The Biology Behind

Combining Innate Immunity With Radiation Therapy for Cancer Treatment
☐☐*Commentary on Mason et. al., p. 361*

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INTRODUCTION

The widely shared goal of cancer immunotherapy is to stimulate an immune response of sufficient quality and magnitude to destroy primary malignancies and their metastases. Cancer immunotherapy has taken many cues from the development of successful antimicrobial vaccines. Antimicrobial vaccines rely on the immune system's capacity to distinguish self-tissues from infectious non-self so that invading pathogens, and the cells they might infect, could be efficiently identified and eliminated, while sparing healthy tissues. The process of discriminating self from infectious non-self is facilitated by the millions (and in some cases billions) of years of evolutionary divergence that separates vertebrates from the pathogens that infect them. This separation has given rise to individual proteins and other generalized molecular structures that serve to distinguish microbes from men. In theory, malignant cells that express protein antigens that either are unique to the tumor, vastly over-expressed by the tumor, or whose expression is at least restricted to a narrow range of self-tissues provides a potential immunologic handle whereby tumors may be specifically recognized and destroyed. In practice, however, it has proven unexpectedly difficult to coax the immune system into vigorously rejecting malignancies, despite repeated demonstrations that tumor-associated antigens can provoke immune responses. In this issue of *Clinical Cancer Research*, Mason et al. (1) shows, using a murine subcutaneous and lung metastasis sarcoma treatment model, that synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs (characteristic of bacterial DNA) could be given with conventional radiation therapy to greatly augment therapeutic efficacy through an apparent immune-mediated mechanism.

CLASSICAL IMMUNOLOGY AND THE ADJUVANT EFFECT

Classical immunologists realized that for a protein immunogen to elicit a response from a vaccinated mammal, it must contain an element of "foreignness" to distinguish it from host tissues. This means that the immunogen protein must either not be produced by the vaccine recipient, or be from a source of sufficient evolutionary divergence to ensure sufficient primary sequence variations between homologous proteins. This requirement was later explained when details were elucidated for antigen processing and presentation by accessory cells (2), recognition of peptide/MHC complexes by T cells via the T cell receptor (3), which is randomly generated in a manner similar to that of immunoglobulin (4), and central or peripheral regulatory mechanisms that delete or inhibit T cell self-reactivity, although preserving the capacity to react against non-self proteins (5, 6). Later studies showed that activation of naïve T cells required not only a foreign antigenic signal supplied by the accessory (or antigen-presenting) cell, but also a second, or costimulatory signal supplied by antigen-presenting cell–expressed surface proteins designated, CD80 and CD86 (7).

Classical immunologists also recognized another vital ingredient for effective immunization, the true biological significance of which as only recently been explained on the molecular level, and that is the presence of an adjuvant during vaccine administration. Adjuvants are, by definition, materials that are added to a vaccine preparation that enhance the vaccine's immunogenicity. One of the most powerful known adjuvants is complete Freund's, which is a suspension of killed mycobacteria in mineral oil. The presence of killed bacteria in the adjuvant clearly enhances immunity against vaccine proteins, but whether this effect occurred primarily through an activation of unknown innate immune mechanisms, or through bystander activation of lymphocytes via strong bacterial antigens, is a subject for discussion.

INNATE IMMUNITY AND PATHOGEN-ASSOCIATED MOLECULAR PATTERNS

Immunologists have traditionally partitioned the immune system into "innate immunity", which encompasses the presumably more primitive elements of the system, including phagocytic, natural killer and antigen-presenting cells, and "adaptive immunity", which encompasses cells bearing randomly generated antigen receptors like T and B lymphocytes. Relatively recently, the late Charles Janeway and his colleagues advanced a remarkable and influential synthesis that described the nature of innate immunity and its relationship with adaptive immunity (8). It also made intriguing predictions regarding the existence of specialized pathogen-associated molecular pattern

