Genomic and proteomic analysis of multiple sclerosis
Opinion

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Multiple sclerosis (MS) and other autoimmune diseases result from the dysregulation of genetic and proteomic programs. In MS, the loss of immune homeostasis leads to aberrant targeting and destruction of the myelin sheath, which manifests as the clinical syndrome of MS. The advent of technologies to perform large-scale analysis of mRNA transcript and protein expression will transform our understanding of the mechanisms underlying the initiation and progression of MS, and will yield new targets for therapeutic intervention.

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Abbreviations

ACTH	adrenocorticotropin hormone
CoA	coenzyme A
CRF	corticotropin-releasing factor
EAE	experimental autoimmune encephalomyelitis
H1R	histamine 1 receptor
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
MBP	myelin basic protein
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
OPN	osteopontin
PAFR	platelet activating factor receptor
RA	rheumatoid arthritis
SCD-1	stearoyl CoA desaturase-1
SLE	systemic lupus erythematosus
Th	T helper

Introduction

Over the past three decades, significant progress has been made towards understanding immunity and autoimmunity. The molecular structures of the T- and B-cell antigen receptors, MHC molecules and a variety of co-receptors have been defined. Signaling pathways that regulate the genetic program of autoreactive lymphocytes have been elucidated. Molecular checkpoints that govern the development of antiviral and tissue-destructive Th1 responses versus allergic and tissue-protective Th2 responses are being characterized and the self-proteins and other self-biomolecules aberrantly targeted by autoimmune responses have been partially identified. Nevertheless, our understanding of the confluence of genetic, environmental and stochastic elements that lead to autoimmunity remain primitive.

Our difficulty in distilling the mechanisms underlying autoimmunity may, in part, stem from past reliance on conventional molecular, biochemical and cellular methodologies that reveal the properties of one or several molecules of interest, but are unable to provide a profile of the genetic and proteomic events underlying autoimmunity. The development of multiplex technologies that enable the large-scale analysis of genomic and proteomic programs will provide critical insights into the mechanisms underlying conditions such as multiple sclerosis (MS) and autoimmunity. Ultimately, a 'systems biology' approach, integrating technology, biology and computation, will be essential to synthesize genomic and proteomic datasets to develop a molecular portrait of autoimmune pathophysiology.

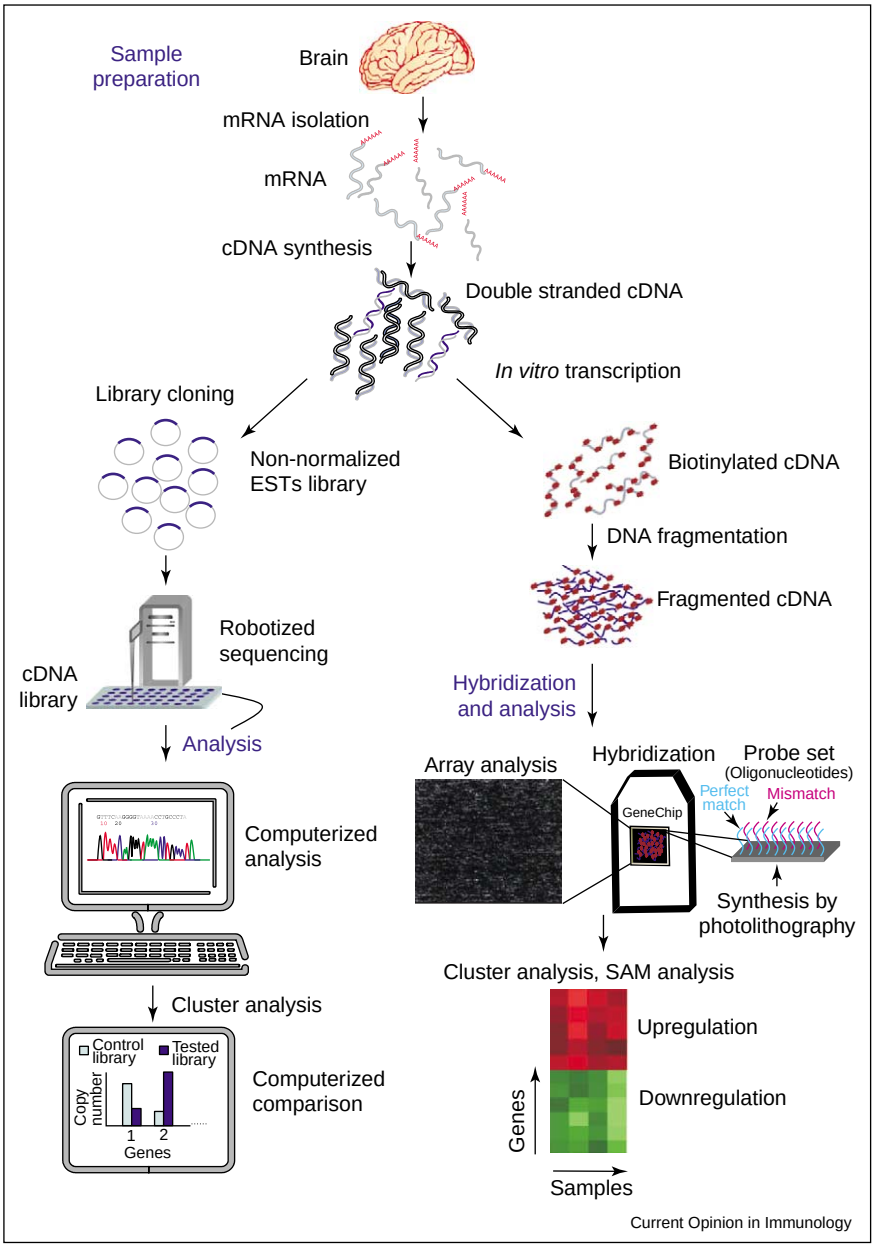
In this review we describe the application of genomic and proteomic methodologies to study MS and its murine model, experimental autoimmune encephalomyelitis (EAE).

