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Molecular basis of applied biological therapeutics

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Introduction

Among the major medical advances of the 20th century was the introduction of biological agents that revolutionized the therapy of diseases affecting millions of patients. Thus, began a new era of highly selective antibody- and receptor-based drugs that target the host-derived pathogenic mediators. These practical advances became possible because of a series of important discoveries in the fields of cytokine biology, monoclonal antibody development and molecular biology. The 7th Axel Key symposium on 'Molecular basis of applied biological therapeutics,' held in Stockholm, Sweden, in September 2010, provided the opportunity to discuss the events underlying these advances. Leading contributors to this field reviewed the science of biological therapeutics and presented their vision for what new developments may follow. The current volume of *The Journal of Internal Medicine* includes a collection of reviews written by these accomplished investigators, who contributed a series of important reviews that highlight the current state of this field and reveal a glimpse at what the future holds in store. The papers are equally valuable as summaries of the events leading to the development of these agents, and as scientific updates on the current status of this important field, which promises to provide some surprisingly new treatments in the future.

Overcoming failure: blocking TNF with antibodies, or shutting it down with erythropoietin

Early work on TNF by tumour biologists raised hopes that administering this cytokine would hold the key to cure cancer. Subsequent discoveries that TNF was both necessary and sufficient to mediate inflammation and organ damage proved that its actions were not tumour selective, as had been hoped. While disappointing in the context of cancer, these findings enabled the development of TNF inhibitors for other diseases mediated by the

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excessive action of this cytokine. Anthony Cerami describes in this volume [1] his discovery of TNF in the context of inflammatory diseases, nearly three decades ago. He and his colleagues unravelled the biology of this inflammatory mediator and developed reagents that neutralized its activity to prevent tissue damage in the preclinical models of disease. These advances ushered in the era of cytokine-based antagonists, including monoclonal anti-TNF antibodies that are now widely used to treat rheumatoid arthritis, inflammatory bowel disease and additional conditions. Dr Cerami then describes his recent work following his discovery that erythropoietin (EPO) confers tissue protective activity by blocking TNF synthesis [2]. These actions are independent of the erythropoietic functions of EPO, because the TNF-inhibiting activities are mediated by EPORII, whereas EPORI mediates erythropoiesis and platelet activation. Phase 2 clinical trials using an EPO peptide to confer tissue protection are underway, suggesting that some day it may be possible to modulate TNF using pharmacological agents that mimic the protective regulatory actions of EPO. Dr. Cerami's review personalizes these stories by placing them in the context of experimental 'failures' that ultimately led to the current use of these agents. He describes with captivating prose the discouragement that follows a research failure and recalls that this can be turned into success using an open mind, and a great portion of stubbornness. In his words, 'I hope that students reading this paper will use the lesson of these examples to help alleviate the pain of failing, and give them courage to keep pursuing their goals.'

Neural reflexes that inhibit TNF: innervated immune responses and development of devices to replace biologicals

Kevin Tracey collaborated with Anthony Cerami in the discovery of TNF's direct inflammatory action and the potential to treat inflammatory disease with monoclonal anti-TNF antibodies. The knowledge that TNF is both necessary and sufficient to cause disease raised an important new question: how does the body regulate the activity of TNF to maintain health? Tracey and his colleagues provided the answer by discovering the inflammatory reflex, a neural circuit that inhibits TNF and other cytokines to prevent the development of tissue injury and organ damage [3]. This surprising finding revealed that fundamental immune responses are controlled by neural circuits. The immune system is now recognized to be an innervated organ, controlled by action potentials, in a manner that is analogous to the heart and other innervated organs. As outlined in this volume, this neural mechanism that suppresses the innate inflammatory response has direct implications understanding the pathogenesis of acute and chronic inflammatory conditions and for the development of devices that may replace biological agents in some patients [4].

The key finding is that the immune system, like all other physiological systems, is continuously monitored and synchronized by neural reflexes regulated by the central nervous system. The inflammatory reflex is comprised of an afferent arc that senses inflammation and an efferent arc, the 'cholinergic anti-inflammatory pathway,' that confers a cytokine-inhibiting signal to the innate immune system in the spleen and other organs. The molecular mechanism is dependent upon action potentials transmitted in the vagus nerve that culminate on the α7 subunit of the nicotinic acetylcholine receptor to inhibit NF-kB nuclear translocation and suppress cytokine release by monocytes and macrophages. In basal

conditions, the cholinergic anti-inflammatory pathway exerts a tonic inhibitory influence on the innate immune response to inducers, and loss of this inhibitory pathway enhances the risk of excessive TNF release and damage. Importantly, Huston and Tracey review clinical evidence that vagus nerve signalling is dysfunctional in patients with inflammatory diseases, with potential to exacerbate inflammatory damage. They also discuss the potential for measuring vagus nerve activity by monitoring heart rate variability as a means to identify patients that may benefit from pharmacological or electrical pacemaker stimulation of the cholinergic anti-inflammatory pathway. A potential future biological use of this knowledge is summarized in the paper: 'similar to tracking haemoglobin A1c levels in patients with diabetes, or blood pressure monitoring in patients with hypertension, heart rate variability monitoring could be used for cytokine-mediated diseases.' It is fascinating to consider these findings from the perspective that neurostimulating devices, which provide a pacing signal to the immune system, may one day augment or replace biological therapeutics.

Successful therapeutic IL-1β targeting in several major clinical disorders

Charles Dinarello, father of the IL-1 field from his early work studying endogenous mediators of fever, now reviews novel advances [5]. Until recently, progress in clinically targeting IL-1β had been hampered by the unavailability of efficacious anti-IL-1β monoclonal antibodies. Studies performed with IL-1 receptor antagonist (IL-1RA) suffer from biological limitations imposed by a short *in vivo* turnover of the molecule and the fact that IL-1β induces its own production. In this volume, Dinarello recounts that this scenario is now improved, and a number of successful clinical studies have been conducted based on a newly available neutralizing monoclonal antibody in diseases including autoinflammatory conditions, gout, diabetes type 2, heart failure, indolent myeloma and additional diseases. Each of these conditions is IL-1β dependent, some of them rather unexpectedly, and antibody-mediated depletion of IL-1β may represent distinctly new therapeutic possibilities.

Dinarello then reviews the basis of IL-1β cytotoxicity in the context of the insulin-producing pancreatic β-cells: high glucose concentrations stimulate IL-1β production from the β-cell itself, implicating a possible role of IL-1β in both diabetes type 1 and 2. Administration of IL-1β mAb to patients with diabetes type 2 resulted in sustained HbA1c reductions apparent even 3 months after a single injection. In addition to improving glycemic control, additional benefit was obtained by evidence that treatment reduced inflammation systemically and in adipose tissue. Large studies in diabetes type-2 are ongoing and planned to evaluate whether prolonged anti-IL-1β mAb therapy will decrease insulin resistance and restore β-cell function. Recent trials with IL-1β blockade in gout also reveal sustained reduction in the number of recurrent arthritis attacks. Strong intra-articular IL-1β production in gout is caused by a combination of monosodium urate (MSU) crystals and saturated free fatty acids (FFA) and not by MSU on its own. Interestingly, MSU initiates IL-1β transcriptional activity and saturated FFA interacting with TLR2 provide a second signal for translation. In the field of osteoarthritis, IL-1β-deficient mice are protected from inflammation-induced cartilage loss, and it is likely that clinical trials for this indication will be illustrative. Taken together, Charles Dinarello presents a strong case here that therapeutic strategies to counteract IL-1β using efficient specific antagonists continues to hold promise in a number of important diseases.

IL-33 as a multitalented cytokine

Iain McInnes and coworkers review the history and biology of IL-33 (also known as IL-1F11) as a newly identified member of the IL-1 family [6]. It is a nuclear protein, primarily expressed in epithelial and endothelial cells. It is released by stressed or dying cells, where it functions as a cytokine and alarmin signalling danger to neighbouring cells. As an alarmin, IL-33 preferentially induces the formation of Th2 cytokines; this is contrasted with another alarmin discussed at the symposium, HMGB1, which preferentially stimulates the production of classical proinflammatory mediators and Th1 cytokines. IL-33 derived from necrotic cells is biologically active, whereas IL-33 released from apoptotic cells is inactive because it is cleaved by caspase 3, 7 and calpain. IL-33 signals via a heterodimer receptor complex (ST2) composed of an IL-33-specific ST2L molecule coupled to the IL-1R accessory protein (IL-1 RAcP). The pleiotropic bioactivity exerted by IL-33 is primarily mediated by interaction with tissue-specific resident macrophages, mast cells and T cells of the Th2 cell lineage. IL-33 has been implicated in the development of atopic diseases, because it facilitates mast cell degranulation. And it induces epithelial hyperplasia and mucus production in the lungs, suggesting a possible pathogenetic role in asthma. Alveolar macrophages activated by exposure to IL-33 from respiratory epithelial cells switch phenotypes to the alternatively activated form (M2) associated with chemokine production, asthma pathology and activation of Th2 lymphocytes with IL-5 and IL-13 release. IL-33 is also overexpressed in the synovial tissue of patients with rheumatoid arthritis, and animal studies indicate that IL-33, acting together with IgG complexes, leads to the degranulation of synovial mast cells and associated IL-17 release, with important pathogenic consequences. Together the results reviewed here indicate that IL-33 contributes to the pathology of asthma, allergy and rheumatoid arthritis.

In contrast to TNF and IL-1, IL-33 may exert protective effects in obesity and atherosclerosis. IL-33 is present in adipocytes and its expression is increased in response to hypoxia and TNF stimulation occurring in the adipose tissue inflammation with infiltrations of proinflammatory macrophages (M1) and Th1 lymphocytes. IL-33 amplifies M2 macrophage differentiation to play a protective role in adipose tissue homoeostasis. IL-33 may in addition decrease the expression of resistin, a molecule that mediates the development of insulin resistance and type 2 diabetes. The IL-33/ST2 pathway also protects against atherosclerosis in animal models and directly inhibits foam cell differentiation in the atherosclerotic plaques. The local vascular formation of Th1 cytokines is skewed towards the production of Th2 cytokines that inhibit arterial inflammation. As elegantly summarized in this review, it will be interesting to see whether the future holds promise for either inhibiting or stimulating IL-33 activity as a strategy to treat clinical disorders.

Antibodies produce behaviour: Pathogenic autoantibodies are NMDA agonists and can be therapeutically targeted in systemic lupus erythematosus (SLE)

Betty Diamond and her coworkers review their important discovery of antibodies that modulate neural function and alter behaviour [7]. This surprising new field, which has

profound implications for understanding the natural history of inflammatory and autoinflammatory diseases, grew from their studies of SLE, a chronic autoimmune disease caused by autoantibodies and immune complexes. Patients express a major class of autoantibodies directed against double-stranded DNA (dsDNA), and they found that many of these antibodies cross-react with protein antigens present on neurons expressing glutamatergic N-methyl-d-aspartate receptors (NMDAR). Of great surprise to many, Diamond showed that these antibodies cross the blood–brain barrier and function as agonists to the NMDA receptor, accordingly mediating severe brain damage, particularly in the hippocampus. Antibody concentrations and activity are measurable in the central nervous system (CNS) of patients with lupus, where they modulate NMDAR activity that impairs cognitive functions, generates mood disorders and may cause neuronal death. The elegant strategy presented here is based on the generation of soluble, monomeric small peptides that specifically bind and occupy the antigen-binding sites of pathogenic antibodies preventing them from interacting with, in this case, NMDA receptors and dsDNA. They have been able to produce a peptide (termed D-peptide) comprised of just five amino acid residues which is target selective and confers therapeutic benefit to prevent neuronal cell damage in a mouse CNS lupusmodel.

