Measuring Immunity: Basic Biology and Clinical Assessment

Michael T. Lotze
Angus W. Thomson
Editors

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To the Institute and Departmental leaders at the University of Pittsburgh: Richard Simmons, Thomas Starzl, Timothy Billiar, Joseph Glorioso, Ronald Herbman and Arthur Levine who have all supported our work both in the laboratory and the clinic.

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Foreword

THE BEDSIDE IS THE BENCH

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A young woman confronted with a diagnosis of systemic lupus erythematosus (SLE) can expect lifelong complications arising from the disease itself, as well as the therapies used to treat this condition. About 50–70 per cent of SLE patients experience inflammation of the kidneys. As such, the young woman can expect to be treated with high doses of corticosteroids, often accompanied by the alkylating agent cyclophosphamide. Unfortunately, the prednisone and cyclophosphamide treatment often results in an initial improvement, but more than 50 per cent of SLE patients will experience a disease flare again within 2 years. Moreover, serious complications of high-dose corticosteroid and cytoxan therapy in SLE patients include osteoporosis, aseptic necrosis, hypertension, diabetes, opportunistic infection, and cataracts as well as gonadal failure, hemorrhagic cystitis and cancer. Clearly, safer and more effective therapies are needed for SLE. Most importantly, there is no way to predict the flares or remission using immunological analyses in affected patients.

Practically speaking, treatment of SLE and other autoimmune diseases remains similar to the therapies used 10 years ago. However, years of elegant work studying immunity and immune-mediated diseases in animal models combined with recent advances in human immunology and genomics offers an unprecedented opportunity to develop new therapies. There is, arguably, no more important concern in moving forward in the development of new immunotherapies than the measurement and quantification of the human immune response. Indeed, with the observed increase in immune-mediated disease and an ever-growing stable of immunomodulatory agents reaching clinical stages of development, the need for reliable indicators of the state of the human immune system has never been greater. The editors of this guide should therefore be congratulated for assembling a highly relevant, and indeed, very timely portrait of our current abilities and future prospects in this respect.

Importantly, if perhaps not unexpectedly, we have come to discover that the human immune system differs in many significant ways from the preclinical animal models used as justification for pursuing new therapies in human studies. A growing body of literature detailing the many examples of therapies that work well in mice but fail to generate similar efficacy in humans (Mestas and Hughes, 2004) underscores the divide between our respective understanding of mouse and human immunology. The scarcity of hard human data on immune mechanisms is truly the Achilles heel of immune-based therapeutic development. Typically, immune-based diseases are diagnosed by measuring a pathological process that has already taken place. This means that the destruction by the immune system is already well underway. Effective monitoring and early detection of these diseases is challenging at many levels, unlike preclinical efforts which can sample the immune response at the site of immune attack (e.g. graft, draining lymph node or inflamed tissue); human sampling is relegated often to the peripheral blood far away from where the action is and rarely before the immune response is already damaging to the target tissue.

Take for example, the case of organ transplantation, where the key clinical challenges are to combat both acute and chronic rejection. At present, the gold standard for diagnosis of organ dysfunction is biopsy, which while accurate, provides its diagnosis only after significant organ damage has occurred. Immunological methods that detect events occurring upstream of the pathology would provide a welcome window of opportunity for earlier intervention. A related issue in organ transplantation is that of clinical tolerance induction. New potential tolerogenic strategies are now entering the clinic, many with the goal of complete immunosuppressive therapy withdrawal. Immunosuppressive withdrawal, however, is more than just the objective of these studies; rather it has been elevated to the status of an endpoint for these trials. Until have a clear description of the immunological properties of tolerance in humans, we are left with only an operational, rather than mechanistic definition of tolerance in humans.

Achieving a therapeutic benefit is the goal of all phase II and III trials and is currently measured using clinical endpoints. Clinical indicators, as currently measured, often do not offer objective quantitative markers for assessments of drug actions. Thus clinical endpoints will greatly benefit from the addition of studies designed to measure human immunity qualitatively and quantitatively. There is a pressing need for new surrogate markers for measuring changes in the immune system.

