

A perspective on new immune adjuvant principles

Reprogramming inflammatory states to permit clearance of cancer cells and other age-associated cellular pathologies

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Aging entails the accumulation of neoantigens comprised of aggregated, oxidized, mutated and misfolded biomolecules, including advanced-glycation end products (AGEs). There is evidence that the immune system can recognize and clear cells fouled by these molecular debris, which contribute to the emergence of cancer and other major age-associated diseases such as atherogenic and neurodegenerative disorders. However, this process may become increasingly inefficient with aging, perhaps in part because of an insufficiency of adjuvant signals normally associated with infection that can program productive inflammatory states and properly orient the immune system toward regenerative healing. Here we propose conceptual foundations for exploring a small set of infection-associated molecules as potential immune adjuvants to reprogram non-productive inflammatory states in aging tissues, and to improve the clearance of cellular pathologies that engender age-associated disease. The proposed adjuvant classes include a subset of D-amino acids used by bacteria to disrupt biofilms; nucleoside derivatives of N⁶-methyladenine, which functions at the core of bacterial dam restriction systems; and derivatives of the galactosyl trisaccharide α -Gal, which invokes the hyperacute response in primates. These foreign amino acids, nucleosides and sugar molecules are generally rare or absent in humans, except in association with infections by bacteria, protists or nematodes. A rationale for exploration of

these candidate adjuvant principles and their chemical derivatives is discussed in terms of their use in generalized strategies to improve the prevention or treatment of cancer and other age-associated diseases, as negative modifiers of aging.

The immune system evolved to protect the host from hostile infections. However, it is also clear that host immunity benefits from the commensal effects of infectious organisms, especially from those that inhabit the digestive system. In confronting foreign antigens, the immune system has evolved molecular systems to interpret some foreign biomolecules that appear widely in infectious organisms as signals to stimulate and orient the inflammatory response. In this role, the Toll-like receptor (TLR) system recognizes pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, flagella and methylated DNA structures that are common in the bacterial kingdom. PAMPs confer potent actions harnessed in some cases historically for vaccine adjuvants, such as the tuberculin TLR3 ligands in Freund's complete adjuvant. Thus, during an infection, the immune system not only encounters foreign antigens but also infection-associated signaling molecules that act as immune adjuvants in the host.

This two-armed system to interpret foreignness informs the ultimate stance of the immune system concerning whether to tolerate or eliminate the foreignness. Sterile and sometimes chronic inflammations created by injury, aging or disease lack adjuvant signals associated with

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Table 1. Characteristics of novel candidate immune adjuvants

Candidate adjuvant	Proposed, molecular targets	Proposed cellular targets	Source	Endogenous function
D-amino acids Leu, Met, Phe, Trp	• Amino acid-tRNA synthetases	T cells B cells Myeloid cells Epithelia Endothelia	Bacteria	Biofilm disassembly
Nucleosides N6-methyladenine (m6A) and derivatives	• Ectonucleosidases CD39, CD73 • ATP receptor P2X7	T regulatory cells Epithelia	Bacteria Protists	DNA modification- restriction (dam restriction)
α -Gal*	• Anti- α -gal • Galectins Galectin-3 Galectin-1	Myeloid cells	Protists Nematodes	Hyperacute response
α -GalCer (α -Galactosylceramide)	• CD1d	NKT cells	Sponge	Unknown

*Galactose- α 1,3-Galactose- α 1,3/4-GalactoseNAc.

infection. In this case, damage-associated molecular patterns (DAMPs), such as heat-shock proteins and HMGB1, that are released with cellular lysis, may interact with TLRs or other receptors to direct inflammatory processes via pathways that are poorly understood but may differ from those engaged by PAMP signals. One major characteristic of sterile inflammation associated with cancer and perhaps aging is a chronic “smoldering” nature which is associated with a subversion of immunity that impedes the clearance of cellular pathology.¹ Through their ability to stimulate the immune system, infection-associated adjuvant molecules may correct deficiencies in the clearing potential that exists, perhaps inherently due to an unselected nature during evolution for responses to age-associated cellular pathologies that emerge only after reproductive processes of the host are complete.

Neoantigens are abundant in aging due to the accumulation of oxidized, mutated, aggregated and generally dysfunctional biomolecules. These neoantigens arouse the immune system, as illustrated by the appearance of macrophages at the sites of atherogenic lesions in the blood vasculature, and cells harboring such debris may be cleared to some extent. However, the absence or insufficiency of infection-associated foreign biomolecules at such sites may be associated with the generation of chronic low-grade inflammatory states that are inefficient in clearing cellular pathologies, permitting the development

of age-associated disease states such as the accumulation of foam cells at vascular lesions that promote thrombotic events.

