CHARACTERIZATION OF AN AUTOIMMUNE

GENE EXPRESSION SIGNATURE

By

Kevin Maas

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Microbiology and Immunology

August, 2006

Nashville, Tennessee

Approved:

Professor James W. Thomas

Professor Thomas M. Aune

Professor Nancy J. Olsen

Professor Mark Boothby

Professor Jennifer Pietenpol

I would like to dedicate this dissertation to my Uncle Ron, who has remained courageous and graceful in the face of overwhelming odds with cancer

ACKNOWLEDGMENTS

to assist me with the collection of these samples. In particular, I would like to thank Dr. Tuulikka Sokka, Dr. Howard Fuchs, Dr. Theodore Pincus, Dr. Victor Byrd, Dr. Sriram Subramaniam, Dr. Al Powers, Janie Lipps, and Patty Enright for the collection of these blood samples. I would like to further thank Dr. Tuulikka Sokka for providing us with editorial comments and access to the early rheumatoid arthritis patients.

On a personal note, I would also like to thank my family, Philip, Justina and Shannon Maas, for their support. They have provided me with encouragement, advice, and love. I would also like to thank my Nashville friends, Roy Barco and Aleksander Stanic, for their discussions regarding scientific aspects of my project. Finally, I would like to acknowledge Yuki Ohi. She has supported me with her love and devotion throughout my years in graduate school.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

TCR- T cell receptor

BCR- B cell receptor

MHC- major histocompatability complex

CIA- collagen induced arthritis

EAE- experimental autoimmune encephalomyelitis

CFA- complete freund's adjuvant

IDDM- type I diabetes

RA- rheumatoid arthritis

MS- multiple sclerosis

SLE- systemic lupus erythematosus

ARF- acute rheumatic fever

NSAIDs- Non-steroidal anti-inflammatory drugs

DMARDs- Disease modifying anti-rheumatid drugs

TREC- TCR recombination excision circle

DSB- double stranded DNA breaks

MRN complex- Mre-11/Rad50/Nbs-1 complex

ATM- ataxia telangiectasia mutant

PIKK- phosphoinositide 3-kinase

CHK2- checkpoint kinase 2

PBMC- peripheral blood mononuclear cells

CONT- control

IMM- immune response

DEG- differentially expressed gene

GNR- γ-radiation non-responder

GPR- γ-radiation partial-responder

PUMA- p53-upreagulated mediator of apoptosis

PARP- poly-(ADP-ribose) polymerase

CHAPTER I

BACKGROUND

Autoimmunity

Basic Paradigms in Autoimmunity: Immune Response to Self-Antigen

Basic concepts in immunology have shaped current theories regarding the mechanisms responsible for autoimmunity. One of the fundamental principles arising from decades of research is that the immune system is able to discriminate self from non-self. VDJ recombination at the TCR (T cell receptor) and BCR (B cell receptor) loci generate a vast polyclonal repertoire. Due to the random nature of this recombination process, the receptors generated may be viable, nonfunctional, or autoreactive. During development, central tolerance is achieved through the combined action of positive and negative selection, resulting in the removal of the nonfunctional and autoreactive lymphocytes. Despite central tolerance, autoreactive clones do survive the selection process and escape into the peripheral circulation. Additional mechanisms, collectively termed peripheral tolerance, prevent autoreactive clones from reacting against self. The mechanisms typically invoked as maintaining peripheral tolerance include anergy (functional unresponsiveness in a lymphocyte), clonal ignorance (lymphocytes remain responsive, but do not recognize autoantigen), and clonal deletion (autoreactive clones undergo apoptosis).

Given that autoimmune disorders arise from immune mediated damage against self, it follows that the mechanisms that allow self:non-self discrimination have broken down. Molecular mimicry, the notion that environmental antigens (such as those from bacteria or viruses) resembling self antigens can promote cross-reactivity against self, has been proposed as a method for instigating loss of tolerance. In the case of T cells, it would be expected that autoreactive lymphocytes recognize and react against self-antigen in the context of MHC (major histocompatability complex). Recognition of autoantigen would ultimately lead to an immune response against self, resulting in end organ damage.

Supporting evidence that foreign antigens resembling self can initiate autoimmunity comes from animal models, such as collagen induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE). CIA, an animal model of RA, is perhaps the most well characterized model. In this model, mice immunized intradermally with Complete Fruend's Adjuvant (CFA, a mixture of oil and *M. tuberculosis* products) and heterologous type II collagen (the major form of collagen present in joint cartilage) develop a monophasic, progressive polyarthritis that ultimately resolves[2]. Alternatively, EAE is an inflammatory demyelinating disease induced in mice through immunization with myelin proteins in CFA. EAE is characterized by ascending paralysis and numbness caused by demyelination and interruption of motor and sensory neurons[3].

Studies examining the susceptibility of mice to CIA support the notion that cross-reactive epitopes, in the context of the strain specific MHC polymorphisms, contribute to the development of CIA. Early experiments demonstrated that not all strains of mice were susceptible to CIA. Genetic studies revealed that the most important genetic interval contributing to CIA susceptibility was the MHC locus[4]. It is now known I-A^q

2

and I-A[r] MHC Class II polymorphisms are responsible for CIA susceptibility in mice, supporting a role for antigen presentation and CD4^{+} T lymphocytes in the disease model[5]. A further role of MHC and T lymphocytes in the disease has been illustrated by prevention of arthritis using blocking antibodies directed against the TCR[6, 7] and MHC II[8].

Further studies examining the immunogenicity of the antigen have revealed that only particular epitopes on type II collage promote autoimmunity. Immunization of susceptible strains of mice with proteolytic fragments of type II collagen reveal that the cyanogen bromide 11 fragment (amino acids 124-402) of type II collagen is capable of mediating disease, with amino acids 260-267 serving as the immunodominant epitope[9]. Additional type II collagen epitopes have also been identified. Similar results, demonstrating a dependence on specific MHC polymorphisms and cross-reactive epitopes, have been found in the EAE mouse model[3].

In general, animal models of autoimmunity provide support for an immunological view of autoimmunity. These models reveal that cross-reactive antigen in the context of disease-associated MHC polymorphisms can promote loss of tolerance. Cross-reactive lymphocytes mount an immune response against self, targeting and damaging end organs, ultimately leading to autoimmune disease manifestations.

Human Autoimmune Disorders: RA, SLE, MS, and IDDM

As a group, the human autoimmune disorders are relatively common, affecting approximately 5% of the population[10]. Autoimmune disorders are thought to arise from immune-mediated attack against self. While the role of MHC and cross reactive foreign

antigens is clear in animal models of autoimmunity, the mechanisms responsible for loss of tolerance are not known for the human diseases[11].

Considerable heterogeneity characterizes human autoimmune disorders, both within a disease and among the different disorders. For example, depending on the type of disease, the average age of onset can range from the teens for type I diabetes (IDDM) (10-20 years of age, Table I) through late adulthood for rheumatoid arthritis (RA) (30-50 years of age, Table I). The major sites of attack for these diseases can be highly organ- or tissue-specific, such as the myelin sheath in multiple sclerosis (MS) or pancreatic β-cells in IDDM to disorders that are more systemic in nature, such as RA and SLE. Epidemiologic and clinical information are compiled in Table I to highlight further differences among these four distinct diseases.

Closer examination further illustrates some of features that are unique to individual autoimmune disorders. For example, SLE is a complex autoimmune disease characterized by the production of autoantibodies directed against components of the cell cytoplasm and the nucleus. Women are affected with SLE approximately 8-10 times more frequently than men (See Table I)[12]. The overall population prevalence of SLE is approximately 0.05 % (see Table I), however there is a racial disparity, with African Americans suffering from SLE with a prevalence as high as 0.4%[13].

Patients typically present with a portion of a spectrum of SLE-associate clinical symptoms, including autoantibodies, skin rashes, arthritis, glomerulonephritis, and hematologic abnormalities[14]. In many cases, the symptoms arise from the deposition of immune complexes (autoantibodies complexed to antigen) in the vasculature and at various sites throughout the body, resulting in inflammation at multiple organ systems.

For example, the deposition of autoantibodies within the mesangium and near the basement membrane of the kidney[15, 16] contributes to the development of glomerulonephritis. Similarly, the deposition of immune complexes and complement at the dermal-epidermal junction[17, 18] can promote skin lesions and rashes characteristic of SLE.

MS is a neuroimmune disorder involving the central nervous system (CNS), which is characterized by infiltration of leukocytes into the CNS, localized inflammation, demyelination, and axon damage. MS predominantly affects women, with approximately 70-75% of MS patients being female (Table I)[19]. The incidence of disease also demonstrates racial disparity, with 90% of MS cases occurring in Caucasians. Interestingly, the incidence of MS displays a striking geographical distribution, with increasing risk zones moving away from the equator[20].

There are two major clinical patterns that occur in patients with MS: relapsing MS and progressive MS. Relapsing MS is characterized by acute neurological attacks, followed by periods of recovery. On the other hand, progressive MS involves the slow onset of neurological abnormalities. In some cases relapsing MS can evolve into secondary progressive MS [21]. Clinically, patients with MS may present with any number of a range of symptoms, with varying degrees of severity, including motor, sensory, and visual disturbances, fatigue, and cognitive loss (Table I)[21]. Regardless of the clinical pattern, many of the symptoms arise from the immune-mediated axonal demyelination and damage in the CNS. This demyelinating damage is believed to occur at discreet inflammatory lesions, termed plaques. Plaques arise as a result of the penetration of inflammatory cells into the CNS[22, 23].

IDDM results from immune mediated destruction of β-islet cells in the pancreatic islets of langerhans. Decreased pancreatic insulin output results in the inability to maintain appropriate blood glucose levels, ultimately contributing to symptoms such as polyuria, polydispia, and weight loss. IDDM typically occurs in individuals during youth, with a peak incidence between 10-20 years of age[24]. Unlike many of the other autoimmune disorders, there is a lack of gender bias in incidence for Type I diabetes. The incidence of Type I diabetes is higher in Caucasian populations than other ethnicities[25]. On a global scale, the incidence of IDDM can vary widely depending on geographical location and ethnicity, however generally speaking European populations tend to be the most predisposed to IDDM, with the Nordic countries (Finland, Sweden, and Norway) displaying the highest incidence. Conversely, countries in Asia and South America appear to have a relatively low incidence of IDDM[26].

Since significant destruction of pancreatic β cells occurs prior to clinical presentation of IDDM, identifying the events responsible for disease initiation in humans has been difficult. Signs of autoimmune dysregulation are believed to occur years prior to the clinical presentation of IDDM. For example, autoantibodies against islet cell antigens, such as insulin[27] and GAD65[28], have been detected in the serum of patients with IDDM. These autoantibodies are present both prior to diagnosis of disease[29] and at

**TABLE I. Comparison of epidemiologic, genetic, and clinical features
for four distinct human autoimmune disorders**

	Epidemiology			Genetics		Clinical Features	
	Onset Age	F:M	Prevalence	Relative Risk		Symptoms	Course
				Sibs	Mono		
SLE	15-40	8:1	≈0.05%	20	250	Glomeruloneph. Skin rashes Cardiovascular	Flares that decrease in activity with age
RA	30-50	2-4:1	≈1%	3-10	20-60	Joint pain/swelling Rh. vasculitis Rh. Nodules Pleurisy	50-70% of patients with progressive disease
MS	15-50	3:1	≈0.03%	20-40	250	Fatigue Cognitive Loss Motor Disturbance Visual Loss Sensory Disturbance	85% Relapsing also with Progressive Variants
IDDM	10-20	1:1	≈1%	15	60	Polyuria Polydipsia Weight Loss Microvascular	Progressive

Data for table compiled from:

 J. Cush, A. Kavanaugh, N. Olsen, C. Stein, S. Kazi, and K. Saag. In J. Pine, Jr. (ed.),*Rheumatology: Diagnosis and Therapeutics.* Williams & Wilkins. Baltimore.1999

Lahita, R.G., Weyand, C.M., Goronzy, J.J.,Brey, R.L., Barohn R.J., and Amato, A.A. (2000). In Lahita, R.G. (ed.), *Textbook of the Autoimmune Diseases.* Lippincott Williams & Wilkins, Philadelphia, pp. 351-375.

Data compiled for four distinct autoimmune disorders, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), and type I diabetes (IDDM). Epidemiological factors include: average age of disease onset (Onset age), ratio of female to male prevalence (F:M), and an estimate of overall prevalence in the population (Prevalence). Epidemiological support of a genetic contribution to autoimmunity (Genetics) is shown by examination of the relative risk of disease (Relative Risk) for either siblings (Sibs) or monozygotic twins (mono) relative to the general population. Clinical features of the individual disorder are also included, with a list of some of the clinical symptoms (Symptoms) and the general disease course (Course).

disease onset[30], suggesting that defects in B lymphocyte tolerance occurs prior to clinical detection of type I diabetes in patients. The accumulation of TH1 CD4$^+$ T lymphocytes in pancreatic islets of patients with IDDM suggests they also play a key role in mediating islet destruction[24]. Studies in NOD mice, a spontaneous animal model for human IDDM, supports the necessity for both B[31, 32] and T lymphocytes[33] in disease initiation.

RA is a progressive, erosive polyarthritis that affects the smaller diarthroidal joints in a symmetric manner. In addition to joint erosion, there are additional extra-articular aspects of the disease that may be present in a subset of patients. These include rheumatoid nodules, pleurisy, and cardiovascular abnormalities such as vasculitis[34]. RA predominantly affects women, and has a prevalence of about 1% throughout the world, with certain Native American populations displaying higher rates of disease (Table I)[14]. Other aspects of RA will be covered in greater detail in the RA section of the introduction.

For all of the autoimmune diseases discussed, the inciting events that initiate disease are not known. The specific antigens that are the target of immune attack are also poorly defined in these disorders. Regardless of these facts, evidence supports the notion that environmental and genetic factors ultimately dictate whether an individual will develop autoimmunity. Environmental factors that may play a role in a predisposition to autoimmunity include diet, climate, and toxins, however the data supporting these notions are incomplete.

It has been further postulated that viruses and bacteria bearing epitopes resembling self antigens may provoke a loss of tolerance against self. Indeed, pharyngeal infection with certain strains of group A streptococci are associated with acute rheumatic

fever (ARF). The onset of ARF occurs after initial infection, and may be accompanied by migratory arthritis of the large joints, possible clinical signs of carditis, and in rare cases involvement of the CNS[35]. Many of the symptoms of ARF are believed to arise from cross-reactive antibodies generated during the immune response to different components of the streptococcal cell wall or membrane[36-39]. Despite the convincing association between an underlying infectious agent and the loss of tolerance in ARF, it has proven difficult to establish an underlying infectious cause for other chronic autoimmune disorders.

The evidence supporting an underlying genetic predisposition for autoimmunity has been more convincingly established. Even in the absence of specific genetic factors, epidemiological evidence suggests an underlying genetic contribution to disease susceptibility. Studies examining the relative risk of disease between two populations, most often siblings or monozygotic twins compared with the general population, support an underlying genetic contribution. The finding that the relative risk of disease is greater than 1 for most autoimmune disorders (Table I) supports the notion that there is a genetic contribution[40-42].

Genetic linkage studies, the quest to identify genetic polymorphisms associated with autoimmune diseases in the population, provide more direct evidence of a genetic contribution to disease susceptibility. A large number of these studies have been performed in MS[43-45], IDDM [46-48], SLE[49-52] and RA[53, 54]. Generally speaking, these studies have revealed that the strongest linkage for susceptibility to autoimmunity resides in the HLA locus, with disease-specific polymorphisms enriched in the different patient populations. As mentioned previously, similar results have been found in numerous

animal models of autoimmunity, and support a role for antigen presentation and T lymphocytes in the pathogenesis for many of these diseases.

In addition to linkage to the HLA locus, genetic screens have identified non-MHC-linked loci that also appear to contribute to autoimmunity. In an interesting study, researchers made comparisons between numerous genomic screens for five distinct human autoimmune disorders, including IDDM and MS[55]. After statistical analysis, the group found that the positive markers from the different studies clustered non-randomly throughout the genome. Eighteen genetic clusters, comprised of markers from at least two different autoimmune diseases, were identified (Fig. 1)[55]. As a control, comparisons of susceptibility loci identified for seven non-autoimmune diseases (such as type II diabetes or schizophrenia) were also compared. No clustering of genetic loci was observed for the non-autoimmune disorders. Additional studies in RA have identified non-MHC loci that also cluster with the previously defined autoimmune clusters[53].

Collectively, these results suggest that there are multiple non-MHC genetic intervals that can predispose individuals to generalized autoimmunity. Epidemiological evidence supports the possibility of a genetic generalized predisposition to autoimmunity. Familial clustering of autoimmune diseases is not uncommon. A susceptible family can contain multiple individuals suffering from more than one type of autoimmune disease[56-58].

Basic Paradigms in Autoimmunity: Genetic Modulation of Immunoreactivity

Despite the striking findings from the human genetic studies, the specific genes and their roles in modulating immune function is poorly understood. However, studies in

animal models of autoimmunity clearly show that genetic susceptibility intervals can

impact global immune function. Similar to the human genomic screens, studies in

spontaneous animal models of autoimmunity have also revealed that multiple non-MHC
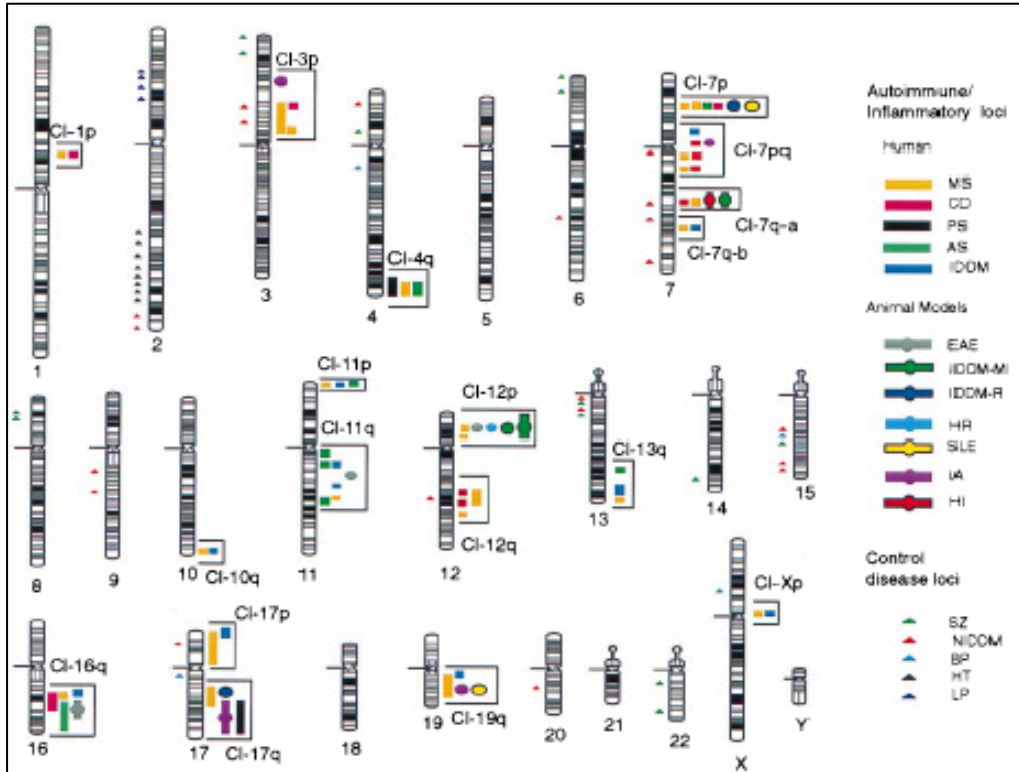
Becker, K., et al. "Clustering of non-major histocompatability
complex susceptibility candidate loci in human autoimmune diseases"
PNAS. 95. 1998. 95 9979-9984

Fig. 1. Clustering of autoimmune candidate loci.   Positive markers for
human autoimmune diseases are denoted by solid colored bars to the right
of the chromosomes. Control human non-autoimmune diseases are
indicated by a small colored triangle to the left of the chromosomes.
Remaining markers represent susceptibility loci from animal models of
autoimmunity. Clusters of susceptibility loci for multiple human
autoimmune diseases are denoted by the boxes encompassing the clustered
loci.

loci can contribute to a predisposition to autoimmunity. One of the animal models
investigated most intensively from a genetics standpoint is the NZM2410 mouse strain, a
spontaneous inbred animal model of SLE. Female mice suffer from more severe disease,
which is characterized by the production of autoantibodies to nuclear components,
splenomegaly, and ultimately fatal glomerulonephritis. Initial genetic analysis of the
NZM2410 strain revealed that three major non-MHC susceptibility intervals, *Sle1*, *Sle2*,
and *Sle3*, contribute to autoimmune susceptibility[59].