A case demonstrating the problems associated with relying on clinical endpoints can be made by looking at the history of immunologic therapies for HIV infection. Antiretroviral therapy has effectively reduced the rate of progression of HIV-infected patients to AIDS to ~2 per cent per year. Thus, trials of additional therapies require large patient populations and/or many years of treatment in order to obtain statistically significant proof of improved efficacy. Furthermore, studies of early HIV infection are virtually impossible without some alternative marker for disease progression because of the long time it takes (up to 10 years or more) for many patients to get sick. Similarly, in the case of cancer, current therapeutic inventions rely on clinical endpoints such as disease progression and death to determine efficacy. These endpoints, although a fair assessment of the clinical efficacy of the therapy, do not provide insights in the immune manifestations of therapy. Is the immune system activated by the therapy, is the tumor resistant to the therapy or does it escape immune surveillance by mutating target antigens?

But perhaps the clinical settings that most appropriately illustrate the need for new technologies and data that allow us to characterize the human immune system are the autoimmune diseases. The diagnosis of specific autoimmune diseases is often problematic due to overlapping pathologies and a lack of clearly distinguishable clinical features between the various diseases. American College of Rheumatology (ACR) diagnostic guidelines rely upon primarily pathologic criteria that, similar to the diagnosis of allograft rejection, present well into disease development – features such as clinical and radiological evidence of tissue damage. The prognostication of specific autoimmune diseases presents an even greater challenge, given that the etiology of many of these diseases remains unclear. In fact, one of the most fundamental questions in autoimmunity remains unanswered: what are the immunological characteristics that distinguish a healthy patient from one with an underlying autoimmune disorder? At present, there are no reliable laboratory-based immunologic methods that are capable of discriminating between a rheumatoid arthritis patient from a healthy control and a multiple sclerosis patient from the same. This 'readout' problem is so severe that in diseases such as type 1 diabetes, current therapeutic interventions rely on clinical endpoints such as hemoglobin A1c to determine efficacy. This metabolic parameter can be influenced by the rigor of glucose control, diet and environmental factors not the quintessential immunology of autoimmune disease. If we have no measurable description of the immunological hallmarks of the disease itself, how then can we begin to assess the efficacy of one therapy over another?

Clearly, our potential for success in the clinic is now limited by our inability to assess the immunological impact of our interventions. Throughout the field of immunology, it is therefore imperative that we develop new biological assays that allow precise and reliable measures of human immunity. The benefits will be enormous: surrogate markers for clinical efficacy providing more relevant, accurate and ethically justified means of assessing new therapeutics; new diagnostic tools that would permit earlier intervention and perhaps even preventative therapies; the ability to move beyond 'one size fits all' medicine towards more individualized therapy; and a wealth of new, direct knowledge of the human clinical experience that will pave the way for improved, second generation therapies. Much of the research elegantly summarized in this book reflects the growing efforts to identify specialized markers that can be used in individual disease settings to distinguish the patient from normal individuals, the responder from the non-responders.

Thus, the papers presented within this volume are a testament to the grand opportunity that lies before us. They serve not only to highlight the progress already achieved towards this goal, but present us with a series of difficult challenges as we move forward. Together they suggest that we have moved into a new phase of development in measuring immunity, one where old approaches might be best discarded in favor of a new paradigm for assay development.

In fact, this new paradigm may be best summed up by the multiple efforts emerging in the academic community, with the primary goal to develop robust standardized assays for measuring human immunity. These efforts include various workshops, as well as the emergence of several large clinical trials consortiums such as the

Immune Tolerance Network (ITN) whose philosophy is 'The bedside *is* the bench'. These consortiums have created organizations with the infrastructure necessary to become the perfect testing ground for many of the assays described within this text, performed in a real-world environment to produce data and ultimately, new tools of extraordinary clinical relevance. And with a growing list of immunologically active agents destined for clinical evaluation, the timing for such a fresh approach is ideal.

Indeed, the emergence of new and improved methodologies provides a solid foundation for the development of new clinically focused immunoassays. High throughput genomics assays, for example, offer exciting new opportunities for identifying new biomarkers and many investigators have already taken up this challenge, with more sure to join them. Federal funding agencies have recognized the import of this approach.

New models are developed, like the ITN, to perform clinical studies on a much grander scale than has likely ever been attempted previously. Infrastructures consisting of core facilities, large relational databases and a combination of mechanistic and discovery efforts will allow comparison studies across diseases, therapies and patient populations under highly standardized protocols and analysis methods in order to answer the simple question – can we distinguish immunologically the diseased from the normal individual as well as the patient that has benefited by the immunotherapy?