There are direct possibilities for considering future therapeutics. It is critical that the peptide is stable in its monomeric structure, as multimeric peptide formations will be immunogenic and boost pathogenic antibody formation. The intriguing results of these preclinical studies indicate that it may be sufficient to block only a subset of dangerous autoantibodies to ameliorate disease, which may avoid systemic immunosuppressive treatment. Additional results of potential clinical development come from the studies of pregnant mice with high titres of anti-NMDAR antibodies. The foetal brain is particularly vulnerable to autoantibodies, and exposure of foetuses *in utero* to maternal antibodies crossing the placenta results in the development of brain damage, altered neural circuitry and functional consequences. Therapeutic intervention by administration of the peptide protected the developing foetal brain from the toxic effects of maternal anti-NMDAR antibodies when mothers were treated with the D-peptide during gestation. As this story unfolds here, one cannot help but be drawn into considering whether this novel therapeutic strategy will be successful in SLE and in a spectrum of other diseases characterized by autoantibodies that alter brain function and behaviour.

Immunotherapy in Alzheimer’s disease

David Morgan reviews work indicating that immunotherapy may prevent or treat Alzheimer’s disease, the most common form of organic dementia affecting 10% of the population over 65 years and 40% over 85 years in the United States [8]. The prevailing hypothesis of pathogenesis is that there is an initial accumulation of amyloid aggregates in the brain, composed of Aβ peptide, a process that seems necessary but not sufficient for disease. A second step is the formation of intraneuronal neurofibrillary tangles generated from hyperphosphorylated structures of the microtubule-binding protein tau and Aβ peptide. Recent advances in imaging technology have enabled the identification of the amyloid brain depositions without a need for biopsies, which is of great help to identify relevant patients for inclusion in future studies. One bit of critical information emerging from these positron

emission tomography studies is that brain deposits precede disease onset by a long period of time, perhaps as long as a decade, theoretically providing a wide therapeutic window for successful intervention. Targeting the Aβ peptide has been a primary goal and common denominator in the development of preventive and curative immunotherapy in Alzheimer's disease. Until now, active immunization strategy against Aβ peptide has shown promising results both in mice and in men, but side effects in some patients included autoimmune brain inflammation [9].

Therefore, Dr Morgan's review addressed alternative approaches. Passive immunization with anti-Aβ peptide antibodies in both mice and men has demonstrated substantial parenchymal amyloid clearance. This was limited by occasional cases of microhemorrhage and vasogenic oedema in some patients, particularly those with the apolipoprotein E4 genotype. Accordingly, identifying antibody variants that retain amyloid clearance with fewer adverse reactions remains a major focus of translational research in this area. The field is now eagerly awaiting results of several ongoing large anti-Aβ monoclonal antibody trials in phase 3 for more information regarding effects on clinical symptoms and side effects. In this compelling discussion, Morgan recounts that a positive outcome would represent a major breakthrough in this devastating condition and an important test of the amyloid hypothesis of Alzheimer's disease.

Dendritic cell-based vaccines as cancer therapy

Karolina Palucka and Jacques Banchereau review the development of therapeutic cancer vaccines based on *ex vivo* generation and antigen loading of dendritic cells (DC) from patients with cancer [10]. After standard cancer therapies have reduced major tumour mass, immunotherapy may offer a possibility to eliminate residual cancer cells to prevent disease recurrence. Now, for the first time, a therapeutic autologous vaccine based on this principle has been used to treat prostate cancer (Provenge) and was approved earlier this year by the FDA. General problems in patients with advanced cancer that preclude tumour elimination by their autologous immune system include endogenous DC dysfunction, expansion and activation of inhibitory tumour-associated regulatory T cells (Tregs), myeloid-derived suppressor cells and suppressive mediators in the tumour micro-environment. The major challenge with *ex vivo* generation of DC is to design conditions to expand DC that will optimally support adaptive immunity expressing strong cytolytic effector functions combined with tumour-relevant homing capacity. Increasing knowledge about DC biology regarding the identification of specialized DC subsets with functional plasticity has advanced this field and provided a rational for the research summarized in this review. As the field advances, there is increasing interest in the possibility that DC vaccines can be developed by identifying and defining tumour-specific antigens. This is a dynamic research field, holding the potential to benefit patients by combining advances in tumour proteomics with a better understanding of DC biology.

Fusokines to treat cancer or autoimmunity

Jaques Galipeau and his colleagues have pioneered the novel field of 'fusokines,' hybrid molecules generated after cloning and fusing two separate cytokine-encoding cDNAs into a

single open reading frame. They have thus far created three different GM-CSF/interleukin fusion cytokines (GIFTs) that act as entirely novel molecules [11]. The biological functions of these GIFTs are both expected and unexpected, results that could not have been predicted from a hypothesis-driven approach using knowledge about their isolated, unfused components. The therapeutic objective of this research is to use the molecules *ex vivo* to differentiate and propagate effector cells from patients prior to administration *in vivo* as personalized cellular therapy in cancer or autoimmune diseases.

Thus far, the work has been restricted to murine experimental models. Combining GM-CSF with IL-2 (GIFT-2) or IL-21 (GIFT-21) can induce immune responses by activating complimentary elements of the antigen-presenting system and the cytotoxic cell system to eliminate cancer cells. Irradiated tumour cells engineered to secrete GM-CSF have previously been demonstrated to induce long-term tumour immunity in experimental cell vaccine models. In the current studies reviewed here, tumour cells or normal cells were transfected with genes encoding for the different GIFTs and the functional outcome studied *in vivo* and *in vitro*. GIFT-2 experiments demonstrated strong anti-tumour activity caused by enhanced $CD8^+$ T-cell and NK cell activity. Control experiments with combined unfused GM-CSF and IL-2 showed only low corresponding activity, which is not remarkable because GM-CSF is known to downregulate NK cell functions. This demonstrated that GIFT-2 had acquired an unanticipated gain of function downstream of its interaction with the IL-2R signalling pathway that rendered NK cells resistant to suppression. Favourable antitumour effects by cellular activation by GIFT-21 were also demonstrated, but via different mechanisms than by GIFT-2. Despite the fact that IL-21 has been shown to eliminate cancer cells via activation of $CD8^+$ T cells and NK cells, GIFT-21 mainly acted via induction of differentiation of monocytes into a unique DC population. These DCs effectively elicited tumour-specific $CD8^+$ responses. The fusokine of GM-CSF and IL-15 (GIFT-15) was also anticipated to mediate cytotoxic tumour responses, but surprisingly turned out to *suppress* immune responses against cancer cells. The result was a rapid differentiation and proliferation of peripheral naïve B lymphocytes into inducible regulatory B cells that secreted immunosuppressive IL-10. In contrast, administration of GIFT 15-activated regulatory B lymphocytes mediated highly beneficial effects in the treatment of a murine model of multiple sclerosis (EAE), which may be pursued as an intervention in other autoimmune diseases.

These results also emphasize a need for a better understanding of intracellular signalling of GIFTs downstream of their respective receptors. Interactions via IL-2, IL-15 and IL-21 receptors have also been explored, but much remains to be learnt, and the contribution of CD131, the signalling GM-CSF receptor, is presently unresolved. Taken together, the authors review a series of novel findings in the GIFT-stimulated cell therapy field, raising interest in the possible future of clinical trials in cancer and autoimmune disorders.

Concluding remarks

Too often today, one reads in the press and scientific literature stories of science, discovery and invention that are oversimplified and distilled into soundbites or single turn of phrase. These snapshots gloss over the false starts, missed turns and arduous work that occurs when

basic discoveries are being pursued in the years prior to the development of some new block-buster clinical success. The reviews included in this volume provide unique insight into how contributions from basic studies of disease pathogenesis can lead ultimately to the development of revolutionary drugs. They also provide a glimpse into the possible origins of what one day may prove to be the next generation of biological reagents and new therapeutic strategies to replace them by targeting endogenous mediators of disease. We are optimistic that the reader will find these reports of ongoing work to be useful to understand the pathway to defining new molecular targets, producing new therapeutic strategies and developing new devices that may augment or replace the current generation of drugs to the benefit of unnamed future patients in the hopes of alleviating their pain and suffering.

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