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Cell-surface and cytokine biomarkers in autoimmune and inflammatory diseases

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Increasing emphasis is being placed on biomarkers as indicators of disease states in patients with autoimmune and inflammatory disorders, such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus. Careful description of the expression of cell-surface markers and cytokines produced by T and B lymphocytes can lead to a more complete characterization of disease activity in patient populations, and serve as an indicator of the patient's response to therapy.

The pharmaceutical industry is faced with developing new and safer treatments for autoimmune and inflammatory diseases in an increasingly challenging environment. On average, ten years and US$800 million are consumed in bringing a new drug to the clinic [1]. Patients with inflammatory diseases, such as rheumatoid arthritis (RA) and psoriasis, have benefited in recent years from new therapies, such as etanercept and infliximab, which target key components of autoimmune pathology. More than 70% of RA patients treated with agents targeting tumor necrosis factor (TNF) will experience a significant reduction in symptoms [2,3]. Similarly, improved immunosuppressants have resulted in greater than 85% retention of graft function one year after kidney or liver transplant (United Network of Organ Sharing combined center data 2004, www.unos.org). This level of success creates a high standard for new therapies to match, which can require large clinical trials to reach a statistically superior clinical endpoint.

By contrast, some autoimmune and inflammatory diseases, such as systemic lupus erythematosus (SLE) or systemic sclerosis (SSC), suffer from a lack of new therapeutics in development. Discovery of more effective therapeutics for diseases like RA or diseases with few therapeutic options, such as SLE, requires innovative approaches to clinical trials. A new paradigm defining drug efficacy is imperative for the industry, the clinician and the patient to identify beneficial outcomes more effectively.

As clinical research progresses, new indicators of disease activity have been identified in peripheral blood and affected tissues by flow cytometry analysis of cell populations and by detection of soluble mediators in serum. Use of these emerging biomarkers can provide insight into disease processes, result in identification of new targets for therapy and provide shorter-term indications of therapeutic success.

This review discusses some of these indicators of disease activity by focusing on autoimmune and inflammatory diseases, such as RA, multiple sclerosis (MS), SLE and type 1 diabetes (T1D). In particular, we focus on cell-surface markers on T and B lymphocytes, as well as circulating or *in situ* secreted cytokines, which might serve as indicators of disease mechanism or disease activity.

Defining biomarkers

Biomarkers must be robust indicators of disease pathogenesis to assess disease states or responses to therapy [2]. Biomarkers closely linked to the mechanism of drug action can be used to measure the *in vivo* potency of new therapeutics. Alternatively, biomarkers can be linked to disease progression or severity. For example, circulating or synovial fluid TNF-α might contribute to arthralgia symptoms suffered by RA patients [3,4], so measurement of TNF-α, particularly in synovial fluid, could be a biomarker for disease progression.

By contrast, surrogate endpoints are specific measurements that substitute for full clinical analysis, whereas clinical endpoints reflect assessments of patients' disease status. For example, decreases in blood pressure can be used as a surrogate endpoint for stroke [2]. Similarly, serum C-peptide levels are a direct consequence of the production of insulin, and thus can serve as a surrogate marker for pancreatic function. Examples of clinical endpoints are American College of Rheumatology (ACR) scores

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for RA, Expanded Disability Status Scale (EDSS) for MS and SLE Disease Activity Index (SLEDAI) scores for SLE. The SLEDAI quantifies disease activity by combining symptoms and clinical endpoints in a numerical scale of disease severity [5]. The ACR score includes clinical measurements of joint swelling, morning stiffness, rheumatoid factor (RF) and radiographic integrity of bone [6]. EDSS scoring quantifies neurological criteria, including mobility, and sensory and cognitive abilities [7].