Although the development of this infrastructure is an enormous undertaking, emphasis on cooperation and working together to create a whole that is greater than the sum of its parts are vital. The time spent in developing rigorously standardized procedures for each assay and meticulously performing routine quality assurance testing will bring enormous benefits in terms of the knowledge gained from this effort: pooling of assay data will be possible between multiple clinical sites operating within the same trial to increase the statistical resolution; assay data can be analyzed in the context of the related clinical information in a multiparametric fashion; longitudinal studies can be carried out with built-in normalization; and as yet undiscovered assays can be applied to archived specimens for cross-analysis at a later time.

The editors of this book have done a remarkably thorough job of covering all the emerging techniques and principles of measuring immunity and they should be congratulated and thanked for what has surely been a tremendous undertaking. The techniques and concepts described in the pages of this book will provide the insights that large networks will apply to the clinical trial setting. I believe that a volume such as this is just what is needed to capture the imagination of the immunology community and may ultimately serve as a fine starting point towards a new paradigm for direct and coordinated investigation of the mechanisms inherent in human immunological diseases.

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Preface

Michael T. Lotze and Angus W. Thomson

An Acte against conjuration Witchcrafte and dealinge with evill and wicked Spirits. BE it enacted by the King our Sovraigne Lorde the Lordes Spirituall and Temporall and the Comons in this p'sent Parliment assembled, and by the authoritie of the same, That the Statute made in the fifte yeere of the Raigne of our late Sov'aigne Ladie of the most famous and happy memorie Queene Elizabeth, intituled An Acte againste Conjurations Inchantments and witchcraftes, be from the Feaste of St. Michaell the Archangell nexte cominge, for and concerninge all Offences to be comitted after the same Feaste, utterlie repealed. AND for the better restrayning of saide Offenses, and more severe punishinge the same, be it further enacted by the authoritie aforesaide, That if any pson or persons after the saide Feaste of Saint Michaell the Archangell next comeing, shall use practise or exercsise any Invocation or Conjuration of any evill and spirit, or shall consult covenant with entertaine employ feede or rewarde any evill and wicked Spirit to or for any intent or pupose; or take any dead man woman or child out of his her or theire grave or any other place where the dead body resteth, or the skin, bone or any other parte of any dead person, to be imployed or used in any manner of Witchecrafte, Sorcerie, Charme or Inchantment; or shall use practise or exercise any Witchcrafte Sorcerie, Charme or Incantment wherebie any pson shall be killed destroyed wasted consumed pined or lamed in his or her bodie, or any parte therof ; then that everie such Offendor or Offendors theire Ayders Abettors and Counsellors, being of the saide Offences dulie and lawfullie convicted and attainted, shall suffer pains of deathe as a Felon or Felons, and shall loose the priviledge and benefit of Cleargie and Sanctuarie …

Witchcraft Act of 1604 – 1 Jas. I, c. 12

We have come quite a long way in the four centuries since the Witchcraft Act was passed during the end of the Elizabethan Age, which limited access to the parts of any body, dead or alive to be used in any 'witchcrafte, sorcerie, charme, or inchantment'. Clearly many of the practices employed and recommended by the strong coterie of authors brought together in this volume would have offended some Elizabethan audiences in 1604! In the same year London was just hearing Shakespeare's Measure for Measure performed on stage for the first time and enabling a 26-year-old William Harvey, who discerned how blood circulates, by admitting him as a candidate to the Royal College of Physicians. Considering the cells and the serologic components circulating within the blood as migratory biosensors and potential measures of immune function within the tissues is a modern interpretation provided by the current retinue of clinical immunologists and pathologists assembled here. A century ago in 1904, Paul Ehrlich published three articles in the New England Journal of Medicine (then the Boston Medical and Surgical Journal), detailing his work in immunochemistry, the mechanism of immune hemolysis and the side-chain theory of antibodies, work which subsequently served as a basis for winning the Nobel Prize along with Elie Metchinikoff. We have since substantially applied measures of the serologic response to pathogens and immunogens but the integration of multiple other assays, particularly cellular assays championed by Metchinikoff, many of them only appreciated and developed in the last decade, into a single readable text has not been previously

carried out. The central goal of Measuring Immunity is to define which assays of immune function, largely based on ready and repeated access to the blood compartment, are helpful in the assessment of a myriad of clinical disorders involving inflammation and immunity, arguably the central problems of citizens of the modern world. This is not a methods manual and should not be perceived as such. Authors were given broad scope and freedom in integrating and assessing the clinical evidence that polymorphisms in genes regulating immune function (Section I), the actual assays themselves (Sections II–V) and how they were applied in clinical conditions (Section VI) might be best illustrated and championed. We are also particularly pleased that new measures and methods, not yet fully realized, are detailed here in Section VII. The greatest value from this work, we believe, is the juxtaposition in one place of the basic science foundations as well as the approaches currently applied and found valuable in the disparate and inchoate regions of clinical medicine.