Large-scale analysis of mRNA transcripts in multiple sclerosis and EAE

Transcripts that are unique to MS plaques, as well as transcripts that are differentially expressed in acute versus chronic MS material, have been identified. We have used two parallel approaches involving either large-scale robotic sequencing of mRNA transcripts from cDNA libraries prepared from brain tissue, or oligonucleotide microarrays (Figure 1; [1,2••,3,4]). The basic goal is to discover transcripts unique to MS plaques, or differentially expressed in acute versus chronic MS material, and then to understand the pathobiology of the proteins encoded by these transcripts.

High-throughput sequencing of cRNA from expressed sequence tags (ESTs), utilizing non-normalized cDNA brain libraries generated from MS brain lesions and control brain, has revealed the most prominent transcripts found in the MS brain [1]. We sequenced over 11 000

Figure 1



Transcriptional profile analysis. We used two parallel approaches to profile mRNA transcripts from cDNA libraries prepared from brain tissue in MS and EAE; the large-scale robotic sequencing of mRNA transcripts (left), and oligonucleotide microarray analysis (right). As depicted, mRNA is isolated from brain tissue and synthesized into double stranded cDNA. For robotic sequencing of mRNA transcripts, this double stranded cDNA is cloned into an expression vector and the resulting non-normalized expressed sequence tag (EST) library is robotically sequenced. Computer analysis is performed to determine the frequency of expression of mRNA transcripts from individual genes. Alternatively, for oligonucleotide array transcriptional profiling the cDNA is *in vitro* transcribed and biotinylated, fragmented, and then the fragmented cDNA is hybridized with oligonucleotide arrays. The resulting oligonucleotide array datasets are analyzed with statistical algorithms including Cluster and SAM (significance of analysis of microarrays) to identify patterns of gene expression associated with MS and EAE.

clones from acute and chronic MS patients and controls, and concentrated the analysis on genes present in both MS libraries, but absent in the control library. This yielded 423 genes, including 26 novel genes. Transcripts for αB-crystallin, an inducible heat shock protein localized in the myelin sheath and targeted by T cells in MS, were the most abundant transcripts unique to MS plaques. The next five most abundant transcripts included those for prostaglandin D synthase, prostatic binding protein, ribosomal protein L17 and osteopontin (OPN).