Standard biomarkers in clinical trials

Although the concept of integrating robust biomarker analysis into clinical trial design is new, clinicians have followed biomarkers for some time. RF (IgM antibody specific for IgG) has been used by clinicians as a diagnostic marker for RA for decades [8,9]. Similarly, rheumatologists have followed serum antibody levels to autoantigens in SLE and RA. Antinuclear antibodies have been used for some time as a component of the diagnostic criteria for SLE. However, antinuclear antibodies are not a homogeneous panel of reactivities, and can vary in titer and pattern of staining, even over time in the same patient. Although signifying an underlying autoimmune process, not all antinuclear antibodies can be linked directly to disease pathogenesis. Antibodies to double-stranded DNA (dsDNA) have been more indicative of ongoing disease process and pathology. However, investigators have reported varying levels of correlation between anti-dsDNA levels and disease severity [10–13]. Differences in laboratory procedures, antigens used to detect anti-dsDNA or differing patterns of symptoms expressed by SLE patients might compound difficulty in correlation. However, it is clear that additional biomarkers are needed for these and other autoimmune and inflammatory diseases.

B cell biomarkers: cell surface receptors and antibodies

SLE affects more than a million adults in the USA and Western Europe [5,10–14]. Patients with this chronic, B-cell-mediated disease can suffer arthralgias, fevers, rashes and eventual end-stage renal disease. Only three classes of drugs are currently approved by the FDA for the treatment of SLE: corticosteroids, such as methylprednisolone; antimalarials, such as hydroxychloroquine; and low-dose aspirin. Clearly, these drugs do not specifically target autoreactive cells but are used by the clinician to suppress a patient's immune system nonspecifically or simply to alleviate discomfort. No new drugs have been approved to treat SLE in the past three decades [14].

Until recently, antibodies to dsDNA or nuclear antigens, such as the Sm antigen and phospholipids, or measurements of complement activation, were used together with SLEDAI scores as indicators of compound efficacy in clinical trials [5,10,11,14]. Changes in clinical scores for an SLE patient can take substantial time after initiation of treatment, and are subject to placebo affect. Clear factors in developing drugs to treat this chronic autoimmune disease are the length of clinical trials and the number of patients needed to overcome these obstacles.

Recently, several clinical studies have evaluated new therapies directly targeting B lymphocytes. Flow cytometry assessments of circulating peripheral B cells have been used to define pathogenic subsets and measure therapeutic efficacy. Co-stimulatory molecules, such as CD80, CD86 and CD40, are important regulators of the immune response, as well as being key indicators of activation on both B cells and antigen-presenting cells [15]. A recent study of co-stimulatory molecules comparing SLE patients with active versus inactive disease found upregulation of CD19 and CD86 to correlate with disease activity [16]. Jacobi *et al.* [17] conducted a more detailed examination of B cell subsets in peripheral blood. Beyond observing an increased frequency of $CD19^+$ B cells, these investigators reported that high levels of CD27 on circulating plasma cells strongly correlated with both increased anti-dsDNA titers and higher disease activity scores.

Recognition of B lymphocytes as important component of disease in RA has emerged over the past ten years [18]. Newer therapeutic regimens target B cells using rituximab, an antibody that recognizes the CD20 molecule expressed on most circulating B cells. Treatment with rituximab produces long-term depletion of peripheral B cells and clinical remission in seropositive RA patients. Surprisingly, symptom remission frequently outlasted the return of $CD20^+$ B cells [18,19]. It is possible that a subset within the CD20 population either is eliminated or returns at a slower rate and this accounts for the dissociation of B cell return and remission. Further characterization of the surface markers on pathogenic B cells, beyond CD20, is required.

Antibodies to islet cell antigens, such as insulin and glutamic acid decarboxylase, have been characterized by endocrinologists studying T1D [20,21]. These studies did not attempt to correlate antibody levels or antigen reactivity with disease severity. Rather, the purpose was to try to predict patients at risk for disease development and to correlate antibody titer and antigen recognition pattern to disease onset. This is particularly crucial in T1D because new-onset patients are primarily children and adolescents, and so use of current immunosuppressants must be weighed against developmental goals, while enabling childhood immunization against infectious disease. The objective of the diabetes research community is to identify at-risk patients by a combination of genetic screening and biomarker expression, enabling ethical early intervention in T1D before complete islet β-cell destruction.