As always the 'conjurations, inchantments and witchcraftes' of our colleagues are what make this volume a ready sanctuary for those seeking enlightenment. The dedication and craftsmanship in their work as well as the exposition here is gratifying to both us and the publishers. Indeed, we recently met with the publishers in London to discuss this work and those planned for the future and considered under the Academic Press/Elsevier banner of 'Building Insights; Breaking Boundaries', particularly reflecting on what the role of the 'Book' was and how it might be more useful for us and our colleagues. Isaac Elsevier first used the Elsevier corporate logo in 1620, just after the Witchcraft Act, as a printer's mark. It shows an elm, its trunk entwined by the tendrils of a vine. A solitary man stands beside the tree, which supports a banner bearing the Latin motto *Non Solus* (not alone). Elsevier published books by outstanding scholars of the day, including Scaliger, Galileo, Erasmus and Descartes. Indeed the contemporary multiauthor authoritative text honors that history and provides a suitable reason for scholarly books. As a given, we believe that there is still substantial value in books, that they provide an authoritative and tightly edited source of integrated information, not easily assessed by perusing the modern literature. By constraining authors to formulate their work in a bounded space with common goals and deliverables, we enable them to indeed build new insights and cross boundaries usually maintained in academic circles, not so different from a Shakespearian drama, distilling human experience derived from a changing world.

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Section I

Fundamentals of the immune response

MHC Class I

Chapter 1

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Self-defence is nature's eldest law.

John Dryden

INTRODUCTION

Although class I MHC proteins were first identified over 50 years ago, their function has only been understood in detail in the past two decades. The three-dimensional structure of the human class I molecule HLA-A2 represented a landmark achievement in the field (Bjorkman et al., 1987a,b). The structure revealed the presence of a binding cleft suggesting antigen binding capability and offered tantalizing evidence of the nature of peptides bound. Shortly thereafter, bacterially produced recombinant class I proteins were re-folded with synthetic peptides which, upon crystallographic analysis, elucidated the molecular details of peptide binding in the cleft (Garrett et al., 1989). In addition to their importance for understanding T-cell recognition, these studies formed the basis for developing class I MHC tetramers, reagents with widespread current use in identifying antigen-specific CD8+ T cells, as will be discussed elsewhere in this volume.

A further seminal discovery was made by Rammensee and coworkers and Van Bleek and Nathenson who first developed methods for extracting peptides from the class I binding cleft (Van Bleek and Nathenson, 1990; Falk et al., 1991). These pooled peptides were analyzed by Edman degradation, resulting in mixed sequences which, nonetheless, revealed some very important properties of class I MHC-binding peptides. The presence of relatively conserved residues at certain positions of all peptides bound to a single type of class I molecule was noted. These were designated anchor residues, based on their role in stabilizing peptide binding. In a leap of insight, highly variable positions within the peptide were proposed to potentially interact with T cell receptors (TCR) and this was later confirmed by crystallographic analyses (Garboczi et al., 1996). The identities and positions of the anchor residues when summarized for an individual class I MHC protein represented its 'peptide binding motif'. This concept has been invaluable for prediction of possible MHC binding peptides within a protein of interest, since without this information, sets of peptides covering the entire protein would need to be tested as potential epitopes. It is now commonplace to use computer-based algorithms, many available on the world wide web, to interrogate protein sequences for sequences corresponding to binding motifs of interest and to base epitope discovery strategies upon such information (Papassavas and Stavropoulos-Giokas, 2002; Hebart et al., 2003; Peters et al., 2003; Saxova et al., 2003).

In this chapter, our current knowledge of class I MHC biology and how this may impact treatment of diseases that involve CD8+ T cell responses will be reviewed. In addition, the importance of the high degree of allelic polymorphism present in class I MHC heavy chains will be discussed. How processing of antigens for class I MHC presentation influences the immune response to be

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generated will also be explored, with emphasis on the molecular mechanisms involved.

