of his theory.

**ANTIGEN IS NECESSARY BUT NOT SUFFICIENT FOR ADAPTIVE INDUCTION**

In 1991, Janeway and postdoctoral fellow Yang Liu used an *in vitro* model to investigate the role of LPS in the induction of T cell activation [10]. In this model, anti-CD3 monoclonal antibody (anti-CD3 mAb) was used to crosslink, cluster, and thus activate the TCRs of a resting T cell, mimicking the action of a specific antigen.

Analogous to conditions of antigen alone, the presence of anti-CD3 mAb alone could not drive T cell proliferation. However, when the experiment was repeated in the presence of splenocytes (a heterogenous cell population derived from the spleen), the T cells showed marked proliferation. To identify the cell providing the second or what then began to be dubbed the “co-stimulatory” signal, Janeway and Liu sorted the splenocytes into their constituent parts and repeated the experiment. Consistent with earlier studies in lymph nodes [11-13], it was the B cell compartment that accounted for the observed T cell response.

This was an unusual but not entirely surprising find. It was known that B cells contributed to the T cell response by presenting antigen, but Janeway was using a system in which signal 1 was provided by an “antigen-independent” stimulus, i.e., the anti-CD3 mAb. Moreover, the B cells themselves had not been provoked with any particular antigen. It appeared as if B cells were offering a signal that had nothing to do with their own unique antigen specificity. To be sure, the researchers repeated their experiment after adding antibodies that would make the BCRs physically inaccessible. Still, the B cells were able to promote the T cell response. Thus, co-stimulation was provided by a “non-specific” molecule carried by all B cells.

Importantly, the researchers also showed that this signal was not constitutively active *but inducible* on B cells after their treatment with LPS. That is, purified B cells were not able to support T cell proliferation unless they first received LPS. The same was observed when macrophages, a cell type belonging to the innate system proper, were used in place of B cells: Only pre-treatment of macrophages with LPS or zymosan (an established, yeast-derived macrophage activator) could induce robust T cell proliferation in the presence of anti-CD3 mAb.

Thus, Janeway, supported by studies from independent groups, had demonstrated that antigen alone was insufficient to trigger the adaptive system. A second signal, which could be provided by B cells or macrophages, was required — and, critically, this signal was only made available after treatment with microbial products such
as LPS or zymosan. It would become clear that the second signal, in this case, was provided by two closely related members of the immunoglobulin gene superfamily, B7.1 (CD80) and B7.2 (CD86). The B7 molecules are expressed by B cells, macrophages, and dendritic cells — collectively known as the antigen presenting cells of the immune system — but only after their exposure to microbial products (Figures 1 and 2). Thus, the first of Janeway’s two-part theory on the induction of the adaptive arm had been borne out. Not yet identified, however, was the receptor by which microbial products were recognized. Impressively, this, too, would come from the Janeway lab.

Because macrophages and dendritic cells had been shown to be the most potent

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1 Working with Richard Flavell, Janeway later implicated co-stimulatory molecules in the pathogenesis of autoimmune disease. In one study, pancreatic β-cells were modified such that they expressed the B7.1 molecule. In a mouse line genetically susceptible to diabetes, expression of B7.1 resulted in earlier and more extensive pancreatic disease [14]. In another study, the presence of CD40 ligand (CD40L) was essential for the expression of B7 molecules in an autoimmune multiple sclerosis-like disease model in mice. The absence of CD40L thus precluded the development of disease, despite immunization with the triggering antigen (myelin basic protein) in Freund’s adjuvant [15].
T cell-activating APCs, Janeway’s lab initiated a hunt for molecules expressed on the surface of these cells that were capable of both binding microbial products and inducing the expression of CD80 and CD86. Such receptors would provide the mechanistic basis for adaptive induction by the innate system.

THE CASE FOR FRUIT FLIES IN HUMAN IMMUNITY

By the mid-1990s, the transcriptional factor NF-κB had been established as the critical activator of innate immune cells [16]. It was also known that the NF-κB signaling pathway was activated by transmembrane receptors that bore a cytosolic domain known as TIR [8]. Working as a postdoctoral fellow in the Janeway lab, Ruslan Medzhitov screened a human splenic cDNA library for genes containing TIR domains. A gene was identified, but instead of an extracellular domain related to other known microbial receptors of the innate system, it contained a leucine-rich repeat (LRR) domain. This was an unexpected find. The LRR domain belonged to a family of proteins found in *Drosophila* [17], among them a receptor called Toll, itself involved in the dorsal-ventral patterning of developing flies.