Could the administration of infection-associated biomolecules complement this inefficiency by reprogramming the inflammatory state stimulated by age-associated neoantigens? In cancer, there is a long history behind this idea, as first recorded in the modern era of medicine by Chekhov in the 1880s with regard to long-observed correlations of infections with tumor regressions. In the US after the turn of the 20th century, Coley investigated this association carefully, eventually settling on a virulent combination of *Streptococcus pyogenes* and *Serratia marcescens* that could trigger dramatic regressions in some patients, including those with advanced metastatic cancer. Controversies about this toxic treatment caused it to fall out of favor by the 1960s, with new laws enlarging the powers of the FDA at that time, allowing it to prevent the use of Coley's toxin outside of trials. More recently, infection-associated molecules underlying the effects Coley observed, which were later better documented, were explored further in cancer patients as immune adjuvants, for example, CpG oligonucleotides that stimulate TLR9 signaling. However, it is now becoming increasingly clear that immune adjuvants can be conceptualized more broadly, to include not only molecules that stimulate the immune system, but also those that relieve immune inhibitory mechanisms. Indeed, advances in

elucidating these mechanisms during the last decade opens opportunities to re-evaluate historical perspectives on the nature and use of immune adjuvants, not only for applications in cancer therapy but also for the prevention or treatment of age-associated diseases more generally, in which tolerance to neoantigens is also apparent.

In cancer studies, protist infections appear to have been much less considered than bacterial infections as a source of foreign biomolecules offering potential immune adjuvant activity. The protist kingdom encompasses all single cell eukaryotes, including “animal-like” protozoa (e.g., cryptosporidia, trypanosomes, toxoplasmae), “plant-like” algae (e.g., rhodophyta, chlorophyta) and “fungal-like” molds (e.g., candida). In developed countries, human infections with protists are not common, due to improved sanitation. Of relevance to cancer, organisms in this kingdom exhibit far greater antigenic diversity and variation than bacteria during their life cycles, and some protists (e.g., parasites such as trypanosomes) exploit antigenic variation as effective strategies to escape immune control, similar to what occurs in cancer immunoevasion. Proposed adjuvant principles discussed in this perspective are listed in Table 1.

D-amino Acids

Recently, an interesting intersection occurred in unrelated studies of immune

modulation and biofilm disruption. Biofilms are the amyloid fibrous structures used by bacteria to organize communities. Disassembly of biofilms by *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* was found to be mediated by a mixture of D-amino acids secreted by many bacteria, including D-leucine, D-methionine, D-tyrosine and D-tryptophan.² Unlike L-amino acids, which are synthesized and used universally in protein translation, D-amino acids appear to be synthesized only in the bacterial kingdom, where their appearance in peptidoglycans that are secreted helps bacteria adapt to changing environmental conditions.³

With regard to D-tryptophan, its identification as a biofilm-disrupting molecule is interesting because of extensive investigations of the immune modifier effects of its simple derivative D-1-methyl-tryptophan (D-1MT), a molecule that has been studied preclinically and clinically as a small molecule inhibitor of the indoleamine 2,3-dioxygenase (IDO) pathway of immune modulation. Based on its tryptophan catabolic activity, IDO is regarded as an immunosuppressive principle that generates antigenic tolerance, but studies in genetically deficient mice indicate that IDO might act more directly as a modifier of the inflammatory microenvironment in cancer, autoimmune disease and infection.⁴⁻⁹ Although the relevant biochemical targets of D-1MT in immune modulation have yet to be understood completely, genetic investigations in mice clearly establish the dependence of D-1MT activity on the IDO pathway.¹⁰ In particular, recent work demonstrates that nanomolar concentrations of D-1MT are sufficient to restore tryptophan sufficiency signaling to mTOR after tryptophan deprivation by IDO (R.M, G.C.P. et al., manuscript submitted). These findings suggest that sensors of D-amino acids might exist to modulate inflammation and immune status by influencing the activation of mTOR, a pivotal player in immune control. The evolution of such sensors in the host would be valuable, since biofilm degradation could exert major impact on the host as a bacterial community switches from a localized organization (e.g., abscess) to a dispersed organization (e.g.,

septicemia). In this context, it is intriguing that leucine amino acid-tRNA synthetase (LeuARS) was defined recently as a sensor of essential amino acid sufficiency for mTOR control.¹¹ Thus, it is intriguing to consider whether D-amino acids used in the bacterial kingdom to modulate biofilms may exert immune adjuvant properties through ARS interactions that could program mTOR-dependent pathways of inflammatory programming and immune activation. Drawing parallels from D-1MT studies, it may be interesting to consider whether the biofilm-disrupting compounds D-leucine, D-methionine or D-tyrosine or their methyl derivatives analogous to D-1MT may function as mTOR regulators or immunomodulators.

N6-methyladenine

Adenosine is a focus of growing interest as a modulator of host immune responses,¹² but variations in the adenine moiety have not been widely considered in terms of their potential immunological impact. In cancer, there is persuasive evidence that the benefits of effective chemotherapy relate in part to the autophagy-dependent release of ATP from dying cancer cells, which can exert an immune adjuvant effect on antigen-presenting cells.¹³⁻¹⁷ Adenosine signaling appears to exert a significant effect on immunoeediting during oncogenesis with potential for therapeutic applications.¹⁸ The purinergic receptor P2X7 is an ATP receptor that has captured particular attention given its overexpression in cancer cells and its implication in cancer progression.^{19,20} Ectonucleotidases CD39 and CD73 that degrade ATP/ADP to AMP and AMP to adenosine, respectively, are widely expressed in tumors and they have been implicated in malignant progression,^{21,22} possibly also upon their expression on tumor-derived exosomes.²³ These molecules may also function on regulatory T cells (Tregs) and vascular cells in the tumor microenvironment.^{24,25}

In the setting of adenosine signaling, the possible immunological impact of N6-methyladenine (m6A) should be considered in the contexts of ribose or deoxyribose forms of adenosine or ATP. m6A is found in the genomic DNAs of bacteria, protists and fungi. In bacteria,

m6A represents the root element in *dam* restriction-modification systems, which regulate virulence and serve as a rudimentary immune system.²⁶ Some protists also employ m6A in DNA as a protective measure against invading infection. However, the precise extent and impact of m6A in eukaryotic DNA is unclear.²⁷ In contrast, m6A appears to be broadly expressed in eukaryotic RNA, where it clusters mainly at 3'UTRs, stop codons and microRNA targets.²⁸ Great interest has emerged in m6A-containing RNAs in studies of obesity, which has a major inflammatory component, because of evidence that major risk conferred by the *FTO* gene is associated with m6A demethylation in cellular RNA.²⁹ From an evolutionary standpoint, it is intriguing to consider whether the impact of adenosine signaling in immunology might relate to the detection of m6A derivatives appearing in bacterial and protist DNA or in certain RNA contexts. In this regard, it is interesting that N6-methyladenine exhibits a shock-like toxicity in mice (unpublished observations). While speculative, m6A represents a rational twist in explorations of how to best exploit adenosine signaling for inflammatory reprogramming.

α -Gal

The trisaccharide galactose- α 1,3-galactose- β 1,3/4-galactose-Nac known informally as α -gal decorates the exterior of all eukaryotic cells, including protists, with the notable exception of humans, apes and Old World primates. In its absence from humans, α -gal plays an important role in triggering the hyperacute immune response, which was originally identified by studies of tissue xenotransplantation.³⁰ The reasons behind the curious evolution of this response are uncertain, but it is tempting to speculate that this trisaccharide plays an important role in immune responses to protist infections, since ~1% of all antibodies present in human serum recognize α -gal epitopes that are presented by any non-human eukaryotic cell or eukaryotic cell-derived virus or parasite. α -gal is unrelated to the galactose moiety found in α -galactosylceramide, a sponge-derived biomolecule that acts as a stimulatory ligand for NKT cells. There is

additional evidence that α -gal can engage the immune system, based on reports of delayed but acute allergic responses in a small number of individuals who consumed red meat or received treatment with the anti-epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab, which is modified with α -gal due to its production from murine SP2/0 cells (which express the α -gal transferase). In both cases, IGEs specific for α -gal were implicated in the delayed anaphylactic response that occurred in these individuals.^{31,32} While the mechanisms underlying these phenomena are poorly understood, they underscore the notion that α -gal can program a robust immune response.

Strategies to harness endogenous α -gal antibodies to generate productive anticancer responses have been pursued, based on engineering α -gal decoration of tumor whole cell vaccines for cancer immunotherapy.³³ Clinical tests based on preclinical findings appear to validate the efficacy of such an approach. Results from a Phase II clinical enrolling 70 resected pancreatic cancer patients treated with a whole-cell allogeneic vaccine termed HyperAcute Pancreas that was generated from a mixed panel of human pancreatic tumor cell lines have been reported recently.³⁴ Significant increases in survival were associated with acute inflammatory responses triggered by vaccine injection, which appeared to be sufficient to trigger cross-presentation of tumor antigens as well as degrade immune escape. Notably, several patients who subsequently relapsed with metastatic disease all exhibited complete responses to salvage chemotherapy.³⁴ This is a remarkable observation given that relapsed pancreatic cancer is invariably insensitive to chemotherapy. While the inflammatory program triggered by HyperAcute vaccines is not well understood, these early clinical findings suggests that α -gal may help to durably retrain the immune system in a manner that can support effective chemotherapy. Another intriguing observation was eosinophilia in most responding patients.³⁴ Eosinophils attack helminth infections (nematodes). Thus, productive inflammatory responses to α -gal may program the immune system to interpret tumor antigens as a parasite infection, to which a robust response can be suitably