CLASS I GENES WITHIN THE MHC REGION

Genetic and physical mapping analyses by many laboratories culminated several years ago in publication of the complete sequence of the human MHC region (Beck and Trowsdale, 2000). The presence of dozens of class I loci, including the well known HLA-A, B and C loci, as well as a number of other class I genes, both functional and nonfunctional, were revealed. Of these, only HLA-A, B and C have been shown definitively to present peptide antigens to CD8+ T cells. HLA-C may have as its primary role interaction with receptors on NK cells that either inhibit or activate lytic function (Fan et al., 1996; Snyder et al., 1999). In contrast, the best known function of HLA-A and -B molecules is to present peptide antigens to CD8+ T cells.

POLYMORPHISM IN CLASS I MHC HEAVY CHAINS

Class I HLA alleles were first identified using antibodies generated in multiparous or transfused individuals and then later using monoclonal antibodies developed by immunizing mice with human cells or purified HLA proteins (Parham, 1983). Serological definition resulted in designation of class molecules such as HLA-A2 or -B7, with numerical names assigned for each locus roughly in their order of discovery. Biochemical analyses using isoelectric focusing revealed additional heterogeneity within the serologic designations and many specificities were divided further into subtypes based on differences in electrophoretic charge (Neefjes et al., 1986). With the advent of widespread DNA sequencing, definitive analyses were soon possible, leading to a great expansion of the number of alleles identified at each locus. For example, HLA-A2, a specificity defined on the basis of antibody reactivity, has been subdivided into 15 alleles as defined by DNA sequencing (Parham et al., 1989). Although some of these alleles are distinguished by non-coding substitutions, others differ at nucleotides that result in amino acid differences, some of which demonstrably alter peptide binding or T-cell recognition.

There are currently identified over 200 alleles at HLA-A and about 400 at HLA-B, with most of the variation in amino acid sequence between alleles present in residues in the peptide binding cleft (Parham et al., 1989). This strongly supports the hypothesis that sequence diversification is driven by the requirement for broad antigen presentation capability, particularly in pathogen-laden environments. Examples of class I alleles that are associated with resistance to certain diseases have been identified, such as that observed in West Africa, where HLA-B53 has been associated with resistance to severe malaria (Hill et al., 1992).

MOLECULAR TYPING OF CLASS I HLA ALLELES

A review of the technical aspects of MHC typing is beyond the scope of this chapter, but some of the principles will be discussed briefly. Primer sets are designed and used for PCR amplification of cDNA to obtain fragments of class I genes, typically those encoding the α1 and α2 domains, where most of the polymorphism resides. After the amplified fragments are applied to a membrane, labeled oligonucleotide probes that can anneal to specific regions of individual class I genes are used in liquid hybridization to detect alleles. Alternatively, additional allele-specific primers are used in a second round of PCR amplification to generate DNA fragments that allow for allele assignment. For both approaches, prior knowledge of class I sequences is necessary and novel or unknown alleles cannot be identified. In the research laboratory setting, it is typically more efficient to identify class I alleles from unknown cells using DNA sequencing of the primary PCR product, rather than establishing secondary screening procedures mentioned above. In a clinical testing laboratory, where multiple samples will be routinely analyzed, the use of secondary screening assays, such as filter hybridization, is more common. There are a number of technologies that are being currently developed to reduce the expense or effort required for molecular HLA testing. Some of these involve the development of membrane or bead arrays that allow for automation of these processes (Guo et al., 1999; Balazs et al., 2001).

CLASS I MHC ANTIGEN PROCESSING PATHWAY

How peptides are generated from protein antigens in the cytosol for delivery to class I molecules has been studied intensively in the past decade. At the forefront in this process is the proteasome, a large organelle with multiple proteolytic activities. Rock and Goldberg and their coworkers first demonstrated that proteasome inhibitors could inhibit class I MHC antigen processing and presentation to T cells (Michalek et al., 1993; Goldberg et al., 2002). This was due to blocking generation of the major supply of peptides required for stabilization of class I molecules and the lack of this peptide pool resulted in their retention in the endoplasmic reticulum (ER). This phenotype was similar to that seen in mutant cell lines that lack the proteins TAP (transporter of antigenic peptides) or tapasin (DeMars et al., 1985; Salter and Cresswell, 1986; Ortmann et al., 1997). These latter proteins are required to facilitate peptide transport into the ER and subsequent class I loading.

The class I biosynthetic pathway can be summarized as follows (Table 1.1). Class I heavy chains are inserted into the lumen of the ER and associate cotranslationally with a second subunit, β_2-microglobulin (β_2m) and with