A few other studies have also analyzed transcriptional profiles in MS lesions. We compared our results with those of Biddison and colleagues [5], who used cDNA microarrays to profile MS lesions. They studied two MS lesions from one brain and found 29 genes to have increased expression in acute MS plaques. These 29 genes were represented on the HuGeneFL chip used in our study [2••], except for α2-chimerin, which was replaced by chimerin. We found 8 of these 29 genes increased in at least two of the four MS samples [2••]. Another recent study by Selmaj and colleagues [6] 'directly compare(d) different regions of multiple sclerosis lesions from lesions displaying different activity from the same individuals'. A comparison of raw datasets from the Selmaj study and the other previously reported analyses [1,2••,5,6] have not, as yet, been performed. The studies by Chabas and colleagues [1], Lock and colleagues [2••], and Whitney and colleagues [5] compared common aspects of MS lesions and the animal model EAE. Other studies have analyzed the transcriptional profiles of EAE lesions [7,8]. It would be useful in the future to study not only a model of acute EAE, but also one of the several other varieties of human and rodent autoimmune demyelinating disease [7,9].

Some early fruits of research into transcriptional profiling in multiple sclerosis

A role for osteopontin in relapses of multiple sclerosis and EAE

In animal models of MS, both our group [1] and Cantor's group [10] have shown that OPN modulates the progression of EAE. EAE was induced in $OPN^{-/-}$ mice and $OPN^{+/+}$ controls using myelin oligodendrocyte glycoprotein (MOG) p35–55 in complete Freund's adjuvant (CFA). EAE was observed in 100% of both $OPN^{+/+}$ and $OPN^{-/-}$ mice treated with MOGp35–55. Despite this, the severity of disease was reduced in all $OPN^{-/-}$ animals, and these mice were totally protected from EAE-related death [1].

The rate of relapses and remissions in these mice was tested. During the first 26 days following induction of disease with MOGp35–55, $OPN^{-/-}$ mice displayed a distinct evolution of EAE, with a much higher percentage of mice experiencing remissions compared to the controls. Although the clinical courses in the two groups were quite different, there were similar numbers and appearances of inflammatory foci within the central nervous system (CNS). Therefore, although OPN does not influence the extent of the inflammatory response, it might critically influence whether or not the course of disease is progressive, or whether relapses and remissions develop. OPN was shown to be pivotal in modulating Th1/Th2 polarization. Th1 cytokine production in myelin-specific T cells was reduced in $OPN^{-/-}$ mice, whereas Th2 production was increased [1]. Finally, DNA immunization with OPN protected mice from developing EAE [11].

Recently, Cantor's group has described concordant results in another model of EAE [10]. Taken together, our results [1,11] and those of Cantor's group [10] indicate that OPN is a potent modulator of autoimmune demyelinating disease. They also challenge the assertion by Blom *et al.* [12] that modulation of EAE is not due to OPN itself, but to genes linked to OPN.

The role for OPN in MS has been augmented by three recent studies using material from MS patients. Further studies have been undertaken looking at OPN polymorphisms and disease course in MS. In 821 MS patients analyzed, a trend for association with disease course was detected in patients carrying at least one 1284A allele in the *OPN* gene, suggesting an effect on disease course. Patients with this genotype were less likely to display a mild disease course and were at increased risk for a secondary progressive clinical type [13]. In MS patients in Japan, polymorphisms in OPN were critical in determining susceptibility to progressive or relapsing MS [14]. Levels of OPN were elevated in the plasma of patients with MS during relapses [15].

Modulation of multiple sclerosis and EAE by genes involved in cholesterol homeostasis

Transcriptional profiling of MS tissue revealed many changes in expression of genes involved in lipid and cholesterol metabolism in comparison to control tissue [2••,3]. Expression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was downregulated in MS tissue, as were the expression levels of other genes encoding critical pathways in lipid metabolism, such as stearoyl-CoA desaturase, acetacetyl CoA thiolase, propionyl CoA carboxylase, and enoyl CoA hydratase. Recently, many groups have explored the potential role of HMG-CoA reductase in MS pathology because of its pleiotropic effects on the immune system, which include downregulating the expression of inducible MHC class II molecules, and blocking leukocyte function-associated antigen-1 (LFA-1) and its interactions with intercellular adhesion molecule 1 (ICAM) [16–18]. The class of drugs known as statins reduce cholesterol synthesis by inhibition of HMG-CoA reductase. Over twenty years ago we showed that inhibition of MHC class II expression could reverse autoimmune disease in several animal models, including EAE, experimental autoimmune myasthenia gravis and experimental autoimmune thyroiditis [19–21]. Recently, promising results in preclinical studies have ignited interest in the potential application of the cholesterol-lowering HMG-CoA reductase inhibitors — statins — in MS therapy [22–24]. At the present time there is one on-going open-label trial testing simvastatin in relapsing-remitting MS, and other trials, using atorvastatin, are being planned.

Neuroendocrine mediation of multiple sclerosis and EAE

The large-scale analysis of gene transcripts in MS lesions revealed that levels of leptin (a neuroendocrine

mediator), melanocortin 4 receptor and adrenocorticotropic hormone (ACTH) receptor are elevated at the site of inflammation in brain [1,2••]. Leptin produces its profound effects on appetite and body weight by altering the balance between the anorectic neuropeptides α-melanocyte stimulating hormone (MSH) and corticotropin-releasing factor (CRF), and the orexigenic neuropeptides agouti-related protein (AGRP) and neuropeptide Y (NPY). Leptin also modulates Th1/Th2 balance, as Th1 responses are defective in ob/ob mice that have a mutation in the leptin receptor [25,26].

The microsomal enzyme stearoyl CoA desaturase-1 (SCD-1) is required for the biosynthesis of the monounsaturated fats palmitoleate and oleate from saturated fatty acids [2••,26]. SCD-1 RNA levels are highly elevated in the livers of ob/ob mice that contain a mutation in the leptin receptor and develop obesity. Indeed, SCD-1 probably plays a decisive role in leptin's metabolic effects. Interestingly, stearoyl CoA desaturase-1 is downregulated in MS brain as well [2••], and both RNA levels and activity of this enzyme are repressed by leptin [25,26]. The role of leptin in autoimmune brain disease, and in the immune system in general, might be mediated by downregulation of this enzyme involved in the biosynthesis of monosaturated fats.

Earlier work had shown that CRF, the key regulator of the stress response in the hypothalamic-pituitary-adrenal axis, or urocortin, a naturally occurring paralog of CRF, acting directly on T cells in adrenalectomized mice ameliorated EAE; antagonists of CRF blocked these effects [27]. Thus, CRF, similar to leptin, is produced by the brain and may act directly on the immune system. Expression of CRF itself can be regulated by cytokines, adding another layer of complexity and a further target for intervention. Another neuropeptide, ACTH, a key mediator of the stress response and produced in the pituitary gland, has been used for over 40 years to treat MS, and the ACTH receptor is expressed in the MS lesion itself [2••].

Links between allergy and multiple sclerosis

Self-antigens can trigger allergic responses, extending Ehrlich's conception of 'Horror Autoxicus', where the immune system attacks various tissues in the human body [28,29]. Large-scale transcriptional sequencing of MS lesions revealed that there are a large number of allergy-related gene transcripts in MS lesions [1,28]. These transcripts include prostaglandin D (PGD), platelet activating factor receptor (PAFR), tryptase, IgFcε receptor and eosinophilic cationic protein. Transcripts for tryptase, PAFR and PGD were elevated in the CNS of animals with EAE [30]. Moreover, histamine 1 receptor (H1R) was elevated on Th1 cells reactive to myelin, and immunohistochemical staining revealed H1R and H2R in inflammatory lesions in the brain. Interestingly, Teuscher and colleagues [31] reported recently that, H1R (GenBank accession no: AF387896) is critical for susceptibility to EAE. We showed that blockade of EAE was possible using inhibitors of H1R [29,30]. Blockade of PAFR also ameliorated EAE [30].

Proteomic approaches

Despite the wealth of information provided by genomic analysis, transcriptional profiling has important limitations. There is growing evidence that relatively frequent discordance exists between mRNA expression and the expression and function of the encoded proteins. This is thought to be due to:

1. Post-transcriptional regulation of protein expression.
2. Post-translational regulation of protein function.
3. The use of alternative splicing of mRNA to generate polypeptides with distinct functional properties.

Furthermore, because autoreactive T and B cells exist in heterogeneous populations at frequencies of less than 1 in 10 000 lymphocytes, transcriptional profiling cannot definitively reveal the specific variable (V), diversity (D) and joining (J) regions expressed by autoreactive lymphocytes. As we enter the post-genomic era, tremendous need exists for technologies that enable large-scale characterization of protein expression and function. The remainder of this review will focus on the development and application of protein arrays to characterize autoantibody responses in EAE and MS (Box 1).