Biomarkers on T lymphocytes

T cells are principal components of many autoimmune diseases, acting as either primary pathogenic cells or acting to help B cells to produce autoantibodies, or in some cases performing both functions. In diseases such as MS and T1D, T cells are principal effectors of neural and endocrine damage, respectively [22–25]. In RA, T cells secrete cytokines that help B cells divide and differentiate into autoantibody-producing cells. T cell-derived cytokines can also activate endogenous or recruited tissue macrophages, which secrete inflammatory cytokines or metalloproteinases that can directly damage synovial tissue. T cell cytokines can also directly cause inflammation and destruction of synovial tissue [26]. Kremer *et al.* have recently reported positive responses to abatacept (CTLA4-Ig) in RA patients [27]. Therefore, it is no surprise that immunosuppressants targeting T cells are active areas of pursuit for the next generation of drug therapies in autoimmune disease. Just as clear is the need accurately to identify and target specific subpopulations of pathogenic T cells, as well as to identify potential disease-ameliorating regulatory T cells (Treg cells).

Given the clear link between T cells and disease onset and progression in MS, several studies have compared biomarkers on

the surface of T cells and disease status. Chatzimanolis and colleagues [28] attempted to correlate the presence of CD45RA$^+$/ICAM-3$^+$-naïve T cells with remission in interferon (IFN) β1b-treated MS patients. Higher levels of CD45RA$^+$/ICAM-3$^+$ T cells in peripheral blood correlated with lower EDSS scores. However, no correlation was observed between the response to IFN-β1b treatment and the percentage of these cells in peripheral blood lymphocytes. Previous work had shown a positive correlation between the presence of CD45RA$^+$/ICAM-3$^+$ cells and response to methylprednisolone treatment in MS [29]. Either the two types of therapy regulate MS by independent mechanisms or this is an example of surface marker modulation by drug therapy, independent of therapeutic disease modulation.

CD40L has been reported to be upregulated on T cells in SLE patients [30]. Increases in T cell expression of CD40L were observed in disease exacerbations [31,32]. Upregulated soluble CD40L has also been reported in active SLE patients [32,33]. Ectopic expression of CD40L might have a role in disease onset and progression [32,34,35]. Although anti-CD40L showed limited efficacy in SLE and was placed on hold for safety concerns [36], CD40L regulation or expression might be a candidate for an SLE biomarker.

Regulatory T cells might be equally effective as biomarkers for autoimmune disease. Still in the early stages of study, interesting observations have been made in correlating the presence of naturally occurring populations of regulatory T cells with inhibition of emerging or active autoimmune disease. Wilson *et al.* [37] reported the disappearance of CD1d-restricted T cells, which express an invariant T cell receptor, immediately before disease onset in T1D patients. Normal levels of these protective cells were reported in the nondiabetic twins of T1D patients. Wang *et al.* [38] reported a decrease in CD4$^+$/CXCR3$^+$ T helper-1 cells, as well as CD4$^+$/CCR4$^+$ cells in the cerebrospinal fluid (CSF) of MS patients successfully treated with intravenous methylprednisolone. Patients in remission also had fewer CD8$^+$/CXCR3$^+$ cells in the CSF. No changes in these populations were reported in nonresponder patients.

The Foxp3 transcription factor is a key element in the identification and activity of CD4$^+$/CD25$^+$ Treg cells [39,40]. Baan and colleagues [41] recently reported Foxp3 expression in the CD25^{bright} population of human T cells, which inhibited mixed lymphocyte reactions. They further demonstrated the presence of Foxp3$^+$ cells in human cardiac allografts. Interestingly, treatment of heart transplant recipients with calcineurin inhibitors or anti-CD25 lowered Foxp3 expression, whereas patients treated with rapamycin maintained levels of this transcription factor. Ehrenstein *et al.* [42] reported compromised function of Treg cells in RA patients. Treg cells isolated from RA patients failed to suppress proinflammatory cytokines produced by monocytes *ex vivo*. Furthermore, patients who responded to infliximab therapy demonstrated a significant rise in peripheral blood Treg cells and a concomitant reduction in C-reactive protein [42]. Given that Foxp3 is an intracellular protein, its use as a clinical biomarker will depend on more sophisticated application of flow cytometry techniques or transcriptional analysis of peripheral blood. The apparent importance of Treg cells could provide a new clinical goal for next-generation therapeutics in autoimmune disease, down-regulation of pathogenic T cells and promotion of protective regulatory cells.

Cytokines and antibodies as biomarkers

Circulating cytokines have been demonstrated to correlate with RA disease activity and to participate directly in disease pathogenesis and symptoms. Principal among these is TNF-α, whose presence in serum or synovial fluid is a strong indicator of disease activity in RA. Neutralization of TNF-α with soluble receptor or antibody decreases ACR scores and improves mobility, in as many as 70% of patients in some studies [3,4,18]. TNF-α is an example of a biomarker that doubles as a correlate for disease activity and a target for therapy. Rosengren *et al.* [43] examined synovial biopsies using ELISA from RA and osteoarthritis patients, for expression of other inflammatory cytokines that might correlate with disease. These investigators found significant elevation of interleukin (IL) 8 and TNF-α in RA biopsy samples as compared with osteoarthritis. IL-1β and IL-6 were detected in both, with a trend toward higher levels in RA. Interestingly, only about half of the RA samples tested positive for the presence of TNF-α protein, despite the demonstrable efficacy of TNF blockade therapy in RA patients.

Elevated levels of serum vascular endothelial growth factor (VEGF) have been reported in RA patients, with successful disease reduction using standard disease-modifying antirheumatic drug therapy being associated with decreased levels of VEGF [44]. Similarly, treatment with anti-TNF antibody plus methotrexate correlated with reductions in VEGF [45].Because this cytokine is associated with angiogenesis, elevated VEGF levels might contribute to pannus formation. The IL-6 pathway has also been implicated in RA pathogenesis, and recent studies have begun to evaluate the efficacy of the anti-IL-6 receptor in RA patients [46]. VEGF has been identified as a potential biomarker for blockade of the IL-6 pathway in a small study [47]. Serum levels of VEGF were statistically reduced after treatment with anti-IL-6R antibody. Patients also showed improved ACR scores. Further studies are needed to determine whether serum levels of VEGF can be a robust biomarker for disease.

Cytokines can either be detected directly as biomarkers for autoimmune diseases, or the 'signature' of the cytokines in peripheral blood mononuclear cells can be detected, as has recently been demonstrated for SLE [48,49]. Some patients with SLE have elevated serum IFN-α, although the latter is difficult to detect and is not a robust biomarker for disease [48]. However, studies comparing gene expression analysis in peripheral blood mononuclear cells from healthy volunteers and SLE patients reveal that genes that are induced by exposure of cells to IFN-α are more highly expressed in the peripheral blood cells of SLE patients [49–51]. Subsets of IFN-inducible genes have been identified in these studies that correlate with disease severity. It has been proposed that expression of these genes or proteins would be markers of efficacy in clinical trials of new drugs for SLE, although this has not been tested. Consensus has not been reached on which genes will be the most predictive.

Commentary and future directions

Currently, clinical trials measure efficacy by assessing the clinical disease status of the participating patients. In the case of autoimmune disease, such assessments frequently rely on the observation skills of the clinician and are reported as scores along a subjective scale. Although such clinical assessments can provide symptom-based assessment of a patient's condition and manage-

ment of the disease, they do not provide mechanism-based objective readouts which can substantiate compound efficacy.

Scoring systems such as the ACR, SLEDAI and EDSS scores rely heavily on physician description of joint inflammation and mobility, or patient motor skills, as well as on subjective assessments by the patient of their overall health and well-being. Certainly, these assessments have utility, and a positive assessment of health and well-being, by both the patient and the clinician, is in fact the goal of drug development. However, collecting such data as the primary endpoint of a clinical trial mandates large numbers of participants, monitored over long periods of time. Furthermore, such semiquantitative measurements are subject to investigator variance and can be difficult to apply broadly across multiple centers.

For modern drug development, it is no longer sufficient simply to measure positive changes in a patient's symptoms. Following clinical biomarkers can give the physician indications of successful treatment while providing additional quantifiable measurements of effectiveness (Table 1). Correlating biomarkers with disease improvement will not only enhance patient management, but will also enable more accurate outcome measurement in clinical trials, potentially decreasing the number of patients required to reach a meaningful outcome, shortening the trial time and lowering the cost of pharmaceutical development.

Future biomarkers could include widespread use of genetic screening to define at-risk patients. The genetic mutation of the human major histocompatibility complex (MHC) HLA-DR molecule, which leads to presentation of islet cell autoantigens in T1D, is a biomarker that distinguishes the genetically-at-risk individual, and which directly participates in the pathogenic response but is not modulated during the course of disease onset or progression. At present, identification of genetically at-risk patients is insufficient to begin preventative treatment. The difficulty in applying these genetic screens is readily apparent in the discordance in disease onset seen in identical twins of T1D patients. Only 50% of affected twins have diabetic siblings [20], indicating that genetic predisposition is necessary but not sufficient for disease onset. Identification of the genetically at-risk, combined with advances in the characterization of pathogenic cell types, might enable ethical therapeutic intervention before disease onset.

Further refinement in the interpretation of clinical biomarkers might include the identification of antigen-specific subsets of circulating lymphocytes and characterization of their activation state. Clinicians might be able to identify autoreactive subsets in peripheral blood lymphocytes by using antigen-specific tetramers [52–54]. Diseases such as RA, MS and T1D have been associated with specific MHC haplotypes [20,25,52,54]. In T1D and MS, specific combinations of MHC haplotype and autoantigenic peptides have been associated with active or nascent disease. Tetramers, which are soluble, engineered protein constructs that combine appropriate MHC and peptide, bind autoreactive cells in peripheral blood [20,52–54]. Clinicians might be able to use simple two- or three-color flow cytometry to quantify autoreactive cells. By combining tetramers with intracellular staining for components of receptor signaling pathways [55], they might further be able to assess the activation state of pathogenic cells, giving both the physician and the drug developer vital information regarding compound efficacy before changes in symptoms become apparent. Similarly, detection of antigen-specific B cell populations could enhance clinical assessment, biomarker development and serve as an indicator of therapeutic efficacy. Advances in the detection of B cell subsets have yielded potentially crucial information identifying pathological subsets in antibody-mediated diseases such as SLE [17]. Combining detection of cell-surface markers such as CD27 with the ability to discriminate antigen-specific B cells could lead to more predictive measurements of autoimmune disease. This will require an enhanced ability to mark antigen-specific cell subsets, as well as a consensus among investigators in a given field as to which antigens are crucial indicators for disease pathology or remission.

Decisions on which biomarkers are most efficacious in the study of autoimmune disease for clinical trials might rest on criteria in addition to sensitivity and disease activity correlation. Peripheral blood is the most readily accessible sample for evaluation of T and B cell activation and circulating cytokines. However, levels of cytokines or subsets of lymphocytes in the circulation might not always be indicative of disease activity in the affected joint or organ. Sampling of affected tissues is feasible, if invasive, in RA and SLE by biopsy of synovia or kidney, although repeated biopsy sampling of the kidney seems unlikely to become routine practice. In T1D and MS, such *in situ* characterization is even more difficult. Future application of biomarkers will not only have to account for disease-specific cell populations and cytokines, but also utilize samples from the sources that are the most predictive.

Care must be taken in the choice of biomarkers. Change in biomarkers expression might be the result of a compound mechanism, rather than disease therapy. The transcriptional promoter region of human CD40L has been reported to be regulated by a cyclosporine A-responsive transcription factor family [56]. Expression of CXCR3 on CD4 cells can be modulated directly by IFN-β [57]. It is possible that the lack of correlation between the actions of methylprednisolone and IFN-β on CD45RA$^+$/ICAM-3$^+$ T cells in MS is the result of the drugs directly modulating a surface marker, rather than a differential action on disease [28,29].