A congenic dissection approach was taken in an attempt to determine the
functional role of these susceptibility loci. Congenic dissection refers to the reduction of
a polygenic system, in this case the NZM2410 strain, into congenic strains each carrying
one or more intervals from the susceptible strain introgressed into a resistant genetic
background (i.e. C57/B6 mice)[60]. Characterization of these congenic strains for
manifestations of autoimmunity has allowed researchers to determine the functional role
of the susceptibility loci either individually or in combination. For example, after 6-9
months of age, B6.*Sle1* mice displays a loss of tolerance toward nuclear antigens, as
measured by the production of autoantibodies against subnucleosomal components[61].
These results implicate *Sle1* in loss of tolerance to nuclear antigen. Studies in B6.*Sle2*
mice revealed that the *Sle2* interval increases serum levels of polyclonal IgM and
increases B lymphocyte responsiveness to *in vitro* and *in vivo* stimuli[62]. T lymphocytes
from B6.*Sle3* mice display signs of T lymphocyte dysregulation, with congenic mice
containing age related increases in the number of activated CD4$^+$ lymphocytes, enhanced

responses to T-lymphocyte dependent antigens, and reduced activation induced cell death (AICD) in response to *in vitro* anti-CD3 stimulation[63].

While each of these genetic intervals appears to contribute to some aspect of immunological dysfunction, no single locus is able to mediate completely penetrant SLE with glomerulonephritis in the B6 congenic strains[60]. However, the cumulative effects of multiple *Sle* intervals is sufficient to cause disease on a B6 background[64]. This series of studies illustrates how genetic regions outside of the MHC locus can impact global immunoreactivity. Individually, each locus impacts an aspect of the immune function, such as tolerance to nuclear antigen, B lymphocyte reactivity threshold, or T lymphocyte reactivity and apoptosis. The cumulative impact of all loci is sufficient to drive the immune system to autoreactivity even on a normally resistant genetic background

Rheumatoid Arthritis

Since RA is the disorder we have studied the most extensively, a separate section is provided to cover basic clinical and science background regarding the disease. RA is classically characterized as a chronic, progressive erosive polyarthritis that targets diarthroidal joints in a symmetric pattern, most commonly affecting the hands and feet[14]. In addition to the cardinal finding of inflammatory arthritis, there are additional systemic manifestations, such as pleuritis, rheumatoid nodules and vasculitis, which may be present in some patients[34].

The most prominent pathologic changes associated with RA occur within the joints of patients (Fig. 2). In active disease, the synovial lining hypertrophies and has been invaded by CD4$^+$ T lymphocytes, B lymphocytes, and macrophages. Synoviocytes,

which consist of fibroblast-like cells and macrophage-like cells, also display signs of

activation and actively produce extracellular matrix components, metalloproteinases, and

proinflammatory cytokines such as IL-1 and IL-6[65]. This heterogeneous inflammatory

tissue comprised of synoviocytes and leukocytes, commonly referred to as pannus, acts as

the major destructive unit in RA resulting in the erosion of subchondral bone. The large

number of neutrophils and elevated levels of IL-1, IL-6, and TNFα, in the synovial fluid

of RA patients further highlights the inflammatory nature of this disease[14].
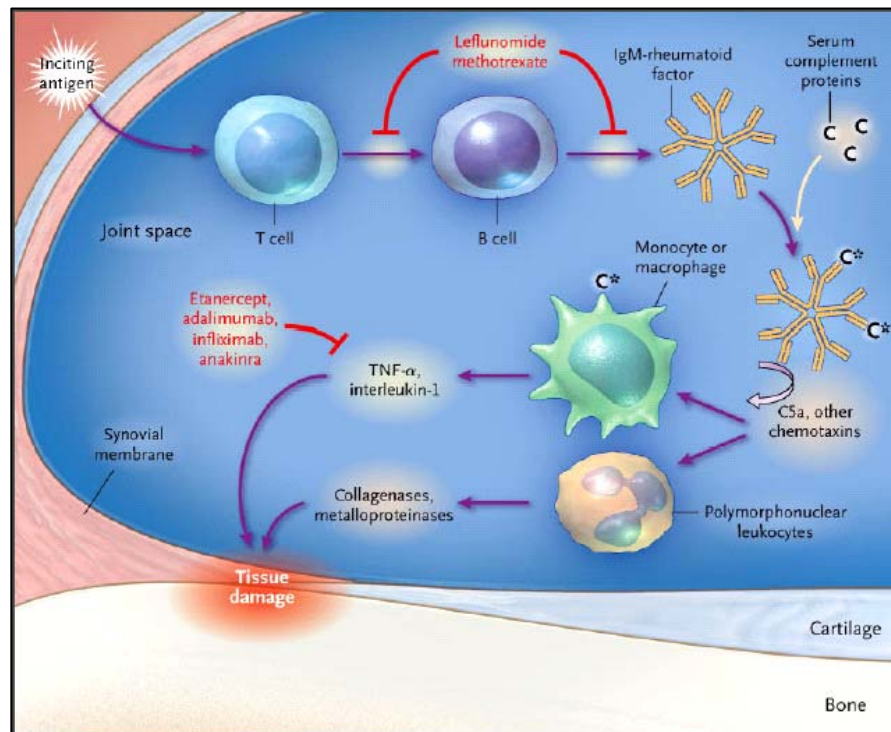
Olsen, N and Stein, M. "New Drugs for Rheumatoid Arthritis." *New England Journal of Medicine*. 350. 2004. p. 2167-2179

Fig. 2. Diagram of the RA joint.

Schematic illustrating the potential role of lymphocytes, rheumatoid factor, complement, and pro-inflammatory cytokines in RA joint damage.

Closer examination of the long-term natural history of RA reveals that the disease does not simply result in irreversible joint damage, but also results in high levels of work disability and increased mortality in patients. Studies show consistent radiographic evidence of progressive erosive disease with time[66-68]. Longitudinal radiographic reports reveal that much of the joint damage occurs within the first 5-10 years of disease onset, highlighting the necessity to correctly diagnose RA patients early in their disease course[69-71]. Progression of RA is also associated with declines in functional status, as assessed by grip strength, button tests, and questionnaire scoring[72, 73]. Work disability is also a major problem in the RA patient population, with disability levels as high as 50-75% in patients 10 years after disease onset[74, 75]. Most striking, reports from diverse locations around the world have documented increased levels of mortality in the RA population when compared to age matched healthy individuals[76-83]. Elevated levels of mortality result from infections, renal disease, cardiovascular disease, and lymphoma[84, 85]. Interestingly, increased mortality in RA patients was predicted by severe RA documented 5-15 years earlier[77, 86]. Patients with poor functional capacity had 5 year survival outcomes. This is comparable to patients with three-vessel coronary artery disease or type IV Hodgkin's disease[87].

The recognition that much of the irreversible joint damage occurs early in the course of the disease and that severe RA is associated with increased mortality[72] has altered strategies for disease treatment[88-90]. Currently rheumatologists use non-steroidal anti-inflammatory (NSAIDS), glucocorticoids, and disease modifying anti-rheumatic drugs (DMARDS) in order to treat RA. While NSAIDs (such as aspirin and COX-2 inhibitors) are effective at decreasing pain and inflammation, they have not proven useful

15

for preventing the progression of erosive arthritis[91]. Low dose glucocorticoids (3 to <10 mg) are also typically given, with lower doses of steroid better tolerated by patients. The DMARDS, or second line agents, such as methotrexate, sulfasalazine, and intramuscular gold injections, have proven to be effective at slowing the progression of RA; in general, the mechanism by which DMARDs alter the course of disease remains largely unknown[91].

Rheumatologists now initiate aggressive DMARD treatments early in the disease course, in an attempt to prevent joint damage, relieve pain, and improve long-term outcomes. Current evidence shows that this fundamental change in strategy promises to be more effective in slowing RA disease progression. Radiographic evidence reveals that continuous, early treatment with DMARDs was capable of slowing the progression of joint erosion[92-94]. Furthermore, studies show that long-term treatment with DMARDS results in improved functional capacity[95] and improves survival rates in RA patients[96-98].

The newest lines of treatment available for RA, the biological agents, are opening new possibilities for treating patients with RA[90]. Scientists are taking advantage of the growing body of knowledge in immunology in an attempt to curb the ongoing inflammatory processes in established RA. Several strategies have been attempted, such as the depletion of T lymphocytes using antibodies directed against cell surface markers, oral tolerization protocols with type II collagen, and cytokine directed therapies[99]. Many of these recent biological therapies are aimed at the immunosupression of branches of the immune response that might play a role in the pathogenesis of RA. Unfortunately, with the exception of the TNFα inhibitors[100, 101], these immunological approaches have been largely unsuccessful due to lack of efficacy or unacceptable adverse side effects.

The difficulty in developing successful treatment arises, in part, from the fact that the underlying mechanism(s) leading to chronic inflammation and damage in RA are still largely unknown. We have attempted to use insights gained from our microarray studies to examine biochemical liabilities in the peripheral blood mononuclear cells (PBMC) of patients with RA. Understanding these defects might help to identify new potential targets for drug design. Keeping this in mind, it is important to understand what is currently known about lymphocyte function in RA.

It is generally believed that RA results from an inappropriate T lymphocyte mediated immune response against antigens present in the joints of patients. Organized lymphoid follicles with germinal centers have been identified in the synovium of patients with RA[102-104]. Since germinal centers represent sites of antigen driven T cell/B cell interaction, their presence supports the notion that antigens are being inappropriately recognized by the immune system. The involvement of T lymphocytes and antigen presentation is further supported by the association of MHC class II HLA-DR4 polymorphisms with the disease[105, 106]. However, examination of synovial T lymphocyte TCR (T cell receptor) sequences for evidence of antigen driven clonal expansion has yielded mixed, inconsistent results among RA patients[104, 107, 108]. While there is evidence of expanded T lymphocyte clones within the synovium or synovial fluid of an individual patient[109, 110], no common expanded TCR sequences have been identified among the RA patient population, suggesting that different lymphocyte populations may be clonally expanded among RA patients[107].

Alternatively, multiple studies provide consistent support for systemic abnormalities in RA lymphocytes. Early observations in the clinical setting revealed that

a portion of RA patients were unresponsive to PPD skin tests compared to control individuals[111]. Further, *ex vivo* studies revealed that RA lymphocytes proliferate poorly in response to tuberculin and a panel recall antigens when compared to control lymphocytes[112, 113]. One possible explanation for these observations is that the chronic pro-inflammatory immunological environment that characterizes RA somehow inhibits T lymphocyte function. In support of this hypothesis, studies have shown that chronic exposure to the proinflammatory cytokine TNF-α can suppress T lymphocyte proliferation and differentiation in response to TCR stimulation[114]. Follow-up studies in mouse hybridoma T cell lines revealed that TNF-α attenuation of TCR signaling is mediated by down regulation of the TCRζ subunit, which ultimately results in impaired TCR/CD3 assembly and cell surface expression[115].

Although chronic exposure to TNF offers one possible explanation for lymphocyte unresponsiveness in RA, recent findings suggest that additional abnormalities in peripheral lymphocyte homeostasis may also account for defective T lymphocyte responses. Examination of the CD4$^+$ TCR repertoire in peripheral blood has revealed a marked decrease in receptor diversity that appears to be independent of the lymphocyte activation/memory status (as assessed by expression of CD45RO)[116]. Furthermore, measurements of recent thymic T lymphocyte emigrants, as assessed by TCR Recombination Excision Circle (TREC) levels, revealed that RA patients contain fewer TREC positive CD4$^+$ T lymphocytes compared to age matched control lymphocytes[117].

Decreases in TREC can be accounted for by decreased thymic output or lymphocyte proliferation in the periphery. The finding that RA CD4$^+$ T lymphocytes

contain telomeres shortened in an age-inappropriate manner, suggests an increased replicative history[116, 118]. Propidium-iodide profiles of DNA content from freshly isolated peripheral CD4$^+$ lymphocytes reveal that a greater number of RA lymphocytes are in the S-G2/M phase of the cell cycle compared to control individuals, supporting the notion of abnormal lymphocyte cell division *in vivo*[116]. Studies examining the functional impact of telomeric attrition revealed that CD4$^+$ lymphocytes from RA patients proliferated less readily *ex vivo* when stimulated with plate bound anti-CD3 for extended periods of time (up to 6-7 weeks)[117], a finding highly reminiscent of the T lymphocyte unresponsiveness initially described in RA[112]. The authors hypothesize that due to perturbations in lymphocyte homeostasis, RA CD4 lymphocytes are approaching immunosenescence, and therefore have exhausted their replicative potential[119].

Although the most thorough examination of lymphocyte homeostasis has been performed in RA, other autoimmune disorders also display evidence of altered homeostasis in the periphery. For example, lymphocytes from patients with SLE [120] and type I diabetes[121] suffer from age inappropriate telomeric loss in a manner highly reminiscent of RA. Although initial observation of patients with multiple sclerosis have not confirmed telomeric shortening, decreased TREC levels were found in MS lymphocytes compared to age matched control individuals[122]. Growing evidence supports the notion that defects in lymphocyte homeostasis, as assessed by proliferation studies, TREC levels, and telomeric erosion, are associated with autoimmunity and may contribute to the development of these diseases.

19

Microarray Technology and Analysis

Recent advances in microarray technology have made it possible to simultaneously measure the levels of thousands of transcripts, providing a vastly more detailed phenotypic picture than possible with gross clinical measurements. The association of differentially expressed genes with a particular autoimmune disease, or shared among disease groups, might provide new insights into the molecular liabilities contributing to the autoimmune condition in patients. Since a considerable amount of the data in this dissertation is derived from microarray analysis, I will provide background into the technology and data analysis.

A general microarray experiment is comprised of a few fundamental steps: 1) the extraction of mRNA from a given source, 2) labeling of the target nucleotide, 3) parallel hybridization of labeled target to immobilized DNA organized in a grid on a solid surface, and 4) imaging and measurement of relative hybridization intensities[123]. Technical variations at each step can impact the reliability and interpretation of a given experiment. One of the major challenges, at least during the early development of microarray technology, was obtaining sufficient RNA for hybridization. Depending on the method of labeling and the type of array used, upwards of 50 μg of total RNA is necessary to obtain reliable hybridization. This amount of RNA has represented a significant barrier to the performance of microarray analysis on biopsy samples obtained from living humans. However the development of techniques allowing the linear synthesis of cDNA has made it possible to amplify small amounts of starting material[124-126]. Several microarray studies have now been successfully performed using RNA isolated from laser microdissected tissue[127] or even from a single cell[128]. As a caveat, it

should be noted that addition of extra amplification steps introduces more technical complexity, ultimately introducing the greater possibility of experimental error.

Technical variations in the labeling of the cDNA target can also impact results. The most common technique for labeling target RNA relies on the incorporation of fluorescent nucleotides into cDNA during reverse transcription. Typically two separate dyes, Cy3 and Cy5, are used to label RNA from an experimental and control source, respectively. Both labeled cDNAs are hybridized simultaneously to the microarray platform, allowing the direct calculation of the ratio of gene expression between the experimental and control conditions. Although common, there are some drawbacks to this technique. For reliable signal detection, relatively large quantities of RNA are needed, thus necessitating large amounts of source material or additional amplification steps. Additionally, Cy3 and Cy5 dyes may not be incorporated equally into cDNA, resulting in possible errors in analysis[123]. As an alternate labeling method, a single probe may be used to label samples. For example, biotinylated or radiolabeled nucleotide may be incorporated into the cDNA target during reverse transcription, and this labeled sample can be directly hybridized to the microarray platform. This technique is more sensitive and requires less starting total RNA (typically around 5-10 μg), making it more amenable to human studies. However, gene expression ratios cannot be calculated from a single hybridization, thus requiring a greater number of microarrays and incurring greater cost. Additionally, consistent internal controls and normalization against background are required to make comparisons among arrays and calculate gene expression ratios.

Two major types of microarray platforms are typically used: cDNA and oligonucleotide microarrays. Probes for cDNA microarrays are typically derived from

PCR products generated from a cDNA library, using either gene- or vector-specific

primers to amplify the particular product[123]. Amplified products are usually spotted onto

a supporting surface, such as glass or nylon membrane, in an organized matrix. The

coding strand is chemically crosslinked to the supporting surface. cDNA microarrays are

more commonly utilized in academic settings, allowing more customizability and

detection of previously unsequenced clones from cDNA libraries[123]. Alternatively,

companies such as Affymetrix produce oligonucleotide arrays. Oligonucleotides from

these arrays are typically generated *in situ* using photolithography on a supporting

surface. The chosen probes typically represent 20-25 mers selected from the sequence of

known target transcripts [123]. Selection of oligomers allows hybridization to specific

regions of a target transcript, however their shorter length may result in false

hybridization. Additionally, since the generation of the probes requires knowledge of the

target sequence, genes with unknown sequence will be missed using oligonucleotide

arrays.

We utilized Research Genetics GF-211 microarrays for our experiments, which

were ideal for addressing constraints of studying human patient samples. The GF-211

filter is a cDNA microarray spotted onto a nylon membrane. The source clones chosen

for amplification and spotting represented approximately 4,300 human genes, around

3,900 were known genes. Total RNA isolated from individual blood samples was labeled

by reverse transcription in the presence of radiolabeled ^{33}P-dCTP. Radiolabeled cDNA

was hybridized to the filters, the image was captured using a phosphorimager, and

digitally processed using Research Genetics Pathways 4.0 software suite. The software

normalized the hybridization intensity for each individual gene relative to both the

background and total genomic cDNA control clones present at regular intervals on all arrays. This normalization process allowed us to make inter-microarray comparisons to determine gene expression ratios between groups of patients. Furthermore, since human PBMCs served as our RNA source, we were restricted in the amount of material we could use for hybridization. The use of radiolabeled probe allowed us to use relatively small amounts of total RNA for hybridization (5 μg), so patient samples were not exhausted by the hybridization process. Furthermore, the Pathways 4.0 software included bundled analytic tools specifically designed to analyzing data from the GF-211 filters.