Concordance of B- and T-cell responses

The ultimate utility of autoantibody profiling hinges on the hypothesis that the specificity of autoreactive B-cell responses correlate with those of autoreactive T-cell responses. Many disease-specific autoantigens are targeted by both B and T cells, including myelin basic protein (MBP) and MOG in MS and EAE [32] The precise epitopes recognized by B and T lymphocytes are shared for certain epitopes. In EAE and MS, B and T cells both recognize the HFFK motif (amino acid one-letter code) within immunodominant MBPp83–99 [33]. Even if discordance exists between the fine specificity of the T- and B-cell responses, the ability of the autoantibody response to identify the specific self-polypeptide(s) against which an individual is autoreacting may be sufficient to identify autoantigen targets and to develop antigen-specific tolerizing therapies.

Box 1 Applications for multiplex autoantibody profiling.

- Prediction of future development of autoimmunity
- Diagnosis of an autoimmune disease
- Prognostication: identification of patients likely to develop more severe disease
- Discovery and definition of autoantigens
- Design and selection of antigen-specific tolerizing therapies
- Monitoring response to tolerizing therapies

The evolution of multiplexed immunoassays for autoantibody profiling

Although there is likely to be controversy regarding the origins of protein microarrays, Geysen, Ekins, Fodor and colleagues [34–36] published several of the earliest descriptions outlining the concept of multianalyte immunoassays. In the early 1980s, Geysen *et al.* [34] developed methods to synthesize peptides on pins to form arrays of peptides. Geysen and others [34] applied peptides synthesized on pins to profile antibody responses in viral infections, and more recently this technique has been used by James, Harley and colleagues [37] in systemic lupus erythematosus (SLE). On the basis of mathematical modeling and confirmatory measurements, Ekins [35] envisioned that immunoassays could be miniaturized and performed on planar surfaces. Fodor and colleagues [36] developed methods to synthesize peptides photolithographically, and described the potential to apply such a system to study antibody responses. Despite the proliferation of studies employing DNA arrays for transcriptional profiling, it was a decade later before analogous studies using protein arrays began to appear in the literature.

In 2001, MacBeath and Schreiber [38] published a seminal paper in the protein array field. They demonstrated that antibodies, antigens and other binding molecules could be printed in ordered arrays on derivatized microscope slides. Shortly thereafter, Haab, Brown and colleagues [39] described experiments in which monoclonal antibodies or their cognate antigens were immobilized in arrays on derivatized microscope slides. The first efforts to produce and apply miniaturized arrays of autoantigen polypeptides were described by Joos and colleagues [40].

Our laboratories refined the methods of MacBeath and Schreiber [38], Haab *et al.* [39] and Joos *et al.* [40] to profile autoantibody responses present in the serum of patients with connective tissue diseases [41••]. We developed protein microarrays containing autoantigens immobilized in ordered arrays on poly-L-lysine-coated glass microscope slides. We demonstrated the detection of distinct autoantibodies that are, in part, diagnostic for eight different autoimmune diseases, including the detection of autoantibodies directed against polypeptides, peptides, protein complexes, ribonucleoprotein complexes, nucleic acids and post-translationally-modified polypeptides. We demonstrated that comparative analyses could be performed using isotype-specific secondary antibodies conjugated to distinct fluorophores. The ability to perform multiplex isotype analysis is likely to facilitate the identification of pathogenic autoantibodies and relevant autoantigens, and may enhance our ability to monitor responses to therapy.

Autoantibody profiling for diagnosis

Autoantibody profiling has the potential to identify individuals at risk of developing disease, as well as establishing the diagnosis of an autoimmune disease. Type I (autoimmune) diabetes mellitus and rheumatoid arthritis (RA) are examples of T-cell-mediated autoimmune diseases for which autoantibodies have clinical utility. In school children, the detection of autoantibodies against combinations of islet cell antigens is predictive of future development of autoimmune diabetes [42]. The detection of autoantibodies against citrullinated peptides are also predictive of RA [43,44]. Proteomic analysis is, therefore, a powerful strategy that may be employed to identify diagnostic autoantibody specificity profiles and to define autoantigen targets in MS and other autoimmune diseases.

Myelin array profiling of autoantibody responses in EAE for prognostication

To create a profile of autoreactive B-cell responses in EAE and MS we generated myelin arrays containing a spectrum of putative myelin autoantigens. Polypeptides and overlapping peptides from MBP, MOG, proteolipid protein (PLP), myelin-associated glycoprotein (MAG), cyclic nucleotide phosphodiesterase (CNPase), αB-crystallin, oligodendrocyte-specific protein (OSP), and golli-MBP were attached in ordered arrays on poly-L-lysine-coated microscope slides. Myelin arrays were probed with sera from mice with EAE. In acute EAE, increased diversity of the autoantibody response predicted increased disease activity in the subsequent disease course [45••]. Mice that developed chronic EAE exhibited extensive intra- and inter-molecular epitope spreading of their autoreactive B-cell responses to target multiple myelin proteins, and increased spreading was associated with a more severe course [45••].

Myelin arrays to guide antigen-specific therapy in EAE

The rationale behind the use of protein arrays to guide the selection of antigen-specific therapy is based on the hypothesis that there is concordance in the specificity of the autoreactive B-cell response with that of the autoreactive T-cell response at the macromolecular level (discussed above). Antigen-specific tolerizing therapies specifically attenuate autoaggressive lymphocytes; a variety of strategies are under development, including the delivery of peptides, altered peptide ligands, polypeptides and DNA encoding autoantigens [46–48]. We applied protein arrays to guide the development, selection and monitoring of antigen-specific tolerizing therapies in EAE [45••,49].

Tolerizing DNA vaccines encoding greater numbers of array-determined autoantibody targets in acute EAE demonstrated greater efficacy in reducing the number of relapses in the subsequent disease course [45••]. Myelin arrays demonstrated that efficacious tolerizing DNA vaccine therapy prevented extensive epitope spreading of the autoreactive B-cell response [45••]. Thus, antigen

arrays have the potential to guide development and selection of antigen-specific therapies, and to provide a surrogate system that can be used to monitor responses to therapy.

Microarrays for autoantigen discovery

Despite extensive efforts to identify autoantigens, the autoantigen targets of many human autoimmune diseases, including MS, remain elusive. Several groups are developing protein arrays in an attempt to discover novel autoantigens. Walter and colleagues [50,51] developed a high-throughput method for bacterial expression and purification of large numbers of polypeptides encoded in cDNA libraries to generate protein arrays. They are applying these arrays to attempt to identify the autoantigen targets in inflammatory bowel diseases.

Post-translational modifications are targeted by autoimmune responses in certain autoimmune diseases, and this possibility significantly complicates the generation of protein arrays capable of identifying autoantibody targets. Examples of post-translational modifications targeted by autoimmune responses include citrulline-modified arginine residues in RA, cleaved polypeptides resulting from apoptosis in SLE, and phosphorylated serine residues in SLE [43,52,53]. Although more robust methods exist to perform large-scale expression of recombinant proteins in bacteria, expression systems using eukaryotic cells may be necessary to produce certain post-translational modifications not made in bacteria. The multitude of potential post-translational modifications of candidate autoantigens is vast, and significantly complicates the generation of antigens for printing on arrays. Finally, certain autoantigens probably exist in complexes with other proteins or biomolecules, or may themselves be nonprotein biomolecules, such as carbohydrates or lipids. Protein arrays are likely to prove useful for identifying elusive autoantigen targets in diseases including MS, RA and psoriasis.

Conclusions

Large-scale transcriptional and proteomic analyses will revolutionize our understanding of MS and other autoimmune diseases. Transcriptional profiling has advanced our understanding of MS and revealed novel therapeutic targets. Therapeutic interventions directed against several of these targets have demonstrated promise in the EAE model. Protein arrays will facilitate identification of autoantibody profiles for disease prediction, diagnosis, tailoring antigen-specific therapies and monitoring responses to therapy. Ultimately, a systems biology approach will be necessary to integrate genomic, proteomic, clinical, and other datasets to generate a molecular portrait of the events underlying autoimmune initiation and progression, and to identify targets for therapeutic intervention.

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- • of special interest
- •• of outstanding interest

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