framed. Extending the concept that α -gal can be employed as an immune adjuvant, the administration of α -gal in the context of an engineered glycolipid has been reported to exert not only anticancer effects but also to accelerate regenerative healing of burn wounds.^{35,36} In the latter setting, macrophages but especially neutrophils were efficiently attracted by a topical liposome formulation of the engineered α -gal glycolipid that increased re-epithelization. These results speak to the potential use of α -gal as an adjuvant beyond cancer in distinct settings of regenerative healing.

In assessing targets for α -gal as an immune adjuvant, there are candidates among the galactose-binding lectins known as galectins, the elevation of which is an established cause of tumoral immune tolerance and progression.³⁷ Direct evidence for galectin-3 binding by α -gal has been presented.³⁸ Galectin-3 modulates T-cell immunity to promote immunoevasion and metastasis in cancer and its immunohistochemical status has been associated with poor prognosis.^{39,40} This galectin has also been implicated in epithelial wound healing,⁴¹ a setting in which α -gal has been shown to exert beneficial effects.^{35,36} Galectin-1 has also been linked to immunoevasion in cancer⁴² and this molecule may also bind α -gal (G. Rabinovich, personal communication). Given the widespread expression of galectin-1 in protists and eukaryotes, its function in hyperacute responses and its promising utility in cancer vaccines, α -gal represents a logical candidate for further exploration as a general immune adjuvant.

“Whole-Adjuvant” Combinations for Exploring the Prokaryotic-Protist Microbiome to Treat Cancer and Age-Associated Disease

Coley’s investigations sought to stimulate immunity to eradicate tumors, but the tools he settled on were derived exclusively from prokaryotes. Would a consideration of protist infections have affected his outcomes? It is evident that intestinal nematodes and prokaryotes interact in the gut and that these interactions can impact on host immunity.⁴³ However, there has been little parallel consideration of the protist

microbiome as a source of immune adjuvant molecules. α -gal may be relevant in this context as an initial opportunity to frame combinations of immune adjuvant molecules that may be evolutionarily relevant but normally absent in hosts in modern times due to improved sanitation and antiprotist medicines. While it is speculative that the candidate adjuvants proposed here may be useful to reprogram inflammation in the context of cancer or other age-associated disorders, a rationale to build new concepts from common infection-associated signals that engage host immunity is logical. Along with α -galactosylceramide (α -GalCer), a ligand for NKT cells⁴⁴ derived from a marine sponge (the simplest multicellular organism beyond protists), one can consider combinations of the amino acid, nucleoside and carbohydrate moieties associated with prokaryote and protist infections discussed above as a “diamond adjuvant” to immunologically pierce the veil of age-associated diseases that present an abundance of neoantigens, but in the absence of infection-associated elements needed for effective immune clearance. Indeed, if the efficacy of chemotherapy and radiotherapy relates to “vaccinating” properties and ATP release (to stimulate adenosine signaling), it is intriguing to consider whether such a “diamond” adjuvant might be combined with low-toxicity vaccines to reduce reliance on high-toxicity therapeutics in cancer treatment. In any case, it is clear that better molecular definitions of the “flavors” of inflammation are needed to conceptualize how to convert “smoldering” chronic inflammations to productive clearing inflammations, so the immune system can respond to neoantigen “foreignness” in an infection-like context that is more evolutionarily relevant.

It is striking to consider the remarkable boost that suitable adjuvants provided to prophylaxis of infectious disease by vaccination in the 20th century. By analogy, similar successes in cancer and age-associated disease might be assisted in the 21st century with novel adjuvants that can permit the immune system to interpret “sterile antigenic” disorders as “infectious antigenic” disorders that immunity has already evolved to handle. With regard to cancer, it is tempting to wonder how

Coley's toxin experiments might have developed further with the inclusion of protists and nematodes in his therapeutic studies of bacterial infections in cancer patients over a century ago.

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Disclosure of Potential Conflicts of Interest

G.C.P. declares a conflict of interest as a compensated scientific advisor, grant recipient and shareholder with interests in New Link Genetics Corporation, which is

engaged in the clinical development of IDO pathway inhibitors and HyperAcute cancer vaccines. G.C.P. is also an inventor on IDO technology patents held by his employer that have been licensed by this company.

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