(PAMP) receptors employed by agents of innate immunity. This view posits that the primary role of the immune system is to distinguish self-tissues from infectious non-self, and to eliminate infection without damaging self. This task is necessary even for relatively simple nonvertebrate organisms that possess innate immune systems, but not adaptive ones capable of generating antigen-specific lymphocytes. Such organisms must nonetheless be capable of recognizing the vast set of antigenically distinct potential pathogens using relatively few receptors. The most economical solution to this problem is the evolution of a small set of receptors that recognize (*a*) generalized PAMPs rather than unique proteins, (*b*) evolutionarily conserved patterns critical to basic microbial physiology and thus unlikely to vary, and (*c*) structures that are absolutely absent from normal host tissues so that a basis of discrimination can be achieved and maintained (8). Candidate patterns proposed at the time this paradigm was advanced included lipopolysaccharide (common to all Gram-negative bacteria), lipoteichoic acid (Gram-positive bacteria), unmethylated CpG DNA (common to all bacteria), double-stranded RNA (a replicatory intermediary for some viruses), and mannans (found in yeast cell walls).

Collectively, these PAMPs are capable of activating cellular components of the innate immune system leading to the secretion of proinflammatory and regulatory cytokines, chemokines, and in the case of antigen-presenting cells, the up-regulation of costimulatory and MHC molecules necessary for the primary sensitization of naïve T cells.

TOLL-LIKE RECEPTORS ARE ARCHTYPICAL PATTERN RECOGNITON RECEPTORS

In a landmark series of experiments that originally sought to determine the role of two genes designated Toll and 18-wheeler in *Drosophila* developmental biology, fruit flies knocked-out for either of these genes displayed remarkable susceptibility to either fungal or bacterial infections, respectively (9, 10). Investigators quickly connected this deficiency with a profound functional lesion in some fundamental pathway governing the innate immune system (invertebrates have no adaptive immune system). The hunt was then on for homologues in the mammalian immune system. Molecular cloning efforts rapidly turned up the first of a family of more than 10 related Toll-like receptors (TLR; 1-10+) with apparent homology to their invertebrate counterparts (11). Evidence quickly accumulated linking individual TLRs to known PAMPs (Fig. 1). For example, double-stranded RNA is recognized by TLR3 (12), the long-sought receptor for lipopolysaccharide turned out to be TLR4 (13), lipoteichoic acid and peptidoglycan is recognized by TLR2 (14), whereas DNA containing the unmethylated CpG motif is recognized by TLR9 (15). Interestingly, the receptors recognizing nucleic acids seemed to be primarily intracellularly oriented, whereas TLRs that recognized bacterial cell wall structures were largely surface-oriented, probably due to differences in the transmembrane and cytoplasmic domains of the TLRs (16). Although fine differences in signaling through TLRs are emerging, there exist great similarities among the pathways activated by the various TLRs (17). In general, engagement of a TLR by its associated PAMP
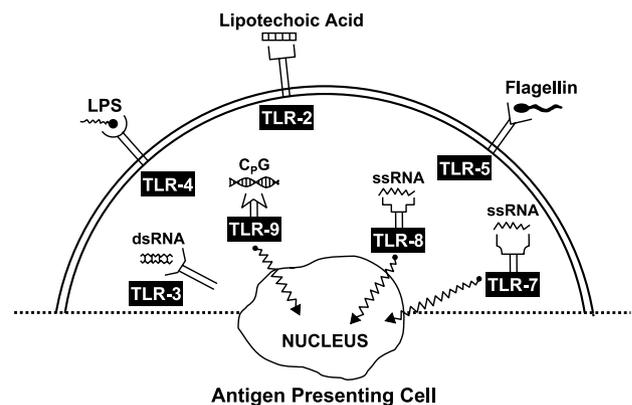

Fig. 1 Toll-like receptors (*TLR*) and their location in human antigen-presenting cells. *LPS*, lipopolysaccharide; *CpG*, unmethylated oligodeoxynucleotides; *ssRNA*, single-stranded RNA; *dsRNA*, double-stranded RNA.