In fact, the most challenging aspect of the microarray experiment is actually analyzing the resulting data in a meaningful way. Researchers using microarray analysis to compare different experimental conditions find that hundreds of differentially expressed genes can be identified, many with functions that are not known in a given experimental context or whose function is entirely unknown. Furthermore, it is difficult to understand which genes are primarily differentially expressed as a result of differences in experimental samples, or represent secondary downstream effects of, for example, the presence or absence of a transcription factor or other factor that determines transcript levels of a gene or group of genes[126]. Because of the nature of the results in microarray experiments, it has been difficult to utilize microarrays to identify primary targets responsible for biological phenomena, an endeavor that has been likened to finding a needle in haystack[126]. Microarrays have proven most successful when utilized as pattern recognition tools. In this sense, gene expression patterns identified by microarray analysis have already shown potential as diagnostic tools. Gene expression profiles have been able to discriminate between subtypes of lymphomas[129, 130] and breast cancers[131].

Clustering algorithms serve as the key tool to identify gene expression patterns that are either shared or discriminate among groups of gene profiles. In the general context of microarrays, these algorithms cluster differentially expressed genes or microarray profiles into groups that share similar expression patterns over a range of conditions. Clustering methods fall into either supervised or unsupervised classes. The supervised class of clustering algorithms involves the analysis of data in which the identity of genes or microarrays is specified prior to computational analysis. These algorithms typically focus on identifying patterns that are capable of discriminating between the specified groups. We have used true supervised clustering only to a limited extent with our datasets. None of these data will be presented in this dissertation, thus I will not cover this class of clustering algorithm. Alternatively, in unsupervised clustering, the algorithm is blind with regards to the identity of the individual gene or expression profile, and instead relies solely on the data to define the clusters[132]. The three most commonly used unsupervised clustering algorithms are hierarchical, k-means, and self-organizing maps. We will discuss aspects of each of these algorithms.

Hierarchical clustering, or pairwise average-linkage cluster analysis, was initially used in the 1950's for phylogenetic analysis. In the context of microarray data, "hierarchical clustering iteratively join(s) the closest elements in the data into a tree structure"[133] "whose branch lengths reflect the degree of similarity between the objects"[132]. Thus differentially expressed genes or microarray profiles which display the highest degree of similarity cluster into the same or nearby branches on the tree. Currently, because of the ease of interpreting the visual data output, and the relative

speed of the algorithm, hierarchical clustering is the most popular method for analyzing microarray data.

Alternatively, k-means clustering identifies k number of centroids (specified by the algorithm's user) that serve as cluster centers. Microarray data points are assigned to each of these cluster centers, such that the total distance between the data points and the cluster centers are minimized. The algorithm then readjusts the centers, compares the dataset against the new center locations, and repeats the process in an effort to obtain the optimal position for the cluster centers. The algorithm is complete once the optimal cluster location has been identified[133, 134]. From a practical standpoint, the user of the k-means algorithm simply indicates the number of centroids, and the software will group the differentially expressed genes or microarray profiles into an equivalent number of clusters.

The self-organizing map (SOM) unsupervised algorithm operates in a manner relatively similar to k-means clustering. Again, the user specifies k numbers of cluster centers. Each of the cluster centers represent a point on a pre-specified two-dimensional geometric grid[134, 135]. Data points are plotted onto this grid in an iterative fashion, until each of the cluster centers lies at the natural center of the data [133]. Practically, the user of a SOM algorithm must specify the grid geometry and k number of clusters desired.

We have utilized EisenCluster, EisenTreeview, and Research Genetic's Pathways 4.0 software packages for our cluster analyses. EisenCluster and EisenTreeview (http://rana.stanford.edu/clustering) were used when examining the degree of similarity between overall expression profiles. For a typical analysis, all three clustering algorithms were utilized (through EisenCluster) to ensure that the clustering did not represent an

artifact of any particular method. Given the current standards of microarray analysis, we typically present hierarchical clustering results. The results for these types of analyses were visualized using EisenTreeview. Throughout our studies we also performed pseudo-supervised clustering to analyze the differentially expressed genes between conditions. For this type of analysis, the average gene expression for a specified group of microarrays was plotted relative to other groups. In this manner, we plotted the average gene expression level for 4,300 genes in control/ immune response individuals, and RA, early RA, SLE, MS, and IDDM patients. Genes that were not significantly different between conditions were removed from the analysis. The remaining differentially expressed genes were subjected to k means clustering with ten centroids. In this manner, we have been able to use standard unsupervised clustering algorithms to identify genes that were capable of discriminating between previously specified patient groups.

p53 and the DNA Damage Response Pathway

Examination of gene expression profiles in patients with a range of autoimmune disorders revealed a gene expression signature that was shared by all the autoimmune patient groups (Results Section). This signature was comprised of 96 over-expressed and 113 under-expressed genes. Most notably, many of the genes that were under-expressed encoded proteins that played key roles in apoptosis and cell cycle progression. Closer examination of the genes present in the autoimmune signature revealed that they were differentially expressed in a manner consistent with dysfunction in the p53-dependent damage response pathways. We have performed studies to examine the function of these pathways in the lymphocytes of RA patients. Since these studies are an important portion

of the dissertation, I will provide background regarding p53 and the damage response pathways. Currently, our studies have only addressed the cellular response to γ-radiation, therefore I will only the cover the pathways necessary to understand this response.

The DNA damage response consists of several overlapping signaling pathways comprised of DNA damage sensors, signal transducers, and effectors, which detect and respond to specific types of DNA damage[136]. The proteins that "sense" and bind to DNA lesions are the most poorly understood portion of the DNA damage response, but are believed to play key roles in distinguishing the type of DNA lesion and in recruiting substrates for subsequent signal transduction events. It is believed that ionizing radiation-induced double strand DNA breaks (DSBs) are bound by the MRN complex (Mre-11, Rad50, and Nbs-1)[137], which co-localizes to DNA breaks and exhibits DNA unwinding and endonuclease activities[138].

Cellular recognition of DSB DNA lesions initiates a signal transduction cascade which ultimately converges on effectors which, depending on the extent of the damage, mediate DNA repair, cell cycle arrest, and/or apoptosis (Fig. 3). The most upstream signaling molecule in the DSB damage response pathway is ATM (ataxia telangiectasia mutant); a high molecular weight member of the phosphoinositide 3-kinase related (PIKK) family of proteins. ATM is preferentially activated by DSB and its activation and substrate recognition requires interaction with the sensor complexes. For example, in response to γ-irradiation, ATM is recruited to the DSB foci with the MRN complex. Cellular studies of samples from patients with the related Nijmegen Break Syndrome, Ataxia Telangiectasia, and AT-like disorders have shown that ATM activation requires a functional MRN complex[139, 140].

Activated ATM phosphorylates a number of substrates, however we will focus on the targets most relevant to our studies. One of the major phosphorylation targets of ATM are the checkpoint kinases, a group of functionally related but structurally distinct Ser/Thr kinases, which rapidly amplify the damage signal and target downstream effectors[141]. One of the major targets of γ-irradiation activated ATM is checkpoint kinase 2 (Chk2), which is phosphorylated by ATM at Thr68[142-144]. This phosphorylation activates Chk2, which then phosphorylates downstream effector targets.

Like ATM, the activated checkpoint kinases share a number of effector targets that play key roles in mediating cell cycle arrest and/or apoptosis in the cellular response to DNA damage. Again, we will only focus on targets of particular interest to our studies. The p53 protein is one of the key targets of activated Chk2. Under normal conditions, levels of cellular p53 protein are relatively low due to MDM2 dependent turnover of p53 through the ubiquitin-proteasome pathway[145, 146]. *In vivo* and *in vitro* experiments indicate that activated checkpoint kinases phosphorylate a series of serines in the N terminal domain of p53[147], a region associated with p53 stability and MDM2 binding[148, 149]. Importantly, Chk2-dependent phosphorylation at Ser20[150] results in DNA-damage dependent stabilization of p53 protein by disrupting MDM2 interactions in certain cell lines. However recent studies have called into question the absolute necessity of Chk2 for p53 protein accumulation in response to DNA damage under all conditions[151, 152].

Increased steady state levels of p53, in addition to numerous other post-translational modifications[149], allows p53 to bind DNA targets in a sequence specific manner[153], and act as a transcriptional regulator of a number of target genes that mediate

cell cycle arrest and/or apoptosis[149]. One of the earliest described genes targeted by p53 transcriptional activity was the cell cycle arrest factor, p21[154]. p21 binds to Cyclin E/Cdk2, inhibiting the complexes activity and arresting the cell cycle at the G1→S transition[155-158]. Due to the nature of p53 regulation and p21 expression, this G1→S transitional arrest typically takes over six hours to be established[159]. Additional cell cycle arrest factors, such as GADD45α[160, 161] are also transcriptional targets of p53.

In addition to inducing the expression of cell cycle arrest factors, p53 also acts to induce the expression of a number of pro-apoptotic targets. Most notably, p53 induces the expression of a number of pro-apoptotic bcl-2 family members, such as Bax [162], Noxa[163, 164], and PUMA[165, 166], thus initiating apoptosis through the receptor-independent pathways. Under certain conditions p53 can also initiate apoptosis through receptor dependent means, by inducing the expression of DR5[167] and/or FAS/FASL [168-170]. Thus, DNA damage initiated post-translation modifications in the p53 protein play a central role in initiating a transcriptional program which culminates in cell cycle arrest, and if the DNA damage is extensive enough, apoptosis.
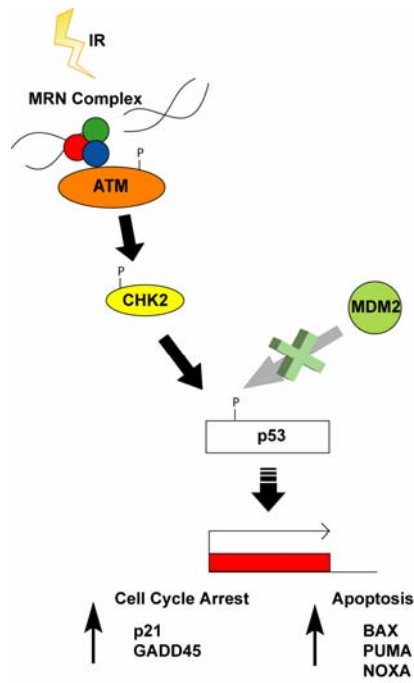
Fig. 3. Diagram of the p53-dependent DSB DNA Damage Response Pathway

Schematic of the p53-dependent DSB DNA damage response pathway,
illustrating the location of DNA-damage sensors, transducers, and effectors in
the cascade.

CHAPTER II

IDENTIFICATION OF THE AUTOIMMUNE SIGANTURE

Abstract

Autoimmune diseases are relatively common, affecting approximately 5% of the human population. These disorders are believed to arise from immune mediated attack against self, however the underlying cause of these diseases is currently unknown. In part, the difficulty in identifying a pathogenic mechanism arises from the considerable heterogeneity both within and among the autoimmune disease groups. We wanted to determine if microarray profiling would allow us to detect common expression signatures either within a particular autoimmune disease group, or among autoimmune disease groups. To accomplish this, we compared differences in gene expression (>4000 genes) in the peripheral blood mononuclear cells of normal individuals to those expressed by individuals with four different autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, insulin dependent diabetes mellitus, and multiple sclerosis). Each individual from all disease groups displayed a similar pattern of gene expression that was highly distinct from the gene expression pattern of the control group. Of note, expression levels of genes that encode key proteins in several distinct apoptosis pathways were markedly reduced in all autoimmune disease groups. Taken together, these data indicate that the pattern of gene expression describes a molecular portrait of autoimmunity that is

constant among individuals with autoimmune disease but is independent of the type autoimmune disease.

Introduction

Autoimmune diseases occur in up to 3-5% of the population[10]. Most represent organ specific immune responses, but some have systemic effects[11]. The site of attack can be highly selective: pancreatic β cells in the islets of langerhans cells for IDDM[171], myelin basic protein in MS[172], or more systemic: synovial lining, lung, heart in RA[173], skin, kidney or heart in SLE[174].

In addition to the distinct targets among these disorders, there is also considerable heterogeneity in the clinical symptoms associated with many of these diseases. For example, patients with RA typically present with symmetric erosive polyarthritis affecting diarthroidal joints. However, RA is also associated with cutaneous, pulmonary, and cardiovascular symptoms[14]. Patients diagnosed with SLE typically suffer from a wide range of symptoms, including rashes, glomerulonephritis, atherosclerosis, and arthralgias[14, 174]. The heterogeneity that characterizes autoimmunity contributes to the difficulty in determining the underlying causes of these disorders. Despite intense efforts in the field, consistent functional, cellular, or molecular defects have not been identified in patients with any of these disorders.

We utilized gene expression microarrays to search for differences in gene expression in PBMC that might be characteristic of all individuals with an autoimmune disorder or might characterize individual autoimmune disorders. We found that gene expression profiles from individuals with autoimmune disease were completely distinct

from those of control individuals. Surprisingly, PBMCs from autoimmune patients exhibited a common gene expression signature that was independent of the type of autoimmune disease and other clinical parameters. Many of the under-expressed genes represented in this autoimmune gene expression signature encode proteins that play key roles in apoptosis, cell cycle regulation, and the induction of peripheral tolerance. The dysregulation of these fundamental cell function pathways may impact the normal immune response by making lymphocytes more susceptible to a loss of tolerance and immune response to self-antigen.

Materials and Methods

Patient Populations

Seven control subjects with no family history of autoimmunity, or evidence of acute or chronic infection were used for our study. Additionally, patients with RA (nine), SLE (nine), IDDM (seven), and MS (four) were enrolled in the study. A clinical diagnosis of each autoimmune disorder was the sole criterion for inclusion. Human subject studies were approved by the Committee for the Protection of Human Subjects of the Vanderbilt University Institutional Review Board.

Sample Preparation and RNA Isolation

All samples were processed within two hours of initial collection. PBMC were isolated from heparinized blood by centrifugation on a Ficoll-Hypaque gradient. After isolation of PBMCs, flow cytometry was used to estimate the relative leukocyte

distribution in representative samples from control, normal immune response (discussed in Chapter 2), and autoimmune disease groups. All groups were composed of about 75% T lymphocytes, 10% B lymphocytes, 5% monocytes, and less than 1% contaminating neutrophils. Tri-Reagent (Molecular Research Ctr. Inc., Cincinnati Ohio) was used to isolate total RNA according to the manufacturer's protocol.

Probe Synthesis and Microarray Hybridization

Total RNA was reversed transcribed with Superscript II reverse transcriptase (Gibco BRL Life Technologies, Rockville, Maryland) in the presence of ^{33}P-dCTP, to yield radio-labeled cDNA probes. The cDNA probes were hybridized to the Research Genetics GF-211 gene filters (Research Genetics, Huntsville Alabama) according to the manufacturer's protocol. After overnight incubation, unhybridized probe was successively washed from the filters using 2X SSC buffer/1 % SDS (2 X 20 minutes) and 0.5X SSC buffer/1 % SDS (1 X 10 minutes). The filters were exposed on a phosphorimaging screen for 24 hours, scanned, and digitally imported for computer analysis.

Clustering Data Analysis of Differentially Expressed Genes

The resulting images were digitally imported and processed using Research Genetic's Pathways 3.0 software package. Data were normalized to yield an average intensity of 1.0 for each clone (4329) represented on the cDNA microarray. Global expression profile comparisons were made using Eisen's Cluster and Treeview software [132]. Microarray gene expression intensities were entered into a tab delimited database and

analyzed using the Cluster software. Genes whose expression levels did not vary significantly could be filtered out prior to clustering. The various datasets were clustered using Hierarchical, K Means, and Self-Organizing Map algorithms[134]

Research Genetics Pathways 3.0 program was used to identify the most differentially expressed genes after immunization and in the different autoimmune disease classes. Microarrays were grouped into one of several conditions: control (healthy individuals before immunization), post-immunization (healthy individuals at different time points after immunization: 3, 6-9, and 19-21 days), and one of the four autoimmune groups (systemic lupus erythematosus, rheumatoid arthritis, insulin dependent diabetes mellitus, and multiple sclerosis). The gene intensities for each condition group were averaged. The expression levels were represented graphically by plotting the natural logarithm of the ratio of gene expression intensity in the experimental group versus the control group for each condition. Genes that did not change significantly (99% confidence, Chen test[175]) over any of the conditions were removed from the database. The remaining genes in the data set were then clustered using an unsupervised K Means clustering algorithm[132][134] with ten centroids. The gene identities, chromosome number, and functions were further analyzed for each cluster using Stanford's GeneCards database (http://genome-www.stanford.edu/genecards).

Clustering Data Analysis of Differentially Expressed Genes

Microarray gene expression raw data for control individuals and autoimmune patients is available at PubMed's GEO site at under accession number GSE3447 and GSE 3459 at http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi.

Results

Gene expression variables were monitored in PBMCs to permit us to focus on changes that occur in both the innate (monocytes and NK cells) and acquired (B and T lymphocytes) branches of the immune system. Reproducibility of our method was first established by performing four hybridizations to separate microarrays using the same RNA sample. We also exposed hybridized filters to phosphorimager screens for two different lengths of time (6 and 24 hrs). Data were normalized and linear regression analysis was employed to estimate reproducibility. The separate hybridizations yielded R^2 values ranging from 0.87 to 0.96. Different exposure lengths of identical filters also produced high R^2 values (0.99). The results indicated that the array data were highly reproducible.

To determine if microarray profiling would allow us to detect common gene expression signatures either within a particular autoimmune disease group, or among the autoimmune diseases, we obtained samples from patients diagnosed with one of four common autoimmune disorders: RA, MS, IDDM and SLE. The relatedness of gene expression profiles in the PBMC from individuals with autoimmune disease was examined relative to profiles from control individuals (CONT) (Fig. 4).

Hierarchical clustering of expression profiles for all the autoimmune groups (MS, RA, SLE, and IDDM) with the controls resulted in separation into three major branches (Fig. 4). One branch consisted of control individuals and two IDDM individuals. All other autoimmune samples were segregated into the other two branches. As evidenced by the dendrogram, the clustering algorithm did not permit discrimination among the different autoimmune diseases based upon gene expression variables. This inability to segregate autoimmune gene expression profiles into disease-specific groups was retained even when invariant genes were statistically removed (genes were removed with standard deviations under 1.5-5 from the mean) from the data set (data not shown). One explanation for the clustering algorithm's inability to distinguish between the
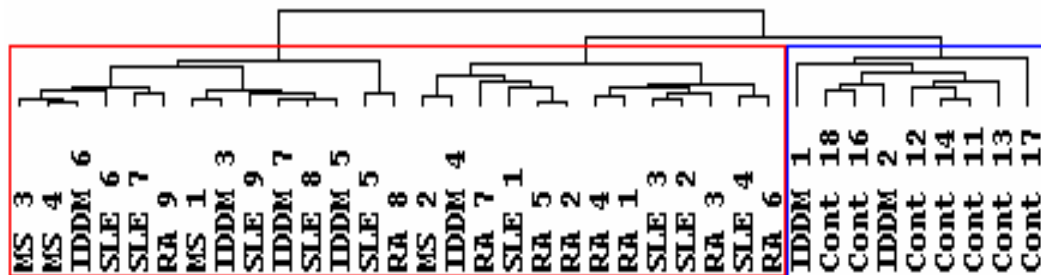
Fig. 4. Comparison of the control and autoimmune classes by cluster analysis. Control, RA, MS, SLE and IDDM groups were analyzed using a hierarchical clustering algorithm based upon expression variables of 4329 genes. Three major branches were apparent from the dendrogram. All control samples segregated within one major branch, with the remaining branches containing mixed autoimmune profiles.

phenotypically distinct autoimmune diseases is that the gene expression patterns associated with these different diseases are relatively similar.

We further analyzed the data set to identify genes that were most differentially expressed in autoimmune disease patients compared to control individuals (K-means clustering, 99% significance, Chen test). Individual samples from the autoimmune groups were segregated based upon disease type (IDDM, MS, RA, and SLE) and were compared to the control gene profiles. The gene expression differences among the groups were plotted as the natural logarithm of the ratio between experimental condition and the control group (Fig. 5 A, B).

Two distinct clusters of differentially expressed genes were identified. The first major cluster (Fig. 5 A) consisted of 127 genes that were over-expressed in all autoimmune disease groups (IDDM, MS, RA, and SLE). The genes in this over-expressed autoimmune cluster represented several distinct functional categories: receptors (*CSF3R*, *HLA-DMB*, *HLALS*, *TGFBR2* and *BMPR2*), inflammatory mediators (*MSTP9*, *BDNF*, *CES1*, *ELA3*, and *CYR61*), signaling/2nd messenger molecules (*FASTK*, *DGKA*, and *DGKD*), and autoantigens (*GARS* and *GAD2*). The second major cluster (Fig. 5 B) contained 181 genes that were strongly under-expressed in all autoimmune groups. Many of these down-regulated genes are known to play key roles in apoptosis (*TRADD*, *TRAP1*, *TRIP*, *TRAF2*, *CASP6*, *CASP8*, *TP53*, and *SIVA*) and ubiquitin-proteasome function (*UBE2M*, *UBE2G2*, and *POH1*). Inhibitors of various cellular functions were also widely represented in this cluster. These included direct inhibitors of cell cycle progression (*CDKN1B*, *CDKN2A*, and *BRCA1*), as well as inducers of cell differentiation (*LIF* and *CD24*). Certain enzyme inhibitors (*APOC3* and *KAL1*) were also represented in this class.

A.

B.

Receptors	Inflammatory
CSF3R, CSF 3 receptor	IFITM3, IFN-induced
HLA-DMB, MHC, DM β	MSTP9, macrophage
HLALS, MHC, class I-like	stimulating
TGFBR2, TGFβ receptor II	BDNF, brain-derived
BMPR2, BMP receptor II	neurotrophic factor
RYR1, ryanodine receptor 1	ELA3, elastase
RARA, retinoic acid	CES1, monocyte/mϕ
receptor	serine esterase
ESRRA, estrogen-like	SPINK2, protease inhbitor
receptor	CRYBA4, crystallin, β A4
ADORA2A, adenosine	CYR61, cyteine-rich
receptor	angiogenic inducer

2nd Messengers	Autoantigens
FASTK, Fas-activate kinase	GAD2
PMAIP4, PMA-induced	GAD65
MPP3, NF$^{\kappa}$B activation	GARS, glycyl-tRNA
RELA, NF$^{\kappa}$B p65	sythetase
LAP18, stathmin	
RAD5IL3, RAD51-like 3	
ABR, gtpase-activating	
Protein for rac and	
Cdc 42	
COPS3	
DGKA, DAG kinase, α	

Inhibitors	Apoptosis
SUDD, extragenic supressor	CASP6, caspase 6
PDE6A, phophodiesterase	CASP8, caspase 8
CDKN1B, cyclin-dependent	APAF1, apoptosis
kinase inhibitor (p27)	activating factor
CKN2A, cyclin-dependent	SIVA, CD-27 binding
kinase inhibitor (p16)	(SIVA) protein
HGS, human growth factor-	TP53, p53
regulated tyrosine	PIG11, p53-induced protein
kinase	NME3, protein expressed in
LIF, leukemia inhibitory	non-metastatic cell 3
Factor	TRAP1, TNFR1 associated
SEMA3B, semaphorin 3B	Protein
BRCA1, breast cancer 1	TRADD, TNFRSF1A-
APOC3, apolipoprotein	associated via death
CIII, lipase inhibitor	domain
CD24, prevents B cell	TRIP, TRAF interacting
Differentiation	protein
KAL1, Kallmann syndrome	TRAF2, TNFR-associated
1 sequence,	factor 2
anti-protease	MAP4K2, mitogen-
	activated protein kinase
	kinase kinase kinase 2

Fig. 5 Cluster analysis of the differentially expressed genes associated with autoimmune disease. Kmeans clustering analysis identified two distinct clusters of genes that were **A**. uniformly over-expressed in all four autoimmune groups and **B**. uniformly under-expressed in all four autoimmune groups. Data are presented as the natural logarithm of the ratio of the immune group (MidImm)/control, or each autoimmune group (IDDM, MS, RA, or SLE)/control. Each individual line in the plot represents the expression ratio for an individual gene for each class (MidImm, MS, RA, SLE, etc.) Expression ratios that did not achieve a 99% significance level excluded from analysis. Below each graph, a partial list of genes is identified by dominant functional category within each of the clusters.

Differences in gene expression between the control and the autoimmune

populations may be attributed to alterations in distribution or activation status of the cells

that make up the PBMC. We performed two types of analyses to test this possibility.

First, we analyzed PBMC preparations for frequency of CD3 (T cells), CD14

(monocytes), CD19 (B cells), and leukocyte alkaline phosphatase (LAP, neutrophils)

positive cells by flow cytometry. All PBMC preparations from both subject groups

contained 75-80 % T cells, ~ 10 % monocytes, ~ 5 % B cells, and < 1 % neutrophils.

Second, we determined if expression levels of genes that are either restricted to a given

sub-population or reflect activation status were differentially expressed in the control

population compared to the autoimmune population (Table II). Expression levels of

these genes varied by less than two-fold between the control and autoimmune groups and

this difference did not achieve statistical significance. Taken together, these data argue

that alterations in composition or activation status of PBMC did not account for the

observed differences in gene expression between the control population and autoimmune

population.

These results point to pervasive changes in gene expression that are relatively

uniform among four phenotypically distinct autoimmune diseases relative to the control

individuals. To examine this in greater detail, we compared expression levels of

individual genes between controls and patients with each of four autoimmune diseases.

We selected ten genes that exhibited the greatest level of under-expression (Fig. 6 A) or

over-expression (Fig. 6 B) at the population level. Expression levels of under-expressed

genes were lower in all autoimmune individuals than in all control individuals (Fig. 6 A).

In contrast, over-expressed genes in the autoimmune population showed greater

individual variation in expression (Fig 6 B). In this group, no individual gene was over-expressed in all autoimmune individuals compared to all control individuals. However, each of these genes was significantly over-expressed in the autoimmune population (P < 0.05).

Since genes under-expressed in autoimmune disease exhibited a more homogeneous pattern of expression among individuals than the over-expressed genes (Fig. 6), we wanted to determine if we could employ this pattern to classify individuals with autoimmune disease and predict if new samples were derived from autoimmune or control individuals. First, we selected 35 core signature genes that were most under-expressed with the greatest statistical significance in the SLE population compared to the control population. Second, in order to give each gene equal weight in the determination we summed the average of the control population and the SLE population for each gene and divided by 2 ([control + SLE]/2). Third, we inspected expression levels of each of the 35 genes in each sample and assigned it a value of 0 if it was less than the average and 1 if it was greater than the average. In this analysis, the maximum possible score was 35 and the minimum possible score was 0 (Fig 7). The range of scores for control individuals was 18-35. Most control individuals achieved a score of 35 (8 of 11). In contrast, the range of scores in individuals with autoimmune disease was 0-5 (SLE), 0-6 (RA), 0-1 (type I diabetes), and 0 (MS) (P < 0.000001). Next, we tested a new set of SLE and RA patients that were not included in the initial data analysis to determine if these individuals would obtain a similar score. The range of scores for this second group of patients was 0-5 (SLE) and 0-6 (RA). These results illustrate that a core set of autoimmune signature genes are capable of perfectly discriminating gene expression

Table II. Expression levels of genes that encode proteins distinguishing among lymphocyte subsets or activation state.

Population:	Control	SLE	RA	IDDM	MS
Expression Level:		Avg +/- Stdev			
T Cell Antigens					
CD3δ	0.7±0.2[a]	0.6±0.4	0.5±0.2	0.5±0.2	0.4±0.2
CD3γ	0.5±0.1	0.6±0.9	0.4±0.1	0.3±0.1	0.4±0.1
CD8β (Tc)	0.8±0.3	0.8±0.2	0.6±0.2	0.5±0.2	0.5±0.2
CD44 (memory)	0.5±0.1	0.8±0.5	0.7±0.4	0.8±0.5	0.7±0.4
CD69 (activation)	0.5±0.2	0.7±0.3	0.6±0.2	0.8±0.3	0.7±0.4
CD62 (L-selectin)	1.3±0.6	1.4±0.9	1.8±1.1	1.7±1.1	1.9±1.1
CD122 (IL-2R β)	0.4±0.1	0.4±0.2	0.5±0.2	0.3±0.1	0.3±0.1
B Cell Antigens					
CD79a	0.6±0.3	0.4±0.2	0.4±0.2	0.4±0.2	0.4±0.2
CD79b	0.5±0.2	0.6±0.3	0.8±0.7	0.8±0.4	0.7±0.3
CD72	0.4±0.1	0.4±0.3	0.4±0.2	0.3±0.1	0.3±0.1
CD22	0.3±0.1	0.4±0.3	0.4±0.4	0.3±0.1	0.3±0.1
Monocyte Antigens					
CD14	0.5±0.2	0.4±0.2	0.3±0.1	0.3±0.2	0.3±0.2
CD163	0.3±0.1	0.4±0.2	0.4±0.2	0.3±0.1	0.3±0.2
CD32 (B /mθ)	0.3±0.1	0.5±0.4	0.5±0.3	0.3±0.1	0.4±0.2
Activation-Induced Antigens					
CD54 (ICAM-1)	4.4±1.8	3.1±2.1	4.3±0.7	4.3±2.2	3.9±1.0
CD38	0.4±0.3	0.3±0.2	0.3±0.1	0.3±0.1	0.3±0.1
CD71	0.2±0.1	0.2±0.2	0.2±0.1	0.2±0.1	0.2±0.1

[a]average expression level of the indicated genes within the different populations.

A Under-expressed autoimmune signature genes

B Over-expressed autoimmune signature genes
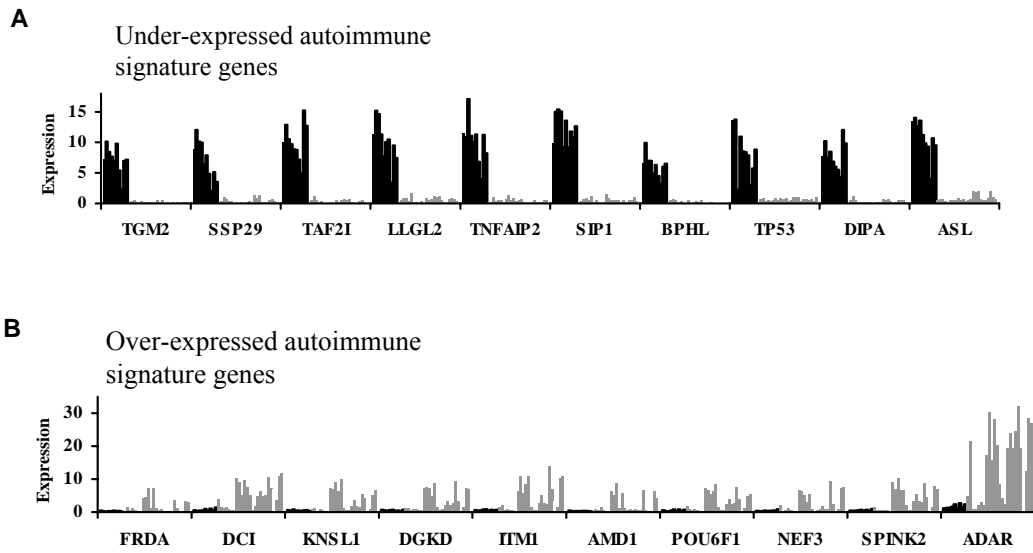
FIGURE 6. Analysis of the most under- and over-expressed genes in the autoimmune population on an individual basis. Expression levels of individual genes were compared among 7 control individuals (black bars) and 25 individuals (gray bars) with an autoimmune disease. The ten most over-expressed (**A**), and ten most under-expressed (**B**) genes were analyzed.
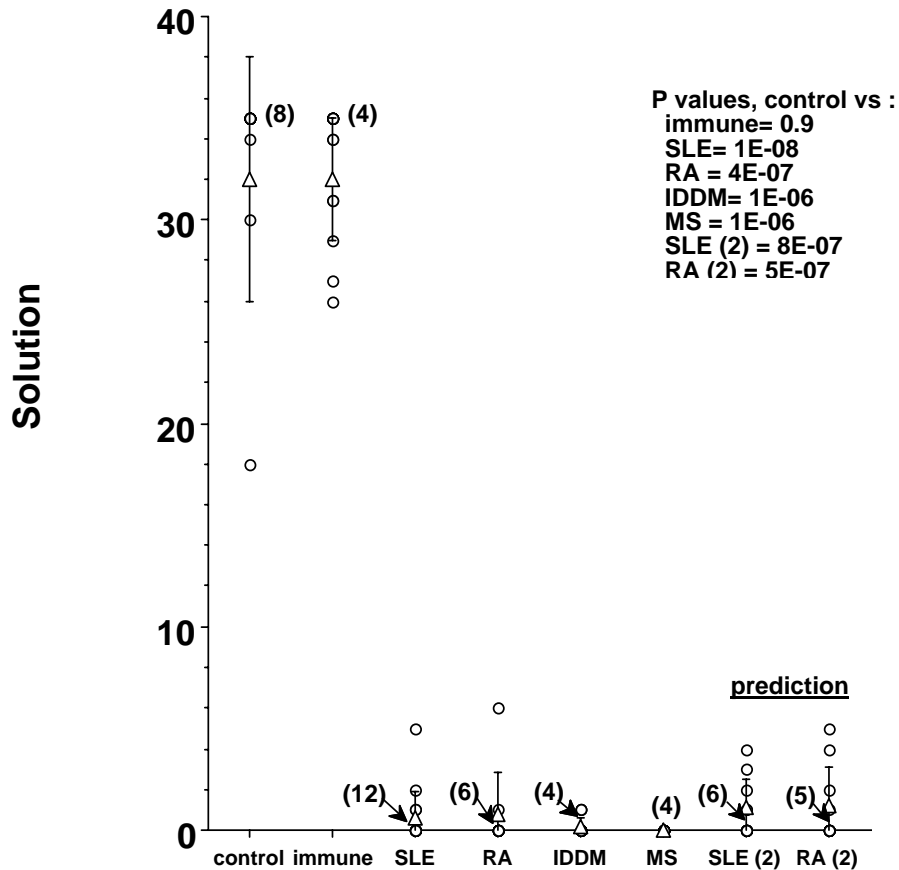
Fig. 7. Classification and prediction of autoimmune disease. The score (Y-axis) is shown for each individual sample analyzed from the different populations (X-axis). Immune response group (immune) will be discussed in Chapter 2. P-values are depicted in the legend. The 35 genes employed to derive this score were (gene symbols or accession #): *TGM2, SSP29, TAF2I, LLGL2, TNFAIP2, SIP1, BPHL, TP53, DIPA, ASL, DKFZp58601922, MAN1A1,* R09503, *LOC51643, BMP8, ORC1L,* W04674, R94175, *CDH1, SUDD, EPB72, CDKN1B, CASP6, TXK, MYO1B, LIF, HSJ2, BRCA1, GUCY1B3, AP3S2,* N68565, *SC65, UBE2G2, SLC16A4,* and *MMP17*.

profiles from control individuals and patients suffering from a range of autoimmune disorders.

The under-expressed autoimmune cluster contained a disproportionate number of genes involved in various apoptosis pathways. Because defects in apoptosis have been associated with autoimmunity[176], we decided to investigate these pathways in greater detail. We selected three major apoptosis pathways: the Fas/FasL, TNFR1, and p53 pathways. Next, the three pathways were reconstructed to illustrate potential blocks in apoptosis resulting from reduced expression of key members of each of these pathways (Fig. 8 A). Both the average expression levels of these genes within each disease class, as well as individual variability within a disease group are shown (Fig. 8 B, C, and D).

In the TNFR I apoptotic pathway, several genes (*TRADD*, *TRIP*, *TRAF2*, and *MAP4K2*) were significantly under-expressed in all autoimmune diseases (RA, SLE, IDDM, and MS) relative to the control and normal immune response individuals (Fig. 8 B). In the FAS/FASL pathway, *CASP6* and *CASP8* were significantly under-expressed in the autoimmune groups (Fig 8 C). The trend of under-expression of apoptosis-associated gene was also found in the p53 pathway, with *TP53*, *PIG11*, *APAF1*, and *GADD45* all differing significantly from the normal immune response and control levels (Fig. 8 D). These data indicate that there are substantial defects in levels of expression of key genes involved in apoptosis in the PBMCs from patients with a range of autoimmune disorders.
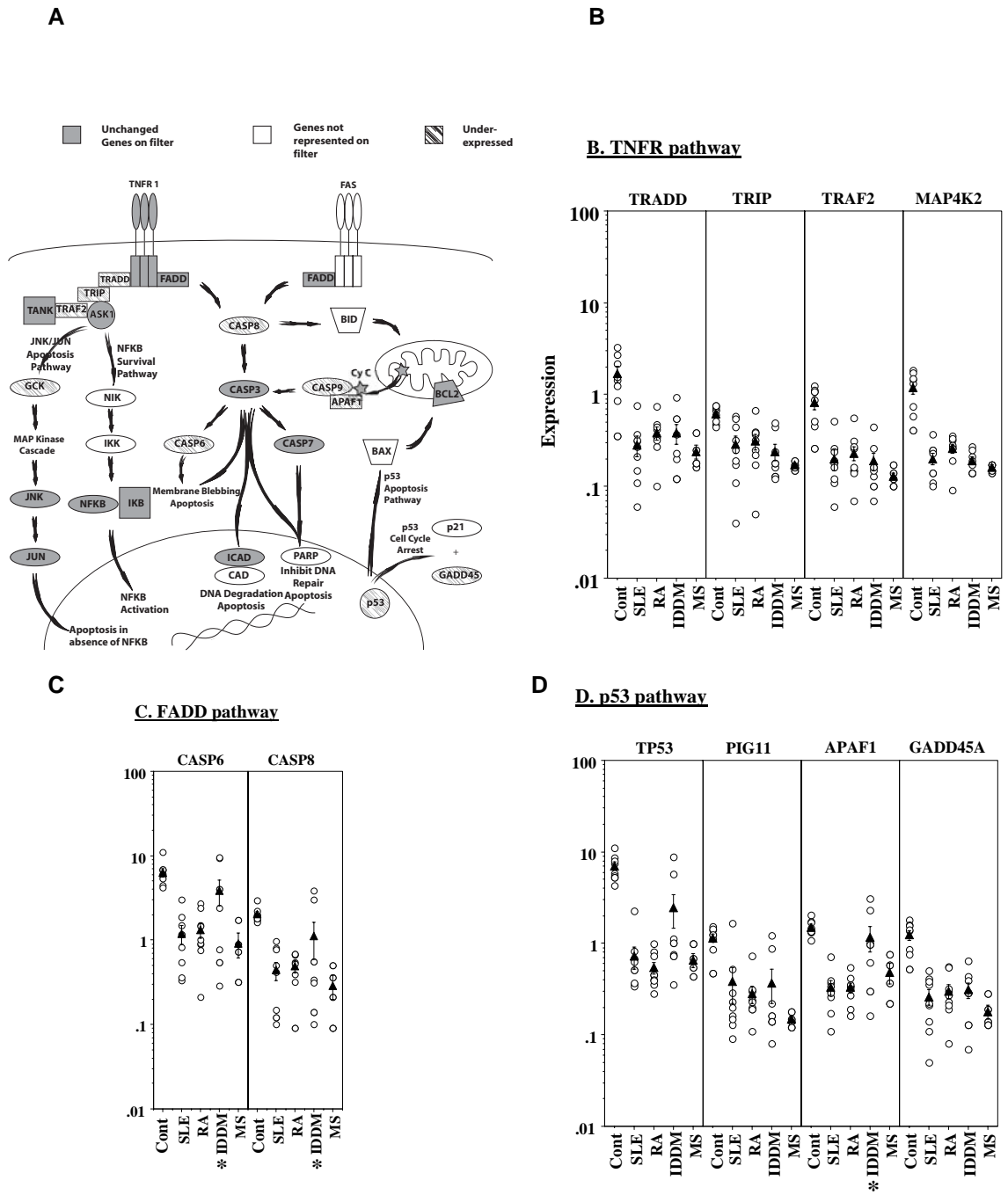
Fig. 8 Expression levels of numerous genes encoding proteins involved in apoptosis in lymphocytes from autoimmune individuals. **A**. An illustration shows the key proteins involved in apoptosis pathways. The key identifies the mRNAs a) for which we do not have expression data (absent from the microarray filter), b) exhibit constant expression levels in the three major classes (pre-immune, immune, and autoimmune), c) are relatively over-expressed in the autoimmune group, or d) are relatively under-expressed in the autoimmune group. **B, C, & D**. Relative expression levels of individual genes illustrated in **A.** are shown for each individual(O) from the immune class and all disease groups within the autoimmune class and the average of the group (•) +/- SEM (error bars). p values < 0.01 for all comparisons of immune vs. each autoimmune group except where noted: * not significant.

Discussion

DNA microarray technology is a powerful tool to compare differences in gene expression under distinct biological conditions. Our study represents the first examination of gene expression profiles in individuals with autoimmune disorders. Given the considerable heterogeneity both within and among autoimmune disease groups, we wanted to determine if we could detect gene expression patterns that were either characteristic of a particular autoimmune disorder or were shared among several autoimmune disorders. To accomplish this we compared four distinct diseases that varied primarily by the target of autoimmune attack. We found that the overall gene expression patterns found in autoimmune disease were distinct and were distinguishable from patterns found in control individuals. Surprisingly, the four autoimmune disease subclasses, RA, SLE, IDDM, and MS, exhibited highly similar profiles of gene expression. This permitted us to identify two clusters of genes that are markedly over-expressed or under-expressed, respectively, in all four autoimmune diseases.

Since our study, several other groups have also used gene expression profiling to examine gene expression patterns in autoimmune disease. Currently, all of the studies have reported that profiles from autoimmune patients can be distinguished from control individuals. In particular, researchers have compared the expression patterns in the PBMCs of adult and pediatric SLE patients[177, 178]. Both groups reported signatures consistent with exposure to interferon α/β in patients with severe disease, supporting the suspected role of the type I interferons in SLE pathogenesis. Additional studies examining gene expression profiles in the peripheral blood of patients with MS have

found, similar to our own study, that many genes that encode proteins that regulate cell cycle progression and apoptosis are under-expressed in MS[179, 180].

Interestingly, animal models of autoimmunity have shown that defects in apoptosis can contribute to autoimmunity. One of the most well known examples of defects in apoptosis contributing to autoimmunity comes from studies in the *lpr*/MRL and *gld*/MRL mice. These mice contain spontaneously occurring mutations in FAS and FASL, respectively[181]. These mutations, on the MRL background, result in development of systemic autoimmunity characterized by production of autoantibodies, and fatal glomerulonephritis[182]. Interestingly, both inactivating and dominant negative mutations in FAS have been found in patients suffering from the rare autoimmune disorder, autoimmune lymphoproliferative syndrome (ALPS, also referred to as Canale-Smith syndrome)[183, 184]. However, consistent defects in FAS or FASL have not been demonstrated in the more common autoimmune disorders[185-187].

Our microarray screening has identified multiple potential apoptosis defects not associated with the FAS/FASL pathway. The linkage of this gene expression pattern to a range of autoimmune diseases suggests that under-expression of these genes may play a key role in the development of disease. Further experiments are needed to verify under-expression of target pro-apoptotic genes in autoimmune patients and to determine if decreased gene expression results in actual functional defects in apoptosis. We will cover studies that demonstrate that RA lymphocytes actually exhibit defects in specific apoptosis pathways in Chapter 4 of this dissertation.

This study is currently the only microarray screen to make comparisons across a range of autoimmune disorders. Given the fact that there is a remarkably consistent

pattern of gene expression despite the nature of the autoimmune disease, several

possibilities may explain the origin of our autoimmune signature. It is generally believed

that autoimmune disorders arise from a loss of tolerance against self-antigens, ultimately

resulting in an immune response against self. One prediction of this hypothesis is that

our autoimmune gene expression signature may recapitulate the immune response to a

foreign antigen. Alternatively, many of the patients studied have suffered from their

respective disorders for a number of years. Therefore, it is possible that the observed

gene expression signature arises from autoimmune disease duration. As another possible

explanation, numerous epidemiological studies have revealed a heritable component to

autoimmunity[40, 42, 188]. Perhaps this conserved autoimmune signature reflects an inherited

trait or traits. We will address each of these possibilities in Chapter 3 of the dissertation.

CHAPTER III

ORIGIN OF THE AUTOIMMUNE SIGNATURE

Abstract

We have observed a highly reproducible gene expression profiles in the PBMCs of patients with a range of autoimmune disorders . Although the underlying mechanism is unclear, we considered three possible explanations for our observations. First, we tested the hypothesis that the autoimmune signature is a recapitulation of the immune response to foreign antigen by examining gene expression changes during a model immune response (influenza vaccination). To address if disease duration contributes to the differential expression of autoimmune signature genes, we compared expression profiles in individuals with established disease (an average of ten years history of disease) to patterns in individuals with early disease (< two years duration). Finally, to determine if our autoimmune gene expression signature reflects an inherited trait, we examined expression signatures in unaffected first-degree relatives of individuals with autoimmune disease. We found that neither differentially expressed immune response genes or disease duration accounted for the observed autoimmune expression pattern. However, gene expression patterns in unaffected first-degree relatives resembled profiles found in individuals with autoimmune disease. A high percentage of differentially expressed genes in unaffected first-degree relatives were previously identified autoimmune signature genes. Comparison of gene expression levels between parent-offspring pairs revealed that expression levels of autoimmune signature genes tended to

have higher correlation levels than other genes on the microarray. Taken together, these results support the hypothesis that the autoimmune gene expression signature reflects an inherited trait or traits rather than a disease process.

Introduction

The break in tolerance to self-antigen is generally believed to represent the critical factor leading to autoimmunity. This model predicts that self-reactive T or B cell clones escape their normal regulatory mechanisms, recognize self-antigen and develop a normal immune response to self-antigen. Following this line of reasoning, we hypothesized that our autoimmune gene expression signature reflected an immune response simply directed against self. To address this hypothesis, we compared gene expression profiles from a normal immune response to profiles associated with autoimmune disease. The response to inactivated influenza vaccine in healthy individuals was used as our model of the normal immune response. Comparison of the most differentially expressed immune response genes did not overlap with our autoimmune gene expression signature, indicating our autoimmune signature did not appear to reflect a normal immune response against an antigen.

The majority of autoimmune patients used for our microarray analysis have histories of established disease over an extended period of time. As an alternate possibility, we hypothesized that the autoimmune expression signature reflected disease duration. To address whether disease duration contributed to our autoimmune signature, we obtained PBMCs from patients diagnosed with RA for less than two years, and compared their profiles to patients with established RA. Examination of the DEGs

(relative to control individuals) revealed that our previously identified autoimmune expression signature was present in the early RA patients, indicating that disease duration did not contribute to this expression pattern.

As reviewed in the introduction, evidence from epidemiologic and genetic linkage studies points toward a heritable component for many autoimmune disorders. To determine whether the autoimmune gene expression signature displayed family resemblance, we examined expression profiles in unaffected first-degree relatives of patients with known autoimmune disorders. Our studies reveal that unaffected first-degree relatives exhibit gene expression patterns distinct from normal control individuals but similar to autoimmune individuals. Many of the differentially expressed genes (DEGs) found in unaffected family members were the same genes that were found in patients with autoimmune disease. Based upon these observations, our data support the notion that the autoimmune signature reflects an inherited trait or traits.

Materials and Methods

Patient Population

Immune Response Studies: Nine healthy control subjects were studied before and after receiving the inactivated influenza vaccine in order to characterize the normal immune response. The same autoimmune patient populations described in Chapter 1 was used for comparison.

Disease Duration Studies: We defined individuals with early RA (ERA) as patients diagnosed with rheumatoid arthritis for less than two years. Eleven patients were

selected that met our criteria for early RA, and had an average disease duration of 1 ± 0.2 years. Profiles from these individuals were compared to the previously examined RA patients. These patients have been diagnosed with RA for an average of 10 ± 3 years. Patient age and treatment regimens were not significantly different between the established RA and early RA groups. Profiles from previously analyzed autoimmune patients (SLE, IDDM, and MS) were used for comparison in the study.

Inheritance Studies: Six previously studied control individuals without active infection or family history of autoimmunity were selected for inclusion in our study. Additionally, previously examined individuals diagnosed with rheumatoid arthritis (n=4) or systemic lupus erythematosus (n=4) served as patients with clinically diagnosed autoimmune disease. All autoimmune patients satisfied established ACR criteria for diagnosis of their respective diseases. Additionally, five families were selected for study. A total of 8 unaffected first degree relatives of patients with autoimmune disease were included in our study. Human subject studies were approved by the committee for the protection of human subjects of the Vanderbilt University Institutional Review Board.

Sample Preparation/RNA Isolation and Probe Synthesis with Microarray Hybridization
As described in chapter 1, Materials and methods section

Clustering Analysis of Gene Expression Profiles
As described in chapter 1, Materials and methods section

Identification of Autoimmune Genes in Differentially Expressed Gene Clusters

Research Genetics Pathways 4.0 program was used to identify the most differentially expressed genes after immunization and in the different autoimmune disease classes. Microarrays were grouped into one of several conditions: control (healthy individuals before immunization), post-immunization (healthy individuals at different time points after immunization: 3, 6-9, and 19-21 days), or an autoimmune group (systemic lupus erythematosus, rheumatoid arthritis, insulin dependent diabetes mellitus, multiple sclerosis, and early rheumatoid arthritis). The gene intensities for each condition group were averaged. The expression levels of each gene were represented graphically by plotting the natural logarithm of the ratio of gene expression intensity in the experimental group versus the control group for each condition. Genes that did not change significantly (99% confidence, Chen test[175]) over any of the conditions were removed from the database. The remaining genes in the data set were then clustered using an unsupervised K Means clustering algorithm[132][134] with ten centroids. The gene identities, chromosome number, and functions were further analyzed for each cluster using Stanford's GeneCards database (http://genome-www.stanford.edu/genecards).

A similar computational analysis was employed for our analysis of unaffected first-degree relatives. In order to identify autoimmune genes present among the most differentially expressed genes in individual profiles, microarrays from previously compiled control groups were separated into two reference conditions: control (control individuals) and post-immunization (6-9 days after immunization with flu vaccine). The gene intensities for each control condition group were averaged. Individual expression profiles for control individuals, RA and SLE patients, and unaffected first-degree family

members were compared against the compiled control conditions. Expression levels were represented graphically by plotting the natural logarithm of the ratio of the gene expression intensity in the experimental group versus the control group for each condition. Genes that did not change significantly (99% confidence, Chen test) over any of the conditions were removed from the database. The remaining genes in the data set were clustered using an unsupervised K Means clustering algorithm with ten centroids. The major under- and over-expressed clusters in the individual profiles were isolated, and the total number of genes in the respective clusters determined. The differentially expressed clusters were restricted to previously identify over-expressed, under-expressed, and core autoimmune genes using Pathways 4.0 Paths command.

Hybridization Intensity Correlation Coefficient Calculations

Parent-offspring pairings were used to estimate the family resemblance of gene expression levels. Five autoimmune parent-offspring pairings were used, with only one individual in a pair having an autoimmune disease. Three control parent-offspring pairings were also included in the study, with no individual having one of the four autoimmune diseases previously studied. The hybridization intensity for each individual gene was used to determine the spearman correlation coefficient between family pairs. Spearman correlation coefficients were calculated for over-expressed and under-expressed autoimmune genes, and for non-autoimmune genes. Pairwise comparison for each of the categories were performed using a permutation t-test.

Results

Immune response and the autoimmune gene expression signatures: Characterization of a model immune response reveals dynamic changes in gene expression

In order to determine if genes differentially expressed during an immune response were found in our autoimmune signature, we began by modeling and characterizing an immune response in healthy individuals. To model a normal immune response, we measured gene expression in PBMCs from healthy control subjects (n=9) before and after immunization with inactivated influenza vaccine. Samples were collected from subjects at three major time points: 3 days, 6-9 days, and 19-21 days after immunization. Total RNA was purified from PBMCs and reverse-transcribed to cDNA in the presence of ^{33}P-dCTP. Labeled cDNA was hybridized to microarray filters and hybridization levels were quantitated by phosphorimager analysis. Data from different hybridizations were normalized to yield an average intensity of 1.0 for each spotted clone (4,329) over the entire array.

A self-organizing map clustering algorithm[132] was employed to compare the control (pre-immunized) to the immunized group based upon similarities in gene expression variables. This method segregated microarrays such that an individual's profile appeared highly similar before and after immunization, as demonstrated by the relative proximity of individual samples (Fig. 9 A). This indicates that total gene expression patterns remain relatively unchanged after immunization. However, this type of clustering analysis does not focus on the most differentially expressed genes. In order to examine distinctions that arise from the most differentially expressed genes, we filtered out genes whose expression level did not vary by more than 3 standard deviations

56

from their respective means. After filtering, expression profiles segregated primarily by

pre- and post-immunization status (Fig. 9 B). This suggests that there are uniform

changes in expression variables of a smaller subset of genes that distinguish pre- and

post-immunization groups.

To identify which individual genes were differentially expressed, we analyzed the

immune response in more detail using K-means clustering to group genes on the basis of

similarity in expression patterns[134]. The immune response profiles were separated into

control (no immunization), early (3 day post-immunized), middle (6-9 days), and late

(19-21 days) post-immunization groups. Expression changes were plotted as the natural

logarithm of the ratio between the post-immunization group relative to the control group
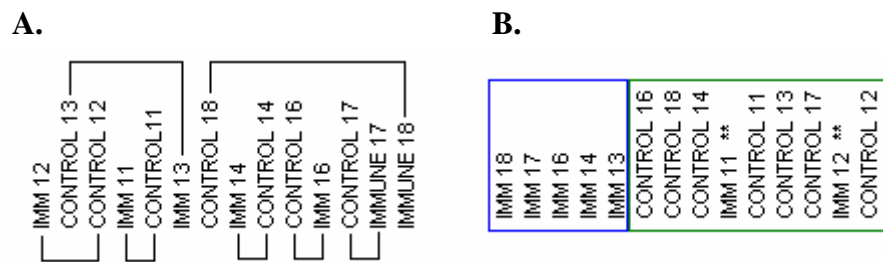
Fig. 9. Cluster analysis of the pre-immune and post-immune data set. **A**. Pre- and post-immune samples were analyzed using an unsupervised self-organizing map algorithm based upon expression levels of 4329 genes. Samples are identified as control (pre-immune) and imm (after immunization). Individuals are designated 11 to 18 and are connected by brackets. **B**. Gene expression levels that did not differ by greater than 3 standard deviations were eliminated from the data set. The clustering analysis was repeated based upon expression levels of the 99 genes that remained after filtering. Groups of profiles that share similar patterns of gene expression are boxed.

(Fig 10 A-C). Genes that did not change significantly over the time course (99% significance, Chen test [175]) were filtered from the data set.

We found three distinct clusters associated with the normal immune response. The first cluster consisted of 304 genes were over-expressed 3 days after immunization (Fig. 10 A). This cluster mainly contained genes that encode proteins involved in key signal transduction pathways in lymphocytes and other cell types (e.g. *PKC*, *PLC*, DAG kinase, MAP kinase, STATs and STAT inhibitors, AP-1 transcription factors, IRFs, and proteins required for proliferation). Genes in this cluster exhibited an increase in expression ranging from 2-12 fold compared to the control group.

The second cluster of 88 late (19-21 days) response genes represented a shift away from signaling and proliferation pathways, towards increased functional activity (Fig. 10 B). Among the late immune response gene cluster, chemokines (*SCYA3*, *SCYA13, and SCYA14*), complement components (*C1S*), IFN-inducible proteins (*IFI35*), and leukocyte homing/adhesion (*ICAM2*) genes were over-expressed. Receptors for serotonin, glutamate, estrogen and retinoic acid were also over-expressed. Increases in the expression level of this group of genes varied from 2-8 fold greater than controls.

The final immune response cluster contained 78 genes that exhibited reduced expression levels over the entire time-course (Fig. 10 C). Strikingly, twelve of the seventy-eight under-expressed immune response genes encoded ribosomal proteins. This represented a decrease in the expression of 32% of all the ribosomal protein-encoding genes present on our cDNA microarray filters. Interestingly, coordinate changes in ribosomal protein gene expression have been linked to differentiation in eukaryotic cells[189] and may reflect differentiation of lymphocytes from a naïve to effector state in

response to immunization. Taken together, these data illustrate dynamic, coordinate changes in mRNA expression that accompany the immune response, in vivo. First, genes were induced that are required for signal transduction and cell proliferation; two key elements of the early immune response. Second, as the immune response against antigen progresses, we observed a shift towards genes that mediate inflammation and lymphocyte effector functions.

Differentially expressed immune response genes do not contribute to the autoimmune gene expression signature

Given the above results, we wanted to compare gene expression profiles of the normal immune response to profiles of autoimmune disease. We initially subjected profiles from immunized individuals and autoimmune patients to hierarchical clustering analysis, in order to examine the degree of relatedness between the two groups based on overall expression patters. Comparison of 6-9 day post-immunized individuals with profiles from patients diagnosed with established RA and SLE resulted in a dendrogram that clustered all immune response gene expression profiles (IMM) into a branch that excludes the autoimmune profiles (Fig. 11A). These results indicate that gene expression profiles associated with the immune response are globally distinct from autoimmune gene expression patterns. Hierarchical clustering analysis comparing the immune response profiles to patients with type 1 diabetes (IDDM) and MS supports the notion that autoimmune gene expression profiles do not resemble the normal immune response (Fig. 11B). Clustering of all the autoimmune groups (MS, RA, SLE, and IDDM) with both the normal immune response and control groups resulted in separation into three major

A.

B.

C.

Signaling	**Inflammatory/Immune**	**Ribosomal & Related**
PRKCZ, protein kinase C zeta	SCYA3, chemokine MIP1a	RPL36A
PRKCSH, PKC substrate	SCYA13, chemokine	RPL24
PLCB3, phopholipase C, β3	SCYA14, chemokine	RPS4Y
INPPL1, inositol phosphate ppase	CD79B, Ig associated	RPS13
DGKZ, DAG kinase ζ	TIAL1, CTL granules	RPL35
GNAT1, G protein	CRYBB2, crystallin	RPS16
GNAL, G protein	ICAM2, adhesion	RPL27A
MAP4K2, MAP kinase cascade	LY6E, thymic shared antigen	RPL5
STAT3	IFI35, IFN inducible	RPS15A
STAT5B	SCG2, secretogranin	RPL11
SSI-1, JAK binding protein	AXL, receptor tyrosine kinase	RPS29
SSI-3, STAT induced STAT inhibitor 3	C1S, complement	RPL30
STATI2, STAT induced inhibitor	FASTK, FAS activated ser/thr kinase	NACA
CDC34, Cell division cycle 34	KLK5, kallikrein 6	PABPC1
TCF3, E2A Ig enhancer binding factor	GAS6 growth arrest-specific 6	EIF3S6
TCF7, T cell transciption	**Other**	**Inflammatory/Immune**
TCF17, Zinc finger protein	AP3D1, adaptor-related protein	B2M, beta-2-microglobulin
FOS, c-fos	GARS, glycyl-tRNA synthetase	CD9
FOSL1, fra-1	ARF5, ADP-ribosylation factor 5	HLA-C
JUND	ESR1, estrogen receptor 1	IFI30, IFN-γ-inducible protein 30
MYBL2, c-myb	GRIN1, glutamate receptor	IFITM1, IFN induced protein 1
IRF5, interferon regulatory factor	HTR2B, serotonin recpetor 2B	LCP1, L-plastin
IRF7, interferon regulatory factor	PCAF, p300/CBP-associated factor	LTA, lymphotoxin alpha
GAK, cyclin G associated kinase		LYZ, lysozyme

Fig. 10 Cluster analysis of differentially expressed genes during normal immune response. Kmeans clustering analysis identified three distinct gene clusters based upon similarities in gene expression variables. These are **A.**, a cluster of 304 early response genes (3 days post-immunization), **B.**, a cluster of 88 late response genes (19-21 d after immunization) and **C.**, a cluster of 78 genes with decreased expression levels over the time course of investigation. Data are presented as the natural logarithm of the ratio of the early immune group/pre-immune group, the middle immune group (6-8 d after immunization)/pre-immune group, or the late immune group/pre-immune group. The individual lines in the plot represents the expression ratio for an individual gene over the early, middle, and late immunization time course. Expression ratios that did not achieve a 99% significance level were filtered from the data set. Below each graph, a partial list of genes is identified by dominant functional category within each of the clusters.

branches (Fig. 11 C). One of the branches consisted primarily of pre-immunized controls and normal immune response samples, while the two remaining branches exclusively contained autoimmune samples.

These results indicate that the gene expression patterns associated with the autoimmune signature are globally distinct from the immune response. However, this type of clustering analysis does not exclude the possibility that a small subset of differentially expressed genes could be shared between the immune response and autoimmune disease groups.
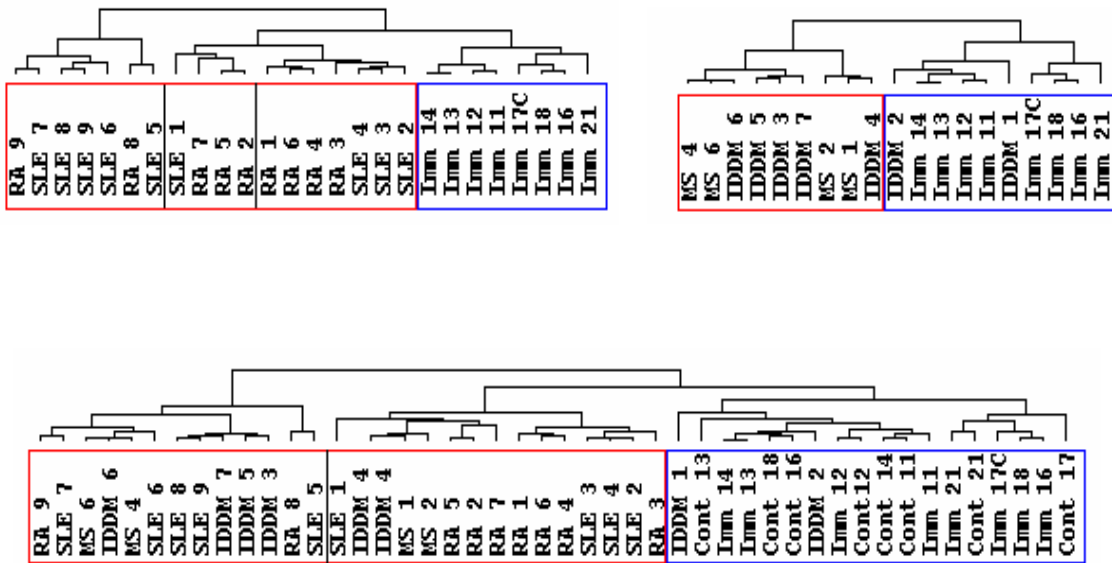
Fig. 11. Comparison of the immune and autoimmune classes by cluster analysis. **A**. The immune (6-9 d post-immunization), RA and SLE groups were analyzed using a hierarchical clustering algorithm based upon expression variables of 4329 genes. Three major branches were apparent from the dendrogram. All immune samples segregated within one major branch. **B**. The immune, MS, and IDDM groups were subjected to cluster analysis as in A. **C.** Hierarchical clustering of all autoimmune disease groups with pre-(Cont) and post- (Imm) immunized control individuals

To address this issue directly, we compared the most differentially expressed immune response genes from our previous K means clustering analysis (Fig. 10) to the most differentially expressed autoimmune signature genes originally described in Chapter 1 (Fig. 5). This comparison revealed that there were two major differentially expressed clusters: a cluster containing 96 genes over-expressed in all autoimmune disease groups and a second cluster of 113 genes under-expressed in all autoimmune disease groups (Fig. 12). Examination of these genes revealed that these differentially expressed genes were the same autoimmune signature genes identified in Chapter 1. We found there was absolutely no overlap in the most differentially expressed middle immune response genes and our autoimmune signature genes. Additional comparisons using the differentially expressed early and late immune response genes also did not overlap with the autoimmune signature genes (data not shown). Taken together, our hierarchical clustering analysis and examination of differentially expressed genes reveal that differentially expressed immune response genes do not contribute to our autoimmune signature.

Disease duration and the autoimmune signature: Early RA gene expression profiles contain the autoimmune gene expression signature

The RA patients that we analyzed in our microarray studies had an average history of autoimmune disease of 10 ± 2 years. These patients contained a high percentage of the autoimmune signature genes differentially expressed in their PBMCs. To address whether disease duration contributed to our autoimmune signature, we obtained PBMCs from patients diagnosed with RA for less than two years (early RA

patients- ERA). We initially utilized a hierarchical clustering algorithm to determine if

the gene expression profiles from ERA patients resembled profiles from RA patients

(Fig. 13). The analysis revealed that the expression patterns for both ERA and RA

patients were distinct from both pre- and post-immunization control individuals.

Additionally, hierarchical clustering grouped the ERA and RA profiles into distinct

branches on the dendrogram, indicating that differences in the overall expression patterns

were capable of discriminating these patient groups. RA9 clustered into the ERA patient

branch. Interestingly, examination of the medical history of RA9 revealed that this

patient has been diagnosed with RA for less than two years.
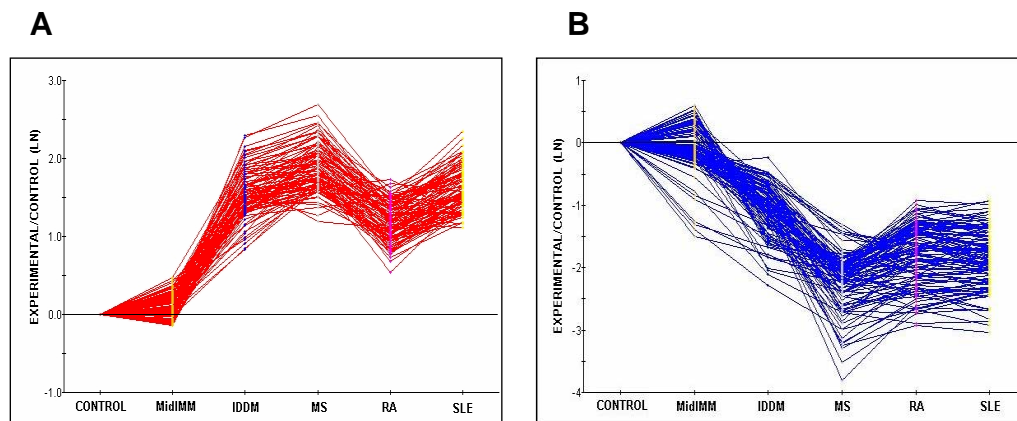
Fig. 12 Comparison of autoimmune signature with differentially expressed
immune response genes Kmeans clustering analysis identified two distinct
clusters (**A** and **B**) of genes that were uniformly differentially expressed in all
four autoimmune groups. Data are presented as the natural logarithm of the ratio
of the immune response group (MidImm)/control, or each autoimmune group
(IDDM, MS, RA, or SLE)/control. Each individual line in the plot represents the
expression ratio for an individual gene for each class (MidImm, MS, RA, SLE,
etc.) Expression ratios that did not achieve a 99% significance level were filtered
from the data set. The genes present in these clusters were all previously
identified autoimmune genes.

Fig. 13 <u>Clustering of Early RA Gene Expression profiles.</u> **A.** Clustering of early RA (ERA) and established RA (RA) patients using the self-organizing map algorithm and **B.** the hierarchical clustering algorithm with complete linkage clustering **C.** Hierarchical clustering of ERA, RA, Imm (post-immunization 6-9d), and Control (Cont) gene expression profiles

Since the hierarchical clustering algorithm detected differences between the expression patterns associated with the RA and ERA groups, we wanted to determine if these distinctions arose through alterations or absence of the autoimmune expression signature in the ERA patients. We addressed this question by examining the most differentially expressed genes in the immunized, ERA, and RA groups relative to control profiles (K-means clustering, 99% significance, Chen test) (Fig. 14). Many of the most under- and over-expressed genes in the established RA group were similarly differentially expressed in the ERA patients. Closer examination of these differentially expressed genes revealed that approximately 90% of the total over- (92/96 autoimmune signature genes) and under-expressed (98 /113 autoimmune signature genes) were previously identified autoimmune signature genes.

The finding that hierarchical clustering can discriminate ERA patients from established RA patients suggests that there are additional differences between these two groups on a gene expression level. Indeed, ERA-specific differentially expressed clusters have been identified, with nine over- and forty-four under-expressed genes present in the ERA group relative to established RA patients[190]. These differentially expressed genes encode proteins with diverse functions, ranging from bone remodeling to oxidative phosphorylation[190]. Additional differentially expressed genes in ERA patients encode proteins previously identified in our characterization of the immune response to viral antigen, suggesting that an active immune response may occur early in the disease course of RA. We believe that this collection of immune response and ERA specific genes account for the segregation of ERA profiles from the established RA profiles.
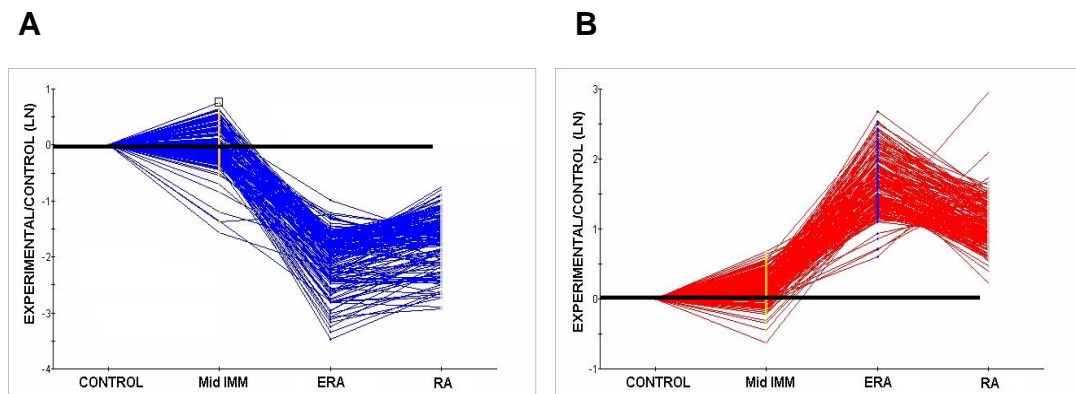
Fig. 14 Analysis of differentially expressed genes in Early RA. Kmeans clustering analysis identified two distinct clusters of genes that were **A.** under-expressed and **B**. over-expressed in the ERA and RA groups. Data are presented as the natural logarithm of the ratio of the immune group (MidImm)/control, or (ERA and RA groups)/control. Each individual line in the plot represents the expression ratio for an individual gene for each class (Control, MidImm, MS, ERA and RA). Expression ratios that did not achieve a 99% significance level were filtered from the data set.

In summary, our examination of the differentially expressed genes in ERA patients revealed that this patient population carries a large proportion of the previously identified autoimmune gene expression signature. Since this signature is present in patients with relatively recent autoimmune disease onset and in individuals with an established history of autoimmunity, it is unlikely that disease duration accounts for the autoimmune signature.

Gene expression profiles of unaffected first-degree relatives of autoimmune individuals resemble individuals with autoimmune disease

To address the possibility that gene expression profiles from unaffected first relatives resembled those of autoimmune patients, we compared their expression profiles to those of control individuals and unrelated autoimmune patients. We used standard microarray analysis to collect gene expression profiles. Initially, a hierarchical clustering algorithm was used to compare unaffected family members to control individuals by overall relatedness of gene expression profiles (using all 4000+ genes). This clustering analysis grouped all control individuals into a single branch and all unaffected family members into separate branches (Fig. 15 A). A representative node of genes reflects the clustering of the overall profiles.

Since unaffected first-degree relatives displayed expression patterns distinct from control individuals, we wanted to determine if gene expression profiles of unaffected family members were also distinct from unrelated patients with autoimmune disease. The same hierarchical clustering algorithm was employed to group individuals and genes by overall similarities in expression patterns. In contrast to the above results, the

program did not group unaffected family members and individuals with autoimmune disease into separate branches (Fig. 15 B).

We were unable to find gene nodes that accurately discriminated between autoimmune patients and unaffected family members, as illustrated by the representative node. Next, we compared individuals in all three groups using the same clustering algorithm. All control individuals were segregated into a single branch and all unaffected family members and autoimmune individuals were segregated into the remaining branches (Fig. 15 C). Unaffected family members and autoimmune individuals did not segregate into separate branches. Rather, each branch with autoimmune individuals also contained unaffected first-degree relatives. As above, we chose a node of genes that reflected the differences in the clustering profiles (Fig. 15 C). Of note, many of the genes present in this node have been previously defined as part of an under-expressed autoimmune gene expression signature[1].

"Autoimmune signature genes" are differentially expressed in unaffected first-degree relatives

The above results suggest that unaffected family members exhibit the same pattern of differential gene expression as individuals with autoimmune disease. To determine if differentially expressed genes from unaffected family members overlapped with autoimmune expression signatures, we performed a k-means clustering analysis on profiles from control (n=6) and immune response individuals (n=6), autoimmune

67

A. Unaffected Family Members & Controls

B. Unaffected Family Members & Autoimmune Individuals

Genes panel A (left to right top dendrogram: F5-GM, F4-M, F3-D, Cont 2, Cont 6, Cont 1, Cont 4, Cont 5, Cont 3, F2-M, F1-M):

Accession	Gene
T95053	PER1
H15431	POLR2D
R51835	–
AA457114	TNFAIP2
R87642	–
N66028	FAP48
W45688	CASP6
AA630082	CDKN1B
N38959	CCT2
R94175	–
H42728	HLA-DMA
H97778	CDH1
H66617	GLG1
AA495974	EBI3

Genes panel B (top dendrogram: F5-GM, F4-M, RA 2, RA 3, RA 1, SLE 2, SLE 1, F3-D, F2-M, F2-F, F1-M, SLE 4, RA 4, SLE 3):

Accession	Gene
AA668527	MADCAM1
AA158244	–
T65407	HADHSC
R94175	–
AA130874	LOC51643
R61332	UBE1
H98636	TNFRSF5
N36882	YES1
H15431	POLR2D
AA490902	GUK1
AA088434	ZNF38
AA180013	HABP2
W69649	MAP2K5
R44870	CFIM
AA489201	SSP29
AA425757	CGTHBA

C. Unaffected Family Members & Autoimmune Individuals

Genes panel C:

Accession	Gene
T54144	SUDD
T49657	KCNK3
N94820	DIPA
R02346	SNRP70
AA171449	BPHL
R73525	EPHX2
AA489201	SSP29
T40541	LLGL2
AA485922	CPNE1
R94175	–
H97778	CDH1
R09503	–
T71887	APOC4
AA485366	PRKAR1
AA158244	–
AA130874	LOC51643
H15431	POLR2D
T65407	HADHSC
R02373	EHHADH
R87642	–
N66028	FAP48
W45688	CASP6
AA630082	CDKN1B
R51835	–
AA458785	GUCY1B3
H94949	TROAP
AA701554	–
AA485871	MYO1B
AA443634	UBE2G2

Fig. 15. Hierarchical clustering of total gene expression profiles. A hierarchical clustering algorithm was employed to group individuals based upon similarities in overall gene expression profiles. Representative nodes are shown to provide sample genes that discriminate between branches. Hybridization intensities for individual genes across the profiles are represented as a range from black (no expression) to red (high expression level). Genes are represented by their gene symbols. Clustering and nodes for: **A.** Control individuals (Cont) and unaffected first-degree relatives (Family Number- F#), **B.** Unaffected first-degree relatives and individuals with autoimmune disease (RA, SLE), **C.** Control individuals, unaffected first-degree relatives, and autoimmune individuals.

patients (RA (n=4) and SLE (n=4) patients), and unaffected first degree relatives (n=6).

We found that the most differentially expressed gene clusters in autoimmune patients

were differentially expressed to the same extent in unaffected family members (Fig 16).

The over-expressed autoimmune/unaffected family cluster contained a total of 127 genes,

and the under-expressed cluster contained 74 genes. Approximately 52% (66/127) of the

total over-expressed and 55% (41/74) of the total under-expressed genes were

autoimmune genes, revealing that a large percentage of the most differentially expressed

genes were "autoimmune signature genes" (Figure 16 A). When the stringency for
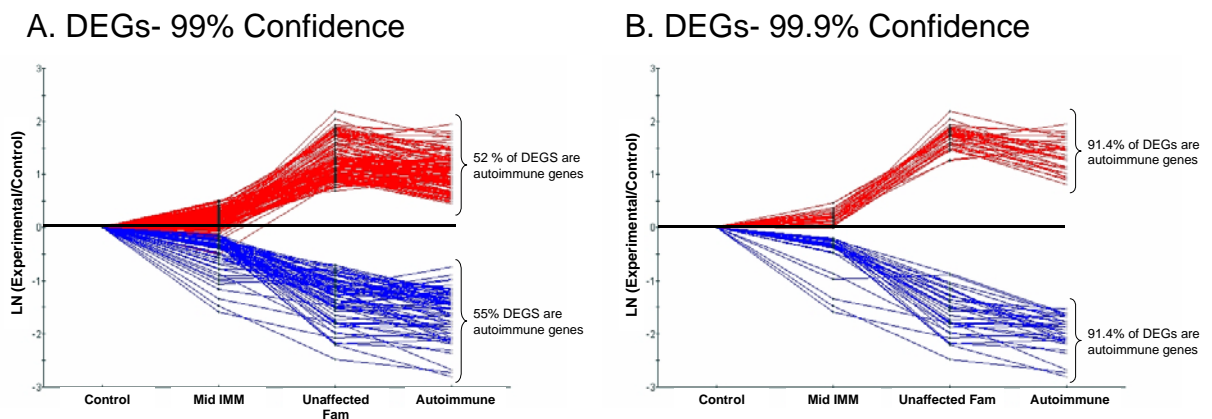
Fig. 16. Percentage of DEGs in unaffected first-degree relatives that are autoimmunity genes. we determined the percent contribution of autoimmune genes to the most differentially expressed clusters identified in unaffected family members compared to random controls. Gene expression data is presented as the natural logarithm of ratio (control/control, immune/control, unaffected first-degree relative/control, and autoimmune/control). **A.** % autoimmune signature genes found in DEGs (P<0.01, Chen test) for unaffected family members. **B.** % autoimmune signature genes found in DEGs (P<0.001, Chen test).

significance was increased to 99.9% (Chen test, P<0.001), over 90% of the remaining

DEGs were previously identified "autoimmune signature genes" (Fig. 16 B).

We also performed analyses to determine the contribution of the autoimmune

signature to differentially expressed genes in individual profiles. To do this, we first used

the clustering algorithm to group genes with similar expression patterns among control

individuals, immune response individuals (an additional control group comprised of

control individuals immunized with inactivated influenza vaccine), and an individual

unaffected family member (Fig. 17-1). The ratio of gene expression

(Experimental/Control, LN) was plotted for each of the 4000+ genes for the control

group, the immune response group and each unaffected family member or autoimmune

individual (RA and SLE). Differences in gene expression among the groups that did not

achieve statistical significance (*P*<0.01, Chen Test) were excluded from further analysis

(Fig. 17-2). Both the major over- and under-expressed clusters were isolated (Fig. 17-3a

and –3b). We further restricted these DEG clusters to the 96 over-expressed and 113

under-expressed autoimmune signature genes (Fig. 17-4a and 4b) identified in the

immune response/autoimmune disease studies (Fig. 12).

Using results from this type of analysis, we determined the percentage of

"autoimmune genes" present in the DEGs of unaffected family members. For example,

after clustering and statistical treatment, the unaffected first-degree relative F1-M had

two major differentially expressed gene clusters: an under-expressed cluster of 246 genes

and an over-expressed cluster of 245 genes. We previously defined the "autoimmune

signature genes" (RA, SLE, IDDM, and MS) as an under-expressed cluster of 113 genes

and an over expressed cluster of 96 genes[1]. When we restricted the DEG clusters in

individual F1-M to autoimmune genes, the under-expressed cluster retained 75 genes and
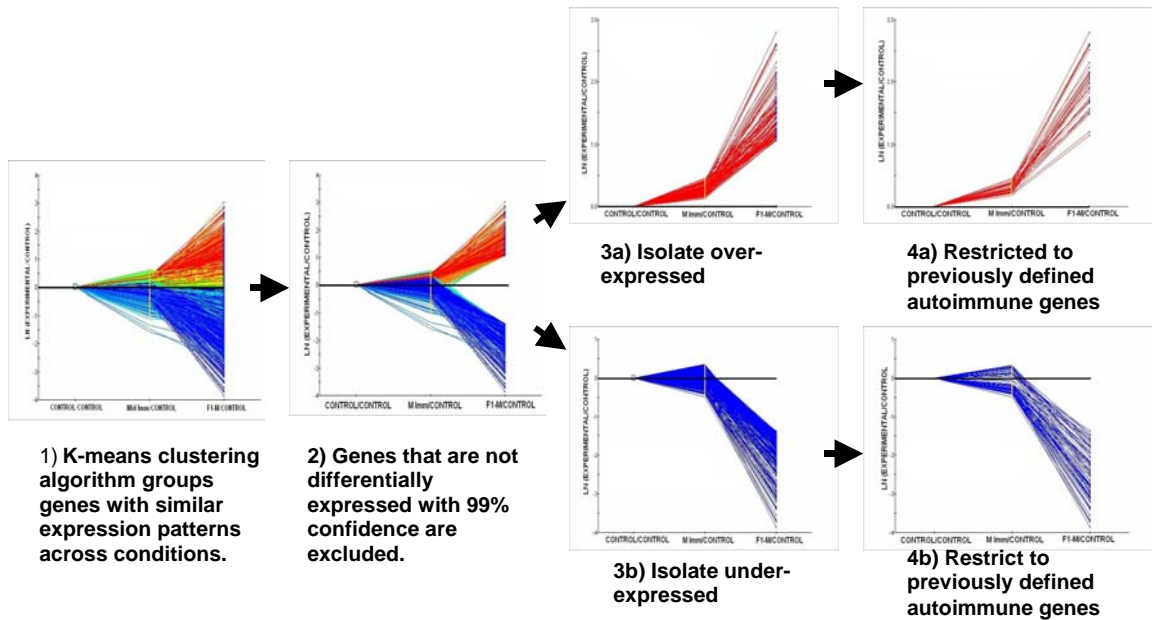
Fig. 17. Schematic of analytic method to determine overlap between differentially expressed genes and autoimmune signature genes. **A.** The average ratio of gene expression relative to the control group was calculated for each group or individual, and expressed on a natural logarithm scale. **B.** Genes that were not significantly differentially expressed ($P < 0.01$, Chen test) were removed from the data set. Remaining differentially expressed genes were grouped into ten clusters using a K-means clustering algorithm. **C.** Over- and under-expressed clusters were isolated for further analysis. **D.** Isolated clusters were further restricted to previously identified autoimmune signature genes. The genes that remained were used to calculate the percent of the total autoimmune signature gene present in the clusters, see Table I.

the over-expressed cluster retained 86 genes. Therefore, after clustering, statistical

treatment, and restriction of non-autoimmune genes, we found that individual F1-M

contained 66 % (75/113) of the under-expressed and 90% (86/96) of the over-expressed

autoimmune signature genes.

Results have been summarized in Table 3 for control individuals, autoimmune patients, and unaffected family members. DEGs in control individuals contained only a small percentage of genes from the autoimmune signature. Conversely, both the over- and under-expressed genes present in individuals diagnosed with autoimmune disease contained significantly higher percentages of the autoimmune signature (47% ± 44, $P<0.02$ and 54% ± 15, $P<10^{-4}$, respectively). Unaffected first-degree relatives contained significantly higher numbers of autoimmune genes represented in the under-expressed cluster compared to control individuals (44% ± 24, $P<0.01$). The over-expressed cluster in family members had a higher percentage of autoimmune genes, but these differences did not achieve statistical significance.

In addition to the total autoimmune signature, we previously identified a core set of thirty genes that were consistently under-expressed in autoimmune individuals [1]. We utilized the same method as outlined above (Fig. 16) to determine the representation of this core of thirty autoimmune genes in unaffected family members (Table 3). Control individuals had few of these core genes present among their most differentially expressed genes (7% ± 10%). Differentially expressed genes for both autoimmune patients and unaffected first-degree relatives were highly represented (90% ± 12% and 76% ± 25, respectively) and were statistically significant compared to controls ($P<10^{-7}$ and $P<10^{-3}$, respectively). There was no statistically significant difference between the percent of these core genes present in autoimmune patients and unaffected family members. Overall, these data demonstrate that genes differentially expressed in individuals with autoimmune disease are also differentially expressed in unaffected first-degree relatives.

Given the fact the core genes were so highly represented in the unaffected family members, we wanted to see how the individual profiles would cluster if we restricted the microarray data set down to the 30 gene autoimmune core. Profiles from control individuals sorted with a remarkable degree of precision to a single branch (Fig. 18), while both autoimmune and unaffected family member profiles segregated into the other branches. This analysis further supports the hypothesis that gene expression profiles of individuals with autoimmune disease and unaffected first-degree family members are highly similar to each other and that both groups are highly distinct from control individuals.

Unaffected family members do not display signs of autoimmune disease, however they contain a high number of autoimmune DEGs. We wanted to determine if there were DEGs that could discriminate unaffected first-degree relatives from individuals with autoimmune disease. Using a variety of approaches, we have been unable to identify combinations of DEGs that successfully discriminate all unaffected family members from all individuals with autoimmune disease (not shown).

Transcript levels of autoimmune signature genes display high levels of family resemblance

The above results indicate that the gene expression profile of a first-degree relative of an individual with autoimmune disease is more similar to an individual with autoimmune disease than an individual without autoimmune disease. This argues that transcript levels of a proportion of autoimmune signature genes may be determined by family resemblance rather than by disease activity. We wanted to use a combination of

Table III. Conservation of autoimmune gene expression

Profiles in unaffected first-degree relatives

| | Gene Expression Clusters | | |
	Over	Under	Core
# Genes:	96	113	29
Sample			
Control			
1	0	3	0
2	1	13	27
3	1	7	3
4	3	5	0
5	6	8	7
6	0	7	3
Avg±StdDev	2±2	7±4	7±10
Autoimmune			
RA1	2	66	97
RA2	14	66	97
RA3	88	65	93
RA4	94	37	80
SLE1	3	63	97
SLE2	4	61	97
SLE3	76	45	97
SLE4	93	27	63
Avg±StdDev	47±44	54±15	90±12
P value	0.02	$< 10^{-4}$	$< 10^{-7}$

(Table continued on next page)

Unaffected 1st degree relative			
F1-M	89	66	97
F2-F	30	54	93
F2-M	39	60	97
F3-D	10	52	80
F4-M	0	4	37
F5-GM	1	26	53
Avg±StdDev	28±34	44±24	76±25
P value	NS	0.01	$< 10^{-3}$

Data resulting from analysis described in Figure 2.
The percentage of autoimmune signature genes
present in the DEG (p<0.01, Chen test) clusters were
calculated for each individual. Over= over-expressed
autoimmune signature, Under=under-expressed autoimmune
signature. The percent of core autoimmune DEGs (Core)
were also calculated for each individual. *P* values for the
autoimmune and unaffected first-degree relative groups
were calculated relative to the control group
averages (student's T test).
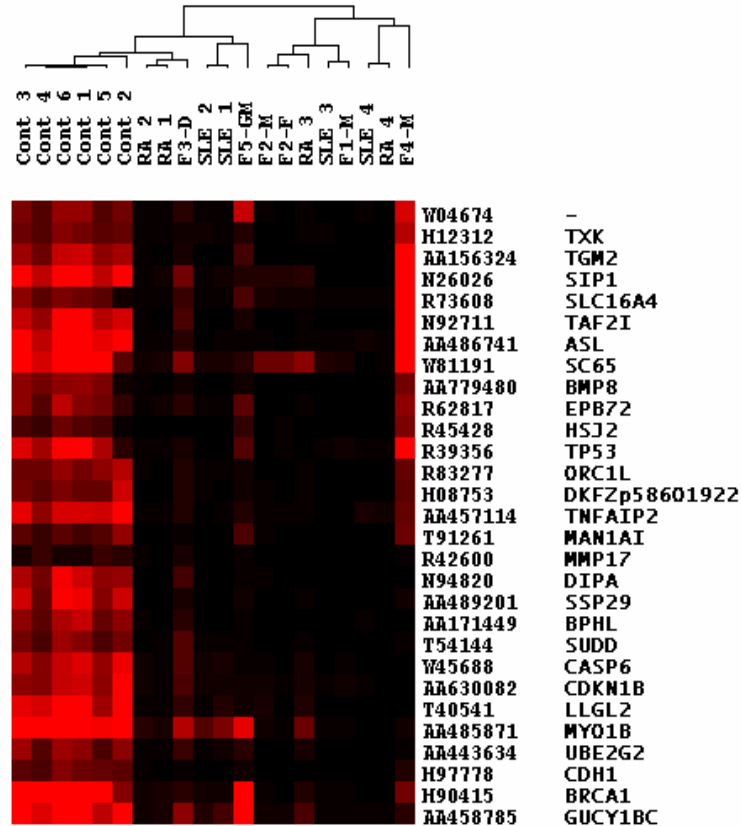
Fig. 18. Hierarchical clustering using core autoimmune genes. Microarray data were restricted to 29 previously identified core autoimmune genes [1]. Profiles for control individuals, unaffected family members, and autoimmune individuals were subjected to hierarchical clustering. Hybridization intensities are represented as a range from black (no expression) to red (high expression).

computational techniques and statistical analyses to determine the degree of gene expression resemblance between family members. We reasoned that if expression levels of autoimmune genes demonstrated a highly family resemblance, they should be similar between a parent and offspring.

We examined the correlation between eight parent-offspring pairings to determine the family resemblance of gene expression levels. For five of these pairings, one individual suffered from a previously examined autoimmune disorder (RA, MS, IDDM, or SLE) while the other individual was unaffected. Neither parent nor offspring had one of the four major autoimmune diseases in the three remaining pairs. Because of small sample size, we utilized the non-parametric Spearman correlation coefficient to determine the degree of parent-offspring relatedness for a give gene. Genes and expression data were divided into one of three major categories: over-expressed autoimmune genes (94 genes), under-expressed autoimmune genes (111 genes), and non-autoimmune genes (3,924 genes). The average Spearman correlation coefficient for each category was calculated. Samples of the autoimmune signature genes displaying the highest average levels of correlation are provided in Tables IV and V.

The Kruskal-Wallis test was used to test the null hypothesis that the correlations among the three gene categories are the same. Since the result did not support the null hypothesis($p < 0.0001$), we performed further pairwise comparisons using a permutation test on t statistics based on the rank score of Spearman correlation to determine which of the categories (over-, under-, or non-autoimmune genes) were significantly different. Pairwise comparisons revealed that, on average, expression levels for both the over- and under-expressed autoimmune genes were more highly correlated between parent and offspring relative to non-autoimmune genes (Fig. 19). These difference were highly significant ($p<0.0001$, for over-expressed autoimmune vs. non-autoimmune and under-expressed autoimmune vs. non-autoimmune, permutation T-test). Comparison of the average correlation for the over- and under-expressed autoimmune genes were not

significant (p=.8279, permutation t-test). These results support the notion that the expression levels of previously identified autoimmune genes display higher levels of family resemblance than the majority of other genes present on the microarrays.

Statistical analysis of data supports validity of analysis

Analysis of microarray data sets can require unfamiliar computational and statistical methods. We wanted to determine if the relationships in gene expression between individuals might be observed by chance. To do so we compared gene expression profiles in 8 control individuals who were randomly grouped into two groups of 4 each (Table VI). We compared this 4 x 4 group of control individuals to a 4 x 4 group containing 4 individuals from our control group and 4 individuals from our unaffected first-degree relative group. All individuals were unrelated to each other. First, we identified the total number of DEGs in the 4 x 4 random control group and in the 4 x 4 random control versus unaffected, unrelated first-degree relatives group. We used ratios of 3-, 4-, 5-, and 10-fold differences in expression to define DEGs. When we performed the comparison in the 4 random controls x 4 random controls group, we identified small numbers of DEGs (15 to 0). None of these differences in gene expression between the two groups of 4 achieved statistical significance (Student's T test). In contrast, when we compared gene expression differences between the 4 random controls and the 4 unaffected relatives group, we identified large numbers of DEGs (208 to 4, depending upon the ratio employed). A larger number of these gene expression differences achieved

Table IV. Most correlated under-expressed autoimmune signature genes

ACC	GENE	Rs	p-value
N68565		0.836378997	0.009651192
AA458785	GUCY1B3	0.841525993	0.008804689
H45618		0.841525993	0.008804689
AA464198	E1B-AP5	0.848500432	0.00773527
AA702422	MJD	0.848500432	0.00773527
AA779480	BMP8	0.848500432	0.00773527
AA778919	P2RY6	0.860621867	0.006081129
R83277	ORC1L	0.860621867	0.006081129
T65407	HADHSC	0.860621867	0.006081129
T97615	CRP	0.860621867	0.006081129
H12312	TXK	0.884864736	0.003493723
R51835		0.884864736	0.003493723
AA033564	DGCR6	0.909107605	0.001751608
AA485355	SSI-1	0.909107605	0.001751608
AA497020	TRAP1	0.909107605	0.001751608
AA844930	GP2	0.909107605	0.001751608
H94949	TROAP	0.909107605	0.001751608
N53169	APOC3	0.909107605	0.001751608
R09503		0.909107605	0.001751608
AA143437	ARHD	0.94547191	0.000388927
AA452353	PPP2R3	0.94547191	0.000388927
AA485922	CPNE1	0.94547191	0.000388927
AA496334	DNM1	0.94547191	0.000388927
AA778663	TNFSF9	0.94547191	0.000388927
AA825491	IRF4	0.94547191	0.000388927
R39356	TP53	0.94547191	0.000388927
R50354	LIF	0.94547191	0.000388927
T50527	GYPB	0.94547191	0.000388927
R40127	RANBP1	0.951290253	0.000278474
AA668527	MADCAM1	0.963486282	0.000118396
AA130874	LOC51643	0.981836214	1.47783E-05
AA457114	TNFAIP2	0.981836214	1.47783E-05
AA486741	ASL	0.981836214	1.47783E-05
H69834	KNG	0.981836214	1.47783E-05
R62817	EPB72	0.981836214	1.47783E-05

List of under-expressed autoimmune genes displaying the highest correlation coefficient in parent offspring pairs. Only genes displaying correlations with greater than 99% significance are shown. Genes are denoted by accession number (ACC) and gene symbol (GENE). The corresponding Spearman correlation coefficient (Rs) and significance (p-value) are shown for each gene.

Table V. Most correlated over-expressed autoimmune signature genes

ACC	GENE	Rs	p-value
AA600189	ADAR	0.836378997	0.009651192
AA134555	HUMGT198A	0.848500432	0.00773527
AA253413	FRDA	0.848500432	0.00773527
AA293218	CSTF2	0.848500432	0.00773527
AA405800	DCI	0.848500432	0.00773527
AA443584	CD8A	0.848500432	0.00773527
AA443638	SNCG	0.848500432	0.00773527
AA481464	PPIB	0.848500432	0.00773527
AA485626	AHCY	0.848500432	0.00773527
AA700876	ORM1	0.848500432	0.00773527
AA701860	FST	0.848500432	0.00773527
AA625888	SPINK2	0.860621867	0.006081129
AA397813	CKS2	0.884864736	0.003493723
AA405987	GKP2	0.884864736	0.003493723
AA504625	KNSL1	0.884864736	0.003493723
AA705069	RARA	0.884864736	0.003493723
AA884167	ANXA13	0.884864736	0.003493723
N63968	POU6F1	0.884864736	0.003493723
AA458507	CSF3R	0.896986171	0.002526122
AA845167	ELA3	0.896986171	0.002526122
AA098896	ESRRA	0.909107605	0.001751608
AA447781	LUM	0.909107605	0.001751608
AA449982	CRYBA4	0.909107605	0.001751608
AA488979	MCRS1	0.909107605	0.001751608
AA521339	GARS	0.909107605	0.001751608
AA709271	NCAM2	0.909107605	0.001751608
AA777187	CYR61	0.909107605	0.001751608
AA815407	RYR1	0.909107605	0.001751608
AA866113	APBB2	0.909107605	0.001751608
AA633811	NFIL3	0.94547191	0.000388927
AA810225	GPR30	0.94547191	0.000388927
AA894557	CKB	0.94547191	0.000388927
W37769	CHGB	0.94547191	0.000388927
AA456830	DGKA	0.981836214	1.47783E-05
AA478273	APEX	0.981836214	1.47783E-05

List of over-expressed autoimmune genes displaying the highest correlation coefficient in parent offspring pairs. Only genes displaying correlations with greater than 99% significance are shown. Genes are denoted by accession number (ACC) and gene symbol (GENE). The corresponding Spearman correlation coefficient (Rs) and significance (p-value) are shown for each gene.
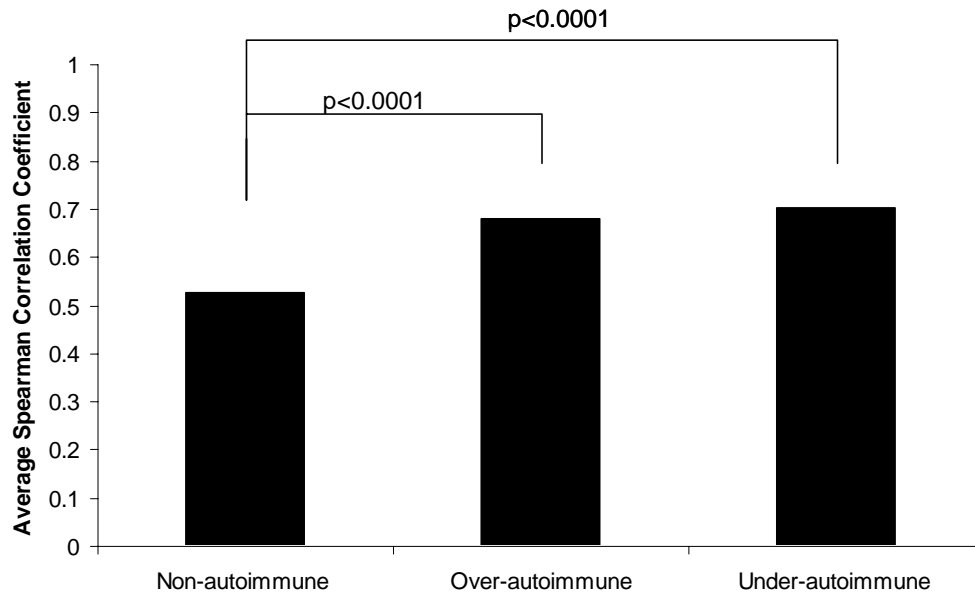
Fig. 19 <u>Comparison of parent-offspring average gene expression correlation coefficients</u>. The average spearman correlation coefficients from 8 parent-offspring pairing for the following categories: non- (Non-autoimmune), over- (Over-autoimmune), and under-expressed (Under-autoimmune) autoimmune signature genes. Significance was established using a permutation t-test.

statistical significance even with this small sample size (108 to 3, depending upon the ratio). The differences between the numbers of DEGs in the 4 random controls x 4 random controls group and the 4 random controls x 4 unrelated, unaffected relatives group achieved high statistical significance ($P < 10^{-4}$). These data argue that it is possible to use simple computational and statistical methods to analyze these complex data sets and that small sample sizes are sufficient to establish statistical confidence.

Table VI. Numbers of DEGs found by comparing random controls to random controls or by comparing random controls to random unaffected 1st degree relatives of individuals with autoimmune disease.

Groups:	Random Controls 4 x 4		Controls vs. Unaffected Family 4 x 4		Statistics χ^2 (P)	χ^2 (P)
DEGs	ALL	$P < 0.05$	ALL	$P < 0.05$	ALL	$P < 0.05$
3-fold	15	0	208	108	77 ($< 10^{-4}$)	104 ($< 10^{-4}$)
4-fold	10	0	78	38	32 ($< 10^{-4}$)	30 ($<10^{-4}$)
5-fold	5	0	45	21	21($< 10^{-4}$)	19 ($< 10^{-4}$)
10-fold	0	0	4	3	4 (NS)	2 (NS)

DEGs are defined as genes that are 3-, 4-, 5-, or 10-fold differentially expressed between groups. ALL is the number of total DEGs and $P < 0.05$ is the number of DEGs that achieve statistical significance in the groups. χ2 analysis was used to compare differences between the 4 x 4 random control group and the 4 x 4 controls versus unaffected family members group. P values were calculated by ANOVA.

Discussion

In this series of experiments, we addressed three possible explanations for the origin of our identified autoimmune gene expression signature. We first examined if the genes that are differentially expressed in autoimmune disease are also differentially expressed during a host immune response against a foreign antigen. We investigated the host immune response to foreign antigen by examining PBMC gene expression profiles at various time points after immunization with influenza vaccine. We found that the immune response was characterized by dynamic changes in gene expression that most likely reflected early signaling and proliferation events, with a shift toward lymphocyte effector functions at later time points. However, comparison of the differentially expressed immune response genes with our autoimmune signature genes revealed that there was no overlap between the two expression patterns. These results imply that our autoimmune signature is not modeled by a host immune response to a foreign antigen.

81

Many patients in our initial analysis of gene expression profiles in autoimmune disease were diagnosed with disease for an extended period of time. As an alternative explanation for our autoimmune gene expression signature, we examined whether the signature changed as a result of disease duration. Hierarchical clustering of gene expression profiles from ERA and RA patients revealed that there were distinguishable differences between the two patient populations. However, additional analysis of the most differentially expressed genes among the ERA and RA patients revealed that the ERA patients contained the autoimmune gene expression signature. Closer examination of the differentially expressed genes in the ERA and RA patients has identified clusters of genes that are ERA specific[190]. These ERA-specific differentially expressed genes allow the hierarchical clustering algorithms to distinguish between the ERA and RA groups. In contrast, the autoimmune gene expression signature does not change as a function of disease duration.

We also examined the gene expression signature of unaffected first-degree relatives of individuals with autoimmune disease. We used several different approaches to compare gene expression profiles between control individuals, unaffected family members, and autoimmune patients. Initial hierarchical clustering of gene expression profiles showed that unaffected family members more closely resembled unrelated autoimmune patients than control individuals. Examination of the DEGs in unaffected family members revealed that a significant portion of these genes were previously defined autoimmune genes. This finding was further confirmed by examining the contribution of the autoimmune signature to the DEGs in individual unaffected family members. Finally, correlation coefficients were derived for gene expression levels from

parent-offspring pairs. Pairwise analysis of the average correlation coefficients revealed that the autoimmune signature genes (both the under- and over-expressed) have significantly higher correlations compared to non-autoimmune genes. These results show that the autoimmune signature displays a high level of family resemblance, and supports the notion that this expression pattern is heritable.

Several recent studies have addressed the genetics of gene expression. Through the combined approach of microarray profiling and genetic linkage analysis, researchers have shown that gene expression patterns are heritable across a range of species [191-195]. In all systems studied, a mixture of *cis* and *trans* interactions between loci have been shown to contribute to the differential expression of genes.

Our current study reveals that all unaffected family members contain a significant number of differentially expressed autoimmune genes. This observation raises the question: how can a signature composed of over 200 differentially expressed autoimmune genes be inherited so readily? Studies examining the association between genetics and the differential expression of genes reveal that *trans* genetic regulation plays an important role in the differential expression of genes. For example, studies found that differential expression of ~ 40% genes is linked to just eight loci in budding yeast, suggesting that *trans* regulation plays an important role in the differential expression of a large number of genes [194]. Similarly, heterozygosity for mutant ATM in human lymphoblastoid cells results in the differential expression of 71 genes compared to controls [196]. Interestingly, examination of our expression signature reveals that approximately 10% of the differentially expressed genes appear to be either direct or secondary targets of p53 (presented in Chapter 3 of the dissertation). This finding leads us to believe that absence

of p53 as a transcription factor may contribute to a significant portion of our autoimmune signature.

The evidence presented here indicates that the autoimmune gene expression signature reflects a heritable trait rather than a disease process. Since screening patients with a range of autoimmune disorders identified this gene expression signature, we believe it reflects a common property of autoimmune disease. Family [56, 197] and genetic linkage studies[53, 55] suggest that there are common genetic regions that are linked to multiple autoimmune disorders. Since gene expression profiles reflect genotype, we believe that this autoimmune expression signature may arise through a heritable trait or traits that predispose individuals to autoimmune disease.

Complementing patient clinical phenotypes with gene expression profiles promises to add increased resolving power to genetic linkage studies. Since gene expression levels are closely related to the biochemical processes associated with disease, microarray profiles offer the most accurate phenotype by allowing discrimination based on subclinical variations[195]. Conversely, our studies have identified a common expression signature present among patients with a range of autoimmune disorders and unaffected family members. In this case, examination of expression data has revealed a common feature that would have otherwise been lost based on traditional clinical parameters. The identification of a possible heritable gene expression signature in the autoimmune patient population serves as an early first step towards understanding differential expression in complex pathological states.

CHAPTER IV

FUNCTIONAL LIABILITIES ASSOCIATED WITH THE AUTOIMMUNE SIGNATURE

Abstract

Patients with autoimmune disorders exhibit highly reproducible gene expression profiles in their peripheral blood mononuclear cells. This profile includes, at least in part, a collection of under-expressed genes that encode proteins that inhibit cell cycle progression and stimulate apoptosis. We wanted to determine if this gene expression profile confers a functional liability upon lymphocytes from autoimmune individuals and measured lymphocyte death after different treatments known to induce apoptosis. T lymphocytes from individuals with RA were resistant to γ-irradiation induced apoptosis, a process known to be dependent upon p53, but were not resistant to apoptosis induced by p53-independent mechanisms. Lymphocytes from RA patients had lower baseline levels of *TP53* mRNA and p53 protein and were deficient in their ability to increase p53 protein after γ-irradiation. A sub-group of RA patients had a second biochemical defect and expressed very low baseline levels of checkpoint kinase 2 mRNA and protein. We conclude that defects in expression of *TP53* mRNA and, in a sub-group, defects in expression of *CHK2* mRNA, lead to severe defects in apoptosis in RA. We hypothesize that this liability may contribute to autoimmunity.

Introduction

We have identified an autoimmune gene expression signature present in the PBMCs of patients with RA, MS, IDDM, and SLE. Many of the under-expressed autoimmune signature genes encode proteins involved in basic cellular homeostatic processes, such a proliferation and apoptosis. One of the most highly under-expressed genes in the signature is *TP53*, which encodes the tumor suppressor p53. p53 is known to play a key role in regulating cell cycle progression and apoptosis.

The tumor suppressor protein p53 is required for the γ-radiation induced apoptosis in thymocytes, TCR α/β T cells, and B lymphocytes in mice and humans[198, 199]. To determine if lack of *TP53* transcript levels in human autoimmune disease had functional consequence, we examined lymphocytes from RA patients for apoptosis defects to γ-radiation and p-53 independent pro-apoptotic agents. We also measured p53 baseline protein levels in PBMCs from RA patients and control individuals. To assess the function of the γ-radiation DNA damage response, we also measured p53 protein levels after γ-radiation. Additionally, we measured levels of upstream regulators of p53, such as Chk2 and ATM, and downstream effectors of p53 function, such as p21 and PUMA.

Our results demonstrate that there are multiple defects in the p53-dependent damage response pathway in lymphocytes from individuals with RA. First, all individuals have markedly lower levels of *TP53* transcript and p53 protein. In addition, defects in expression of *Chk2* message and Chk2 protein are also found, but these defects occur in only a portion of the RA population.

Materials and Methods

Patient populations

Control individuals (n=38), with no current chronic or acute infections or family history of autoimmunity, and individuals meeting the ACR clinical criteria for rheumatoid arthritis (n=46) were included in this study. Both control and autoimmune populations had approximate female/male ratios of 3:1. Age ranges (22-55 yrs) and racial distributions of both groups were similar. Human subject studies were approved by the Committee for the Protection of Human Subjects of the Vanderbilt University Institutional Review Board.

Sample processing and PBMC isolation

PBMC were isolated from heparinized blood by centrifugation on a Histopaque gradient (Sigma, St. Louis, MO). Isolated PBMCs were washed twice in HBSS.

Analysis of cell viability by flow cytometry

PBMC were suspended at 1×10^6 cells/ml in complete media (RPMI 1640 medium, 10% fetal calf serum, glutamine, and penicillin/streptomycin). Cells were untreated or treated with different apoptosis inducing agents: 10 gray ionizing γ-radiation, 5 μM dexamethasone, 1 μM staurosporine, or 100 J/m2 UV radiation. Pilot experiments with PBMCs from control individuals were used to establish optimal doses and time points for each apoptosis-inducing agents. At appropriate time points, cells were harvested and washed with FACS buffer (10% BSA in PBS with 0.2% sodium azide) and

incubated with FITC- and PE-labeled antibodies against CD3, CD4, CD14, CD 19, and CD45RO (Becton-Dickinson, San Jose, CA). Cells were washed and suspended in 500 µl FACS buffer supplemented with 2 µl of 0.5 µM 7-AAD (Molecular Probes, Eugene, OR) as a viability marker prior to flow cytometric analysis. Samples were analyzed for 1 minute at high flow rate. 7-AAD positive cells were excluded from analysis and the total numbers of remaining lymphocytes were tabulated to determine viability.

RNA isolation and quantitative PCR

Tri-Reagent (Molecular Research Ctr. Inc., Cincinnati Ohio) was used to isolate total RNA from PBMCs. 5 µg total RNA was reverse transcribed with Superscript II reverse transcriptase (Gibco BRL Life Technologies, Rockville, Maryland) to prepare cDNA. cDNA was also prepared from the HCT116 cell line to construct relative standard curves. *TP53* (*TP53* Forward Primer- 5' CTG TCC CTT CCC AGA AAA CCT 3', *TP53* Reverse Primer- 5' GGC TGT CCC AGA ATG CAA GA 3') and *GAPDH* (*GAPDH* Forward Primer- 5' CCA CCC ATG GCA AAT TCC 3', *GAPDH* Reverse Primer- 5' TGG GAT TTC CAT TGA TGA CAA) specific primers were used to amplify cDNA samples with SYBR-green PCR master mix (Applied Biosystems, Foster City, CA). Fluorescence was monitored using an ABI PRISM 7000 detector (Applied Biosystems, Foster City, CA). Relative quantities of *TP53* and *GAPDH* transcripts in control and patient samples were calculated using a standard curve derived from the HCT116 cell line.

Western blot analysis

Whole cell lysates were prepared in 1X PBS, 1% NP-40, 0.5% Na Deoxycholate, 0.1% SDS plus a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). For each sample, equal amounts of total protein were electrophoresed and transferred to Immobilon P membranes (Millipore, Bedford, MA). Membranes were blocked in a 5% non-fat milk, 0.1% Tween 20 in PBS and probed with combinations of the following primary antibodies diluted in 1% non-fat milk 0.1% Tween 20 PBS solution: p53, Chk2, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), p21 (Oncogene Research Products, San Diego, CA), PUMA (Abcam, Cambridge, MA)**,** PARP-1 (Cell Signaling Technologies Inc., Beverly, MA), and PCNA (Calbiochem, San Diego, CA). Membranes were washed three times with PBST, and probed with goat anti-mouse HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) in 1% non-fat milk PBST. Membranes were washed 3 X 20 minutes with PBST.   The ECL Plus chemiluminescence kit (Applied Biosciences, Foster City, CA) was used to visualize bands.

Western blot luminescent intensity analysis

Multiple exposures of films were captured using the Fluor-S-Max imaging system (BioRad, Hercules, CA). Both background chemiluminescence and chemiluminescent intensities for individual bands were measured. Background chemiluminescence was subtracted from all band intensities. Images of bands that were overexposed (as detected by Fluor-S-Max software suite) were excluded from analysis. Intensities of samples were normalized relative to baseline HCT-116 levels to make inter-blot comparisons and compensate for differences in exposure time.

RT-PCR and semi-quantitative PCR analysis

cDNA prepared from PBMC RNA from control individuals or RA patients was used for Chk2 (*Chk2* Forward Primer- 5' AGC AGT CTC ATG GCA GCA G 3', *Chk2* Reverse Primer- 5' AAT GCC TTA GGA TAA ACT GAC TGA 3') and GAPDH (*GAPDH* Forward Primer- 5' CCA CCC ATG GCA AAT TCC ATG GCA 3', *GAPDH* Reverse Primer- TCT AGA CGG CAG GTC AGG TCC ACC) amplifications. Thirty cycles were used for initial RT-PCR amplification of samples. Semi-quantitative PCR was performed on control and RA samples. PCR reactions were carried out as described above, with the exception that cDNA was serially diluted and amplifications were for thirty-five cycles.

Statistical analysis

Results are expressed as mean ± S.E.M. Groups were compared for statistical significance using student's T-test with a *P* value of <0.05 considered significant.

Results

Defