

REVIEW

Proteomic biomarkers for autoimmune disease

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Autoimmune diseases affect 3% of the world population, yet the diagnosis and classification of autoimmune diseases remain based on clinical examination combined with traditional laboratory tests and imaging studies. The development of genomic and proteomic technologies provides an unprecedented ability to identify novel biosignatures to diagnose, classify, and guide therapeutic decision making in patients with autoimmune disease. In this article, we review recent advances in proteomics technologies and their application to autoimmune disease.

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1 Introduction

DNA microarray technology was developed over a decade ago, and recently gene transcript profiles that may provide utility for the management of patients with autoimmune disease have been described. Such profiles are derived from PBMC and include interferon inducible gene clusters in systemic lupus erythematosus (SLE) [1], and RAGE and phosphatase PTPN22 signaling pathways in rheumatoid arthritis (RA) [2, 3]. Nevertheless, the clinical utility of these gene expression profiles remains to be determined. The proteins encoded by these genes are believed to be responsible for mediating disease, and direct proteomic measurement of their expression and function could provide greater utility for monitoring disease. Indeed, preliminary data indicate that interferon-inducible serum protein signatures correlate with disease activity in SLE and might predict future activity scores [4].

Recent advances in proteomics technologies enable large-scale profiling of proteins in tissues and serum from patients. Application of these technologies to autoimmune

diseases enables identification of protein biomarkers for diagnosis and prediction in the clinical setting. Examples of important outcome parameters in autoimmune disease include disease severity, progression to disability, and response to therapy with a given disease-modifying drug (Fig. 1). However, reliable markers for the prediction of these outcomes in autoimmune conditions are lacking [5, 6].

This review highlights recent advances in the application of proteomics technologies for biomarker discovery in autoimmune diseases, with emphasis on RA, a prototypical autoimmune disease. For in-depth descriptions of the technologies touched upon in this article, we refer the reader to the respective chapters of this special issue, and to a string of excellent reviews published over the past 12 months [7–10]. Proteomic screening for and discovery of novel biomarkers will enable development of high-quality multiparameter assays with superior performance characteristics to guide diagnosis and management of patients with autoimmune disease.

2 Management of patients with autoimmune diseases: Unmet clinical needs

Autoimmune diseases comprise a wide variety of systemic or organ-specific inflammatory diseases, characterized by aberrant activation of immune cells to target self tissues. Autoimmunity arises from complex interactions of genetic factors and environmental factors, and there is substantial hetero-

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Abbreviations: DMARDS, disease modifying antirheumatic drugs; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus

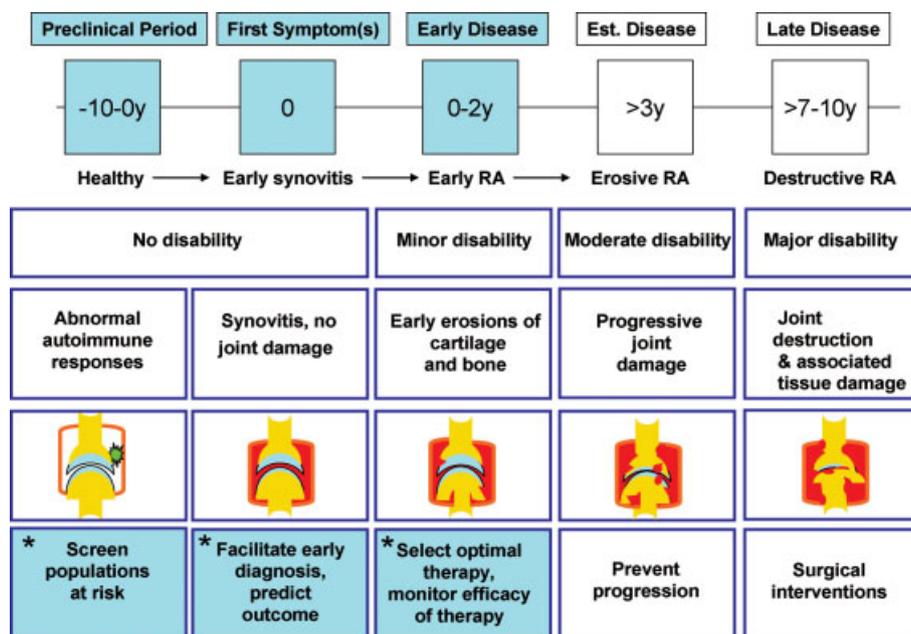


Figure 1. Disease course in RA. Disease progression, disability, structural damage and long-term outcome in rheumatoid arthritis (RA) are depicted in the schematic. Indicated in blue boxes are clinical checkpoints where accurate bioassays for outcome prediction and planning of interventions are most desired. The schematic shows a synovial joint, with cartilage in pale blue, synovial membranes in orange/red, and bone in yellow. Est. = established; RA = rheumatoid arthritis.

genity in the clinical manifestations, disease courses and outcomes among patients [11]. The etiology and pathogenesis of autoimmunity remain poorly understood.

Autoimmune diseases are manifested by a “coordinated immunological attack” [12] directed against self molecules (autoantigens) that are mistaken by the immune system as non-self. Alterations in genes that control pathways regulating self tolerance are critical in the pathogenesis of these diseases [13]. However, disease ultimately manifests itself on the protein level, and many events are not amenable to study by RNA transcript profiling. In contrast, key processes in autoimmune disease, such as PTM and autoantibody production are directly addressable by proteomic studies. For example, citrullination has been identified as a crucial PTM of major autoantigens in RA [14], and antibodies targeting citrullinated proteins and peptides can readily be detected on autoantigen microarrays [15].

The physician who manages patients with autoimmune diseases is confronted with multiple challenges: (i) establishing a diagnosis early in the course of disease; (ii) identifying patients likely to develop more severe disease; (iii) selecting therapies to which a given patient will respond; and (iv) balancing limited health care resources and the high costs of biological disease modifying antirheumatic drugs (DMARDs). Together, these issues constitute a great socio- and pharmaco-economical challenge [6].

3 Clinical checkpoints in the diagnosis and management of autoimmune disease

A schematic depicting the chronology of autoimmune disease is presented in Fig. 1, exemplified by the disease course in RA. Heterogeneity in clinical presentation and disease

course are common, confounding the early diagnosis and prediction of disease severity. Initial disease manifestations and laboratory tests are generally nonspecific and do not allow for a precise diagnosis. Frequently, the patient must be followed for months to years by clinical examination, laboratory tests and radiographs to establish the diagnosis. With the recent approvals of multiple biological disease-modifying agents, this early disease period may represent an important window for therapeutic intervention. Delay in diagnosis, and thus institution of the most appropriate therapies, results in increased tissue damage. RA needs to be treated early and aggressively to prevent cartilage damage [16]. At several clinical checkpoints, optimal interventions are delayed due to a lack of accurate biomarkers (Fig. 1).

4 Proteomic technologies as biomarker discovery tools

Proteomic technologies hold the potential to revolutionize clinical care by providing tools for the discovery of biomarkers for diagnosis, prediction of disease course, guiding therapeutic selection, and monitoring response to therapy. Nevertheless, tremendous work remains to develop, refine, validate and apply proteomics technologies to identify biomarkers in autoimmune disease. We will now highlight several proteomics technologies and their application to autoimmune diseases, including (i), 2-DE and MS for autoantigen and biomarker discovery; (ii), autoantigen microarrays to characterize autoantibody responses; (iii), antibody array technologies to profile cytokines and other biomolecules; (iv), RP protein array studies to analyze phosphoproteins; and (v), flow cytometric analysis of phosphoproteins.

5 2-DE MS for protein biomarker discovery

Investigators have explored 2-DE and MS approaches to elucidate differentially expressed proteins in autoimmune disease. Protein extracts from tissue or blood are separated in two dimensions on SDS-PAGE. Proteins were then visualized by CBB or SYPRO Ruby staining, followed by analysis of protein expression patterns using spot detection software. Differentially expressed proteins were then subjected to in-gel digestion with trypsin, followed by SELDI-TOF or MALDI TOF MS analysis and a search to definitively identify proteins. Employing this method, disease-specific patterns of protein expression were described for RA in comparison to reactive arthritis and osteoarthritis [17], and to ankylosing spondylitis [18]. Although descriptive, these studies spotlight a handful of differentially expressed proteins in autoimmune diseases that should be scrutinized more closely for their potential roles in pathogenesis. Other popular methods such as 2-D-DIGE have great potential to complement above-described methods, and have recently been used in the setting of autoimmune disease biomarker discovery [19].

Since disadvantages of 2-DE include poor reproducibility, lack of sensitivity, low throughput and considerable work load (see below), a simple alternative to the 2-DE method, BioChips[®] (CIPHERGEN, Fremont, CA), became popular. Among the many protein chips offered to researchers, the CIPHERGEN system became very popular for clinical biomarker discovery due to its minimal sample quantity requirements and label-free detection by MS. CIPHERGEN Biochips[®] provide miniaturized protein-binding surfaces that enable high-throughput separation of proteins with certain physical properties.

CIPHERGEN Biochips were successfully used to identify a characteristic protein signature in tear fluid of patients with Sjögren Syndrome (SS), an autoimmune condition, allowing the distinction of SS from other dry-eye syndromes [20], for the identification of a novel biomarker in RA [21], and recently, for the identification of a protein biosignature associated with response or non-response to conventional therapy in systemic onset juvenile idiopathic arthritis (SOJIA) [22]. In this latter pilot study, a serum protein profile of 23 proteins was associated with response to conventional treatment. Additionally, 9 proteins showed differential expression in patients with active disease who responded to conventional therapy in comparison to patients with active disease who did not. Although interesting, these observations are too preliminary to conclude that a serum protein profile exists for prediction of treatment response in SOJIA. Of note, the major peak that was identified in this study represented SAA, a very abundant acute-phase protein.

5.1 Fractionation to enhance 2-DE MS-based biomarker discovery

The major limitations of proteomic studies using serum and synovial fluid, and to a lesser degree cerebrospinal fluid, include (i) the wide dynamic range of proteins found in a

complex biological sample, and (ii) the propensity of acute phase proteins to obscure other serum proteins of interest. These limitations in part explain the inability to discover low abundance serum proteins [9, 23]. Efforts are in progress to address these limitations, as discussed elsewhere [9].

To address some of these limitations, Liau and colleagues [24] recently used size exclusion fractionation prior to MS analysis. To improve resolution, sensitivity and dynamic range, and therefore the number of peaks for MS analysis, the authors used size-exclusion fractionation of hyaluronidase-treated synovial fluid samples that were first depleted of abundant proteins, followed by multidimensional LC/LC-MS/MS. After a subset of potential markers was selected from 418 identified proteins within the 40-kDa fraction of the proteome of the synovial fluid, the authors went on to demonstrate that a profile of protein biomarkers was significantly associated with erosive joint disease.

Another interesting approach to address the limitations of quantitative serum proteome analysis was recently introduced by Pan and colleagues [25]. The authors used an LC-MALDI-TOF/TOF-based platform for the analysis of peptide arrays, whereby peptides were selected based on knowledge obtained from previous proteomic discovery experiments. Their method enables accurate quantification of previously identified biomarkers in serum by comparing a native peptide unique to the protein of interest in the serum specimen with a spiked isotope-labeled stable reference peptide, allowing the calculation of an abundance ratio. Low-abundance molecules in complex biological samples were easily detected and quantified. This method represents a promising tool for quantification of biomarkers in clinical applications, and should facilitate high-throughput screening of large numbers of samples, as required for biomarker validation.

5.2 2-DE MS analysis for the discovery of novel autoantigens

2-DE, immunoblotting and MS can be applied for the discovery of autoantigens. Proteins are transferred to NC membranes after 2-DE separation, followed by Western blotting of the membrane using autoimmune serum or other body fluid as matrix for capture antibodies. After detection of the bound antibodies, the membrane can subsequently be used as a template to locate and excise even low abundant proteins for MS analysis. Examples include the discovery of novel autoantigens in celiac disease [26], multiple sclerosis in both serum [27] and cerebrospinal fluid [28], and lupus [29], as well as the discovery of PTM variants of proteins proposed to be autoantigens such as citrullinated alpha-enolase in RA [30].

Together, these studies substantiate the feasibility of MS for biomarker identification in autoimmune disease. A spectrum of potential new biomarkers in a variety of autoimmune diseases has been reported. However, only very small numbers of samples were analyzed in these studies, and larger validation studies are needed to establish the clinical relevance of those markers and their suitability for clinical assay development.

6 Autoantigen microarrays for profiling autoantibodies

A hallmark of many autoimmune diseases is the presence of high-affinity, high-avidity autoantibodies. Antibodies have long been used for the diagnosis and classification of autoimmune diseases [31]. Autoantigens represent the fraction of the tissue proteome that is targeted by aberrant immune responses in autoimmunity, and multiplex analysis of autoantibody responses against spectra of candidate antigens represents a powerful screening tool to delineate biomarker signatures in autoimmunity.

Our laboratory pioneered the development of autoantigen microarrays and generated connective tissue disease-arrays to study a variety of autoimmune diseases [32], myelin sheath proteome-arrays to study multiple sclerosis [33], and arthritis antigen arrays to study RA [15]. Recently, Zhen and colleagues [34] described smaller-scale glomerular proteome arrays to study SLE. Candidate and putative antigens are robotically printed onto array substrates, and the arrays are probed with immune or control sera. Serum autoantibodies that specifically interact with their cognate ligand are detected using fluorophore-conjugated anti-human secondary antibodies. Patterns of reactivity defined subsets of patients in RA and SLE. Autoantibody targeting of citrulline-containing epitopes was associated with more severe RA, whereas targeting of native, unmodified peptides and proteins occurred predominantly in RA patients with mild disease (Fig. 2). Distinct autoantibody signatures were also correlated with proinflammatory cytokines (manuscript submitted) and the presence of the shared epitope polymorphism in the major histocompatibility complex (MHC) class II gene, the single most important genetic marker of severity and outcome in RA [15]. In a recently published study, glomerulome protein microarrays, containing 30 known protein antigens, enabled identification of clusters of antigen reactivity associated with higher SLE disease activity scores (SLEDAI), while increased IgM antibody poly-reactivity correlated with less active disease [34]. These data suggest that glomerulome antigen arrays are useful for molecular disease subtyping in SLE.

Elegant solutions to the important problem of determination of specificity of antibodies of unknown specificity are whole-proteome microarrays. High-density human protein microarrays (>5000 human proteins) are now commercially available to study protein-protein interactions (Immune Response Biomarker Profiling ProtoArray[®], Invitrogen, Carlsbad, CA). Proteins included on ProtoArrays[®] are expressed in a baculovirus system. Thus, advantages include the presence of certain PTMs, while important limitations are the absence of the PTMs phosphorylation and citrullination, which are known autoimmune targets in SLE and RA, respectively, and the absence of alternatively spliced protein species on such arrays.

Above studies corroborate the notion that autoantibody profiling using antigen arrays is well positioned to become an anchor technology for the development of multiplex autoantibody-based biomarker assays for use in clinical medicine.

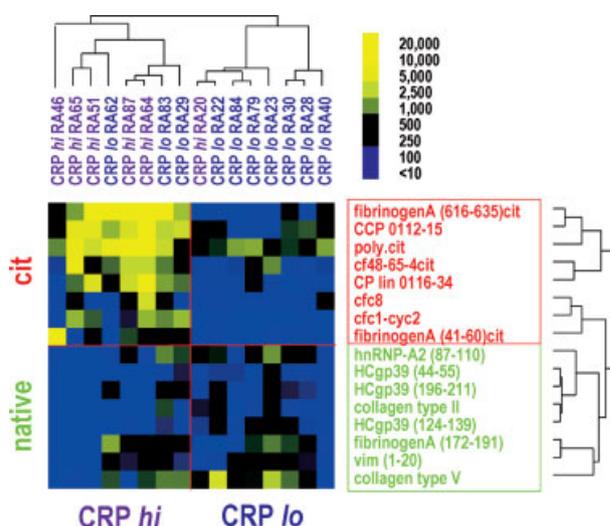


Figure 2. Proteomic identification of subgroups of RA patients likely to develop more severe vs. less severe arthritis. Arthritis antigen arrays were applied to profile autoantibody responses in a cohort of early RA patients. A statistical algorithm identified antigens differentially targeted in patients predictive to have a severe disease course [C-reactive protein (CRP) levels >1.5 mg/dL] and patients predicted to have mild disease course (CRP levels <0.5 mg/dL). Citrullinated epitopes (in red font) were preferentially targeted in the CRP^{high} subgroup (severe RA), whereas native, unmodified antigens (in green font) were preferentially targeted in the CRP^{low} subgroup (mild RA). Hierarchical clustering was applied to arrange the patients and significance analysis of microarrays (SAM)-identified antigen features. The labels to the left of the cluster image indicate the general locations of citrullinated antigens (red font) and native antigens (green font). This figure was reproduced from Hueber *et al.* Reproduced from [15] with permission.

7 Combining proteomics profiling and transcriptional profiling

Few studies have compared transcriptional and proteomic data in autoimmune disease. Lorenz *et al.* [35] screened patients with a diagnosis of RA and osteoarthritis in search of differentially expressed candidate molecules on the transcriptional and protein level. The authors profiled >12 000 genes by Affymetrix RNA chips and 791 proteins by a multi 1-D SDS-PAGE Western blot format that employed validated mAb. Concordant expression patterns were observed for 16 molecules, illustrating the substantial discordance rate of gene and protein expression. Moreover, only 33% of the antibodies recognized their cognate antigen on Western blots.

Gering *et al.* [36] attempted to identify specific pathways relevant to the development of diabetes in the nonobese diabetic (NOD) mouse model. The authors examined the transcriptome (by Affymetrix chips) and proteome (by 2-DE analysis followed by PMF of differentially expressed proteins) of splenic lymphocytes of NOD mice and normal control mice (C57BL/6) at two time points. A Bayesian network

analysis was performed, which allows the mapping of networks by graphically grouping molecules around 'nodes' into functional categories such as "apoptosis", "cell differentiation", etc. The overall theme that emerged was the presence of two clusters centered on nodes representing the oncogenes MYC and MYCN, on both the genomic and proteomic level, as principal discriminators between the diabetic mice and healthy control mice. The use of a systems biology approach to identify functional networks may provide important insights into pathogenesis. Although beyond the scope of this review, Bayesian network analysis represents a powerful mathematical tool to provide mechanistic underpinnings of disease states, and guide the selection of disease-state specific biomarkers [37].

8 Antibody arrays for biomarker profiling

Multiple academic investigators and companies are developing antibody arrays for the multiplex measurement of secreted proteins and markers of inflammation in blood and other body fluids. Such markers include cytokines, chemokines, metalloproteinases, soluble receptors, growth factors and other molecules. So far, only few studies have reported distinct cytokine profiles in RA [38, 39] and relapsing poly-chondritis [40], using bead-based multiplex cytokine assays. Early studies focused on measuring serum cytokines for monitoring treatment responses in patients treated with novel biological DMARDs such as the anti-TNF- α blocker Infliximab [41]. Several smaller studies followed where combinations of cytokines were measured in a low throughput format to monitor response to conventional DMARDs [42] and biologicals [43]. Nevertheless, to date no compelling set of cytokine biomarkers has emerged from these studies. It remains to be determined whether cytokine profiling will prove an effective approach to monitor response to treatment in autoimmune disease. Standardizing protocols for sample handling and processing will facilitate comparison of proteomic data from antibody array platforms. We anticipate that cytokines and other secreted proteins in blood will become an important category of biomarkers in autoimmune disease.

9 RP protein arrays

RP protein arrays have been adopted to analyze the proteome of clinical biopsies and cancer tissue specimens [44]. The seminal study on serum proteome profiles in patients with ovarian cancer [45] ignited efforts to integrate proteomic data analysis into cancer trials, such as IRB approved molecular profiling protocols in trials designed and conducted by the FDA-NCI Clinical Proteomics Program.

RP protein arrays were originally described for the study of frozen tissue samples [46], and have recently been adopted by Chan *et al.* [47] for the multiplex analysis of signaling

pathways in CD4⁺CD25⁺ T cells, a key lymphocyte population relevant to maintenance of peripheral tolerance in autoimmune diseases [48]. The authors spotted lysates of purified murine CD4⁺CD25⁺ T cells, unstimulated or stimulated with interleukin-2 at various time points, onto NC-coated microscope slides, whereby each spot represented the proteome of about 20 cells. The arrays were then probed with 62 different phospho-specific antibodies, and after incubation with horseradish peroxidase-conjugated secondary antibodies, differences in phosphorylation events were detected and quantified. These experiments revealed differential STAT phosphorylation in activated CD4⁺CD25⁺ regulatory T cells over time as compared to unstimulated CD4⁺CD25⁺ cells and CD4⁺CD25⁻ cells. Their findings shed light on a novel downstream pathway that may play roles in T regulatory cell function. This important proof-of-principle paper opens new avenues for the large-scale, multiplex study of signaling events in immune and other cells relevant to autoimmune disease.

10 Flow cytometry single-cell analysis of phosphoproteins in signal transduction

Profiling disease state-specific events in the proteome of single living cells represents a powerful approach to delineate patient-specific biomarker profiles. This technology is being developed and explored in the laboratory of G. Nolan (Stanford). Irish *et al.* [49] described isolation and analysis of single myeloma cells from patients prior to chemotherapy, including stimulation of cancer cells with cytokines followed by analysis of intracellular phosphorylation events by multi-parameter flow cytometry using panels of phospho-specific antibodies. Cryptic signaling pathways were identified in stimulated but not in unstimulated cells. Moreover, Bayesian network analysis revealed phosphoprotein networks unique to the stimulated tumor cells. These data suggest that disease state-specific signaling dysregulation is revealed upon stimulation of cells, but remain hidden when cells are characterized under steady-state conditions. Cell populations with the potential to reveal biomarker profiles in RA include cartilage-invasive fibroblast-like synoviocytes (FLS), immune cells in the synovial fluid, and monocyte subpopulations in the peripheral blood. Studies in autoimmune disease including SLE and RA are now under way (G. Nolan, personal communication).

11 Concluding remarks

Before multi-parameter assays will become available for use in clinical practice, several important tasks will need to be achieved. These include the identification of high-quality biomarkers using a variety of technology platforms. Candidate biomarkers will need to be validated in independent, large clinical sample sets, including prospective clinical trials and in

multiple independent clinical laboratories. While more complex than whole-serum proteome analysis, proteome analysis of cell populations and single cells will likely provide superior biomarkers for certain diseases. Further, single-cell protein mapping may reveal biomarkers that directly reflect pathogenic mechanisms or drug efficacy in the target cells. Multiple proteomics technologies provide the potential to identify biomarkers with utility for personalized medicine and targeted therapy. Ultimately, the development and use of such proteomic biomarkers for diagnosis, assessing prognosis and guiding therapy will revolutionize the care for autoimmune disease patients.

12 References

- [1] Baechler, E. C., Batliwalla, F. M., Karypis, G., Gaffney, P. M. *et al.*, *Proc. Nat. Acad. Sci. USA* 2003, **100**, 2610–2615.
- [2] Batliwalla, F. M., Baechler, E. C., Xiao, X., Li, W. *et al.*, *Genes Immun.* 2005, **6**, 388–397.
- [3] Gregersen, P. K., *Nat. Genet.* 2005, **37**, 1300–1302.
- [4] Bauer, J., Baechler, E. C., Batliwalla, F. M., Ortmann, W. A. *et al.*, *Arthritis Rheum.* 2005, **52** (suppl), S276 (abstract).
- [5] Scofield, R., *Lancet* 2004, **363**, 1544–1546.
- [6] O'Dell, J. R., *N. Eng. J. Med.* 2004, **350**, 2591–2602.
- [7] Utz, P. J., *Immunol. Rev.* 2005, **204**, 264–282.
- [8] Fathman, C. G., Luis Soares, L., Chan, S. M., Utz, P. J., *Nature* 2005, **435**, 605–611.
- [9] Coombes, K. R., Morris, J. S., Hu, J., Edmondson, S. R. *et al.*, *Nat. Biotechnol.* 2005, **23**, 291–292.
- [10] Balboni, I., Chan, S. M., Kattah, M., Tenenbaum, J. D. *et al.*, *Annu. Rev. Immunol.* 2006, **24**, 391–418.
- [11] Firestein, G. S., *Nat. Immunol.* 2003, **432**, 356–361.
- [12] Steinman, L., *Cell* 1996, **85**, 299–302.
- [13] Goodnow, C. C., Sprent, J., Fazekas d., S., Groth, B. *et al.*, *Nature* 2005, **435**, 590–597.
- [14] Vossenaar, E., van Venrooij, W., *Arthritis Res. Ther.* 2004, **6**, 107–111.
- [15] Hueber, W., Tomooka, B. H., Kidd, B. A., Lee, B. J. *et al.*, *Arthritis Rheum.* 2005, **52**, 2645–2655.
- [16] Weinblatt, M. E., *Cleve Clin. J. Med.* 2004, **71**, 409–413.
- [17] Sinz, A., Bantscheff, M., Mikkat, S., Ringel, B. *et al.*, *Electrophoresis* 2002, **23**, 3445–3456.
- [18] Tilleman, K., Van Beneden, K., Dhondt, A., Hoffman, I. *et al.*, *Proteomics* 2005, **5**, 2247–2257.
- [19] Ryu, O. H., Atkinson, J. C., Hoehn, G. T., Illei, G. G. *et al.*, *Rheumatology (Oxford)* 2006, in press.
- [20] Tomosugi, N., Kitagawa, K., Takahashi, N., Sugai, S. *et al.*, *J. Proteome Res.* 2005, **4**, 820–825.
- [21] Uchida, T., Fukawa, A., Uchida, M., Fujita, K. *et al.*, *J. Proteome Res.* 2002, **1**, 495–499.
- [22] Miyamae, T., Malehorn, D., Lemster, B., Mori, M. *et al.*, *Arthritis Res. Ther.* 2005, **7**, R746–755.
- [23] Diamandis, E. P., *Mol. Cell. Proteomics* 2004, **3**, 367–378.
- [24] Liao, H., Wu, J., Kuhn, E., Chin, W. *et al.*, *Arthritis Rheum.* 2004, **50**, 3792–3803.
- [25] Pan, S., Zhang, H., Rush, J., Eng, J. *et al.*, *Mol. Cell. Proteomics* 2005, **4**, 182–190.
- [26] Stulik, J., Hernychova, L., Porkertova, S., Pozler, O. *et al.*, *Proteomics* 2003, **3**, 951–956.
- [27] Almeras, L., Lefranc, D., Drobecq, H., De Seze, J. *et al.*, *Proteomics* 2004, **4**, 2184–2194.
- [28] Dumont, D., Noben, J. P., Raus, J., Stinissen, P. *et al.*, *Proteomics* 2004, **4**, 2117–2124.
- [29] Thebault, S., Gilbert, D., Hubert, M., Drouot, L. *et al.*, *J. Immunol.* 2002, **169**, 4046–4053.
- [30] Kinloch, A., Tatzer, V., Wait, R., Peston, D. *et al.*, *Arthritis Res. Ther.* 2005, **7**, R1421–1429.
- [31] van Mühlen, C. A., Tan, E. M., *Arthritis Rheum.* 1995, **24**, 323–358.
- [32] Robinson, W., DiGennaro, C., Hueber, W., Haab, B. *et al.*, *Nat. Med.* 2002, **8**, 295–301.
- [33] Robinson, W., Fontoura, P., Lee, B., de Vegvar, H. *et al.*, *Nat. Biotechnol.* 2003, **21**, 1033–1039.
- [34] Zhen, Q. L., Xie, C., Wu, T., Mackay, M. *et al.*, *J. Clin. Invest.* 2005, **115**, 3428–39.
- [35] Lorenz, P., Ruschpler, P., Koczan, D., Stiehl, P. *et al.*, *Proteomics* 2003, **3**, 991–1002.
- [36] Gerling, I., Singh, S., Lenchik, N. I., Marshall, D. R. *et al.*, *Mol. Cell. Proteomics* 2006, **5**, 293–305.
- [37] Sachs, K., Perez, O., Pe'er, D., Lauffenburger, D. A. *et al.*, *Science* 2005, **308**, 523–529.
- [38] Hitchon, C. A., Alex, P., Erdile, L. B., Frank, M. B. *et al.*, *J. Rheumatol.* 2004, **31**, 2336–2346.
- [39] Raza, K., Falciani, F., Curnow, S. J., Ross, E. *et al.*, *Arthritis Res. Ther.* 2005, **7**, R784–795.
- [40] Stabler, T., Piette, J. C., Chevalier, X., Marini-Portugal, A. *et al.*, *Arthritis Rheum.* 2004, **50**, 3663–3667.
- [41] Charles, P., Elliott, M. J., Davis, D., Potter, A. *et al.*, *J. Immunol.* 1999, **163**, 1521–1528.
- [42] Kraan, M. C., Smeets, T. J. M., van Loon, M. J., Breedveld, F. C. *et al.*, *Ann. Rheum. Dis.* 2004, **63**, 1056–1061.
- [43] Pittoni, V., Bombardieri, M., Spinelli, F. R., Scivo, R. *et al.*, *Ann. Rheum. Dis.* 2002, **61**, 723–725.
- [44] Espina, V., Mehta, A. I., Winters, M. E., Calvert, V. *et al.*, *Proteomics* 2003, **3**, 2091–2100.
- [45] Petricoin, E., Ardekani, A. M., Hitt, B. A., Levine, P. J. *et al.*, *Lancet* 2002, **359**, 572–577.
- [46] Paweletz, C. P., Charboneau, L., Bichsel, V. E., Simone, N. L. *et al.*, *Oncogene* 2001, **20**, 1981–1989.
- [47] Chan, S., Ermann, J., Su, L., Fathman, G. C. *et al.*, *Nat. Med.* 2004, **10**, 1390–1396.
- [48] Sakaguchi, S., *Annu. Rev. Immunol.* 2004, **22**, 531–562.
- [49] Irish, J. M., Hovland, R., Krutzik, P. O., Perez, O. D. *et al.*, *Cell* 2004, **118**, 217–228.