passes a signal through the intracellular Toll/interleukin (IL)-1 receptor signaling domain to an adaptor protein (such as myeloid differentiation factor 88). Myeloid differentiation factor 88 then transduces the signal to a member of a family of IL-1 receptor–associated kinases. When IL-1 receptor–associated kinases are activated, a cascade of event ensues leading to the activation of tumor necrosis factor receptor–associated factor 6. This in turn activates IκB kinase, an activator of the proinflammatory nuclear factor NF-κB, as well as the activation of mitogen-activated protein kinase kinase kinase, which in turn activates c-*Jun* NH$_2$-terminal kinase, p38 kinase, and extracellular signal-regulated kinase kinase (Fig. 2). The activity of these proteins cooperatively leads to the induction of a host of proinflammatory cytokines (such as tumor necrosis factor-α, IL-6, and IL-8) as well as the enhanced expression of T cell costimulatory molecules on the surface of antigen-presenting cells (11).

BRIDGING INNATE AND ADAPTIVE IMMUNE RESPONSES

With the discovery of pattern receptors such as the TLRs, and their attendant specificities and signal transduction pathways, the findings of classical immunologists can at last be fully reconciled with modern molecular immunology. It would now seem that three separate types of signals are required to fully engage both innate and adaptive immunity, and allow their cooperation to produce optimized immune responses. The first signal, in chronological order, is provided by a PAMP. The pattern receptors, such as a TLR on the surface of a cellular component of the innate immune system (such as a dendritic cell), interacts with a molecular pattern of microbial origin, for instance, bacterial lipopolysaccharide. This signals the dendritic cells to secrete proinflammatory cytokines and chemokines and also greatly enhances the expression of costimulatory and MHC molecules. This signal is probably equivalent to the signal supplied by adjuvants such as complete Freund's adjuvant during artificial immunization. At the time of pathogen contact, the dendritic cells could also acquire protein antigens from the microbes that they then process and present on their surface in

conjunction with highly expressed MHC molecules. Dendritic cells that are thus activated also tend to migrate to draining lymph nodes where they have the opportunity to interact with T lymphocytes. Here, the dendritic cells concurrently supply the T cells with the other two signals, the antigen-specific signal (MHC/peptide) and costimulation (via CD80 and CD86). These two signals allow T cells of the appropriate antigen specificity to proliferate and attain effector function, which may include cytotoxicity and the capacity to supply "help" to B lymphocytes for antibody production. Thus, the first signal recruits the innate immune system, whereas the other two signals, supplied by innate immunity, recruits and directs adaptive immunity.

ELICITING IMMUNE RESPONSES AGAINST TUMORS

As stated above, the primary role of the immune system is to distinguish self-tissues from infectious non-self, and to eliminate the non-self, although generally sparing normal host tissues. Because most proteins found in tumor cells are identical to ones found in normal tissues, most possible antigenic specificities have probably been either eliminated by thymic selection or suppressed by peripheral tolerance. Opportunities exist, however, among the subset of proteins either uniquely expressed by tumors or narrowly restricted to particular tissues so that the potentially negative impact of

Fig. 2 Signaling pathways involved in Toll-like receptor activation. *TLR*, Toll-like receptors; *PAMP*, pathogen-associated molecular pattern; *LPS*, lipopolysaccharide; *CpG*, unmethylated oligodeoxynucleotides; *ssRNA*, single-stranded RNA; *dsRNA*, double-stranded RNA; *LTA*, lipoteichoic acid; *IL-1*, interleukin 1; *IL-1RI*, interleukin 1 receptor; *MyD88*, myeloid differentiation factor 88 adapter protein; *IRAK*, IL-1 receptor–associated kinases; *TRAF-6*, tumor necrosis factor receptor–associated factor 6; *IKK*, I κ B kinase; *MAP3K*, mitogen-activated protein kinase kinase kinase; *NF-κB*, proinflammatory nuclear factor NF-κB; *JNK*, c-*Jun* NH$_2$-terminal kinase; *p38*, p38 kinase; *ERK*, ERK kinase; *P*, phosphorylation.

thymic selection or tolerance may be minimized. However, under the best of circumstances, this provides only one of the three signals required for maximized immune responses (antigenic signal). It is very unlikely that a strong response can develop from this one signal in the absence of costimulatory and proinflammatory signals, and such signals are unlikely to be maximized without contact with a PAMP.

A logical way to enhance immunity against tumors is, therefore, to construct sensitization schemes where signals simulating microbial infection are supplied. The immune system is thus "deceived" into believing it is under microbial attack, and will respond with utmost vigor. Mason et al. (1) have used intratumoral or peritumoral injection of CpG DNA oligonucleotides, a powerful TLR9 agonist, to supply such a signal. TLR9 is expressed in mice on both plasmacytoid and myeloid dendritic cells (18, 19), on macrophages, and on B cells. As such, it can be expected to induce a host of proinflammatory cytokines such as tumor necrosis factor-α, IL-12, and IL-6, as well as enhanced expression of costimulatory molecules on dendritic cells. Plasmacytoid dendritic cells are also uniquely adapted to secrete high levels of IFN-α when exposed to TLR9 agonists (19).

TLR AGONISTS AS RADIATION SENSITIZERS

Although most radiation sensitizers have been aimed at increasing oxygen radical production in radiated tumor tissue, Mason et al. (1) utilize a TLR agonist to take advantage of several critical changes in tumor tissue induced by radiation therapy. In the experiments presented by Mason et al., administration of CpG ODN alone was insufficient to induce significant antitumor responses. Instead, radiation therapy and CpG ODN administration needed to be paired to observe dramatic antitumor effects. CpG ODN, when combined with radiation therapy, decreased the effective dose of radiation therapy needed to induce tumor regression. Animals cured of tumors using combination therapy were more resistant months after curative therapy to autologous tumor challenge than mice that were cured with radiation therapy only. It therefore seems apparent that a specific, and probably T cell–mediated immunity, is responsible for tumor rejection.

We have discussed how the administration of CpG ODNs likely contributes to this enhanced immunity. The role played by radiation therapy, however, is less definable at this time, and may be multifactorial. However, some clues are beginning to emerge. Irradiation of tumor cell lines induces several changes in expression of critical molecules involved in immune recognition and killing by T cells, including increased MHC molecule expression, increased adhesion molecule expression, and increased expression on tumor cells of the death receptor Fas (CD95; ref. 20). The role of the latter has been shown in a transgenic carcinoembryonic antigen tumor model, where elevated Fas expression in the tumor induced by radiation therapy contributed to the enhanced regression of tumor following anti–carcinoembryonic antigen vaccination (21). It remains unknown whether the enhanced response to radiation therapy reported by Mason et al. results from altered expression of these critical molecules, and these questions will undoubtedly be investigated as this type of therapy moves forward.
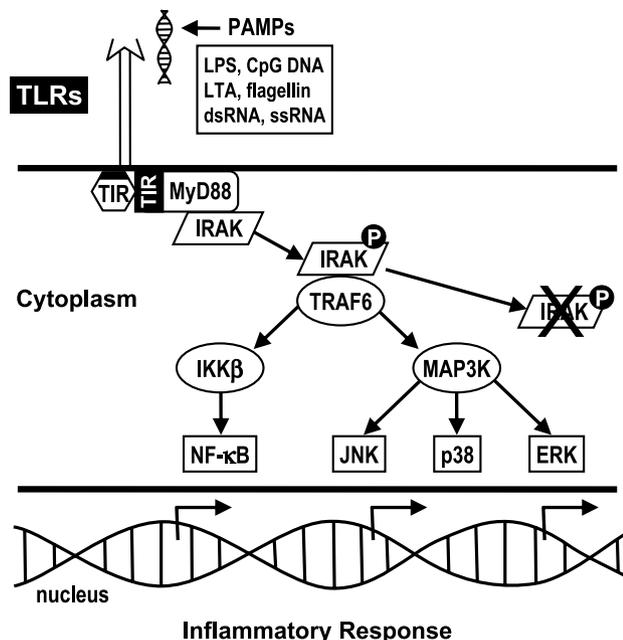
Indeed, additional mechanisms may also be involved. For example, radiation could damage and kill tumors directly, leaving the surviving cells more vulnerable to immune attack. Irradiation could also act by releasing tumor antigens for uptake by dendritic cells or other antigen-presenting cells, enhancing the antigenic signal, as CpG enhances costimulation. Evidence for this possibility (21) was shown by the development of T cell responses to p53 and gp70 tumor antigens after a carcinoembryonic antigen vaccine was combined with radiation therapy in treating an experimental colon cancer. It is also possible that radiation may curb a suppressor mechanism that otherwise inhibits strong antitumor responses.

CONCLUDING REMARKS

Vaccines and immunotherapeutic strategies directed against cancer should consider at least three separate types of signals which must be supplied to generate maximized immune responses; one directed at the innate immune system (PAMP), and the other two directed at the adaptive immune system (antigenic and costimulatory signal). Mason et al. have shown that local injection of CpG ODN, a TLR9 agonist, can dramatically enhance the therapeutic efficacy of radiation therapy through an apparent immune-mediated mechanism. This is a finding of practical importance, and it could be visualized that combinations of cancer vaccines with TLR agonists may induce tumor-specific CD4+ and CD8+ T cells, whose migration and killing activity would be enhanced by radiation therapy (Fig. 3). Combining radiation therapy and TLR agonists may reduce the amount of radiation therapy required to eradicate tumors, thus acting as an ''immunosensitizer''.

Important questions that must be answered by future studies include the mechanisms of immunity and how radiation therapy enhances this immunity. Another question relevant to future translation is the ability of CpG to work effectively in humans. DNA-based vaccines in humans have thus far fallen well short of murine preclinical studies, possibly due to differences in optimal CpG motif recognition (22). Also, TLR expression differences exist between mice and humans (18) with mouse plasmacytoid and myeloid dendritic cells expressing TLR9, whereas with humans, only plasmacytoid dendritic cells do (19). The biological implication of this difference remains unknown.

Interestingly, single-stranded RNA has recently been reported to act as a PAMP by signaling through TLR7 and TLR8 (23). These TLRs are not only expressed on myeloid dendritic cells, but their stimulation leads to secretion of high levels of IL-12 (24), a cytokine shown to enhance tumor recognition and killing of tumor by CD8+ T cells (25). Thus, efforts should not be wedded to a single TLR ligand, but to the general strategy of providing the correct signals to the innate and adaptive immune system to maximize responses. The selection of TLR ligand should be tailored to the biology of the organism to be vaccinated as well as to the quality of immune response desired. These strategies will benefit the development of more ''traditional'' vaccines as well as innovative combination therapies such as described by Mason et al.

Fig. 3 Schematic of combined cancer vaccine, Toll-like receptor agonist, and radiation therapy in the treatment of a malignant tumor.

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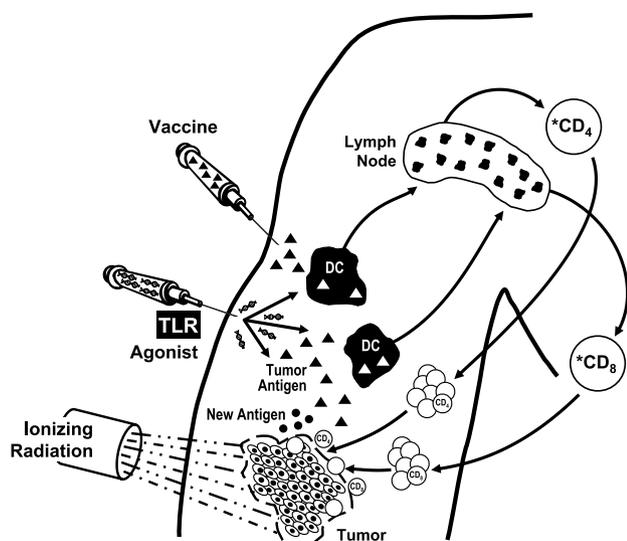
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