TABLE 1

Emerging biomarkers in autoimmune and inflammatory diseases[a]

Disease	Marker (source)	Change in disease	Refs
RA	TNF-α (synovial fluid)	Increased	[3]
RA	VEGF (serum)	Increased	[44]
SLE	CD27$^+$/CD20$^-$ B cells (blood)	Increased	[17]
SLE	CD154$^+$ B cells (blood)	Increased	[35]
MS	CD45RA$^+$/ICAM-3$^+$ T cells (blood)	Decreased	[28]
MS	CD4$^+$/CXCR3$^+$ T cells (blood, CSF)	Increased	[38]
T1D	Vα24 JαQ T cells (blood)	Decreased	[37]
RA, T1D or MS	Antigen-specific tetramers (blood)	Increased Increased Increased	[52] [53] [54]
RA	Treg cells (blood)	Decreased	[42]
RA?	Intracellular FoxP3 (blood)	Decreased?[b]	[40]

[a] Abbreviations: MS, multiple sclerosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; T1D, type 1 diabetes; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

[b] Hypothesized from decreased numbers of Treg cells in RA patients.

An understanding of biomarkers on subsets of cell populations could elucidate which patient populations will respond to a given therapy. Patients with RA and SLE are being treated with rituximab in an attempt to control the B cell aspects of their disease. As with RA patients, the majority of SLE patients receiving anti-CD20 therapy demonstrated clinical improvement, concomitant with maximal B cell depletion [18,58,59]. Also, as with RA patients, some rituximab-treated SLE patients sustained clinical benefit after the return of $CD20^+$ cells in the periphery, and some patients attained a therapeutic outcome, despite a minimal effect on anti-dsDNA antibody levels [59]. CD20 is expressed on most circulating B cells but is low or absent on plasma cells [18]. Jacobi *et al.* [17] demonstrated a strong correlation between levels of $CD27^+$/$CD20^-$ plasma cells and SLE disease activity. The comparative effectiveness of rituximab in inducing disease remission might indicate a role for different subsets of B cells in these related rheumatic diseases. Further characterization of autoimmune lymphocyte subsets will yield further clarity and enable more specific therapy in RA and SLE, as well as other autoimmune diseases such as MS and T1D, where the clinical role of B cells is suspected but less well defined.

Emerging technologies in gene-expression profiling will have an expanded role in defining which biomarkers are relevant in a particular disease state. Studies using samples isolated from peripheral blood or affected tissue continue to reveal new proteins, which correlate with disease susceptibility, onset and activity [60,61]. Whether gene expression monitoring (GEM) will be a platform for clinical trials and clinical practice will depend on the continued development of the technology. The advantages of the technology lie in the quantitative nature of the readout and the relatively small sample required to profile the patient. As with serum cytokine and cell-surface marker measurements in peripheral blood, it remains to be seen if GEM analysis of peripheral blood lymphocytes is clinically relevant. Less technically demanding readouts of systemic inflammation, such as serum cytokine profiles, might provide information in situations where GEM analysis is unavailable. Again, serum cytokine analysis might not be a true indicator of disease activity for any one analyte, with tissue expression of an inflammatory mediator being more indicative of pathology. Multianalyte technologies such as Luminex™ or Cytometric Bead Array™ can measure patterns of cytokine expression from serum or body fluids, which might prove more robust indicators of systemic inflammatory disease. The combination of these multianalyte patterns with GEM analysis could offer the clinician and the researcher more sensitive and reliable gauges for disease and therapeutic assessments. Combinations of biomarker measurements might also lead to further categorization of patient populations, based on patterns of gene, cytokine and cell-surface marker expression that might give a better profile of the patient's disease subtype. This could lead the way to a more tailored or personalized therapeutic approach, with the physician adjusting therapy on the basis of a patient's inflammatory profile.

Other indicators of disease modulation in future clinical trials could rely on indicators such as serum levels of soluble forms of cell-surface molecules, such as, soluble vascular cell adhesion molecule 1, soluble intercellular adhesion molecule 1 and sE-selectin [62–64].

Although the potential for using biomarkers as indicators of clinical disease state and pharmaceutical efficacy is great, the field is in its infancy. Crucial issues remain to be addressed and standardized. Which biomarkers are most relevant for a particular disease, which sample source(s) enable the most robust predictions, and how storage, transportation, stability and consistency of samples affect the readouts are all issues which must be dealt with if the potential for biomarker use in clinical trials and disease management is to be realized.

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