It was enigmatic that a receptor involved in *Drosophila* axis development would contain a cytosolic inflammation-related motif such as TIR. However, in the summer of that same year, an independent group working from Strasbourg, France, demonstrated that loss-of-function mutations in the Toll receptor left adult flies highly susceptible to fungal infection and death [18]. They also demonstrated that Toll gain-of-function mutations led to the constitutive expression of critical antifungal genes. Moreover, the downstream pathways on which Toll relied were homologous to the mammalian pathways that activated NF-κB via TIR domains. Thus it appeared that in addition to its patterning function in *Drosophila* embryos, Toll mediated critical immune responses in the adult fly (Figure 3).

These findings galvanized the Janeway laboratory, which had already observed that a constitutively activated form of the gene identified by Medzhitov potently activated NF-κB, released a variety of pro-inflammatory cytokines, and, importantly for Janeway’s theory, upregulated the expression of the co-stimulatory molecule CD80 in human cell lines [8]. Janeway and Medzhitov would later publish this finding in a 1997 issue of the journal *Nature* [19].

CONCLUSION: THE IMMUNE SYSTEM, REVISED

The tenets of Janeway’s theory were made complete when Godowski and colleagues showed that a human homologue of the Toll receptor recognized LPS and initi-
ated the critical NF-κB signaling pathway [20]. In that same period, a number of human receptors with Toll homology were identified [21,22], which collectively came to be known as the Toll-like receptor (TLR) family. To date, 12 mouse and 10 human TLRs have been identified, each recognizing a unique but conserved microbial pattern and each capable of upregulating co-stimulatory molecules among antigen presenting cells (Table 1).

In just 10 years, Janeway and his colleagues exposed the immunologist’s dirty little secret. In doing so, they revised the working model of the immune system. It is now clear that antigen presenting cells recognize conserved microbial components, collectively dubbed PAMPs for “pathogen-associated molecular patterns.” These PAMPs serve as ligands for a broad class of proteins referred to as pattern recognition receptors (PRRs), of which the TLRs are a subset. When a PRR on an APC binds to its corresponding PAMP, the cell begins to efficiently present antigen (signal 1), upregulate the expression of co-stimulatory molecules (signal 2), and elaborate cytokines (now known as “signal 3”) that guide the formation of adaptive cells uniquely poised to respond to the inciting pathogen. Thus, the adaptive system is mobilized against antigens in the context of infection, and this defense is specifically targeted to niches the pathogen calls home.

In addition to advancing the basic science of immunology, Janeway’s revelation on induction of the adaptive system has had important clinical implications, spurring an era of rational vaccine design that exploits PRRs. Immunologists today recognize that effective vaccines will stimulate APCs. Known PRRs, like those within the TLR family, are specifically being triggered by adding or conjugating PAMPs to antigens of interest. For example, peptidoglycans and other skeletal cell wall components in the Bacillus Calmette-Guérin (BCG) vaccine are recognized by TLR2 and TLR4 to generate protective immunity against Mycobacterium tuberculosis [23]. Similarly, vaccines against Haemophilus influenzae type b, once a leading cause childhood meningitis, can be conjugated with outer-membrane proteins from Neisseria to elicit effective adaptive responses, a phenomenon dependent on TLR2 [24]. Moreover, the adjuvant properties of recently developed DNA vaccines, which contain unmethylated CpG clusters, are thought to be mediated by TLR9 [25].

Table 1. The family of Toll-like receptors and their ligands in the human.

| Toll-like receptor                  | Ligand                        |
|------------------------------------|-------------------------------|
| TLR-1:TLR-2 heterodimer            | Triacyl lipopeptides          |
| TLR-2:TLR-6 heterodimer            | Diacyl lipopeptides           |
| TLR-3                              | Double-stranded RNA           |
| TLR-4                              | Lipopolysaccharides           |
| TLR-5                              | Flagellin                     |
| TLR-7                              | Single-stranded RNA           |
| TLR-8                              | G-rich oligonucleotides       |
| TLR-9                              | Unmethylated CpG DNA          |
| TLR-10                             | Unknown                       |

All TLRs are thought to act as homodimers, unless otherwise specified. Table adapted from Janeway’s Immunobiology, Seventh Edition [26].
Janeway was of that rare breed of scientists whose theoretical contributions are as well-known as their experimental ones. In his lifetime, he contributed to more than 290 publications, but his ideas undoubtedly influenced and informed many fold more. The field of immunology continues to benefit from his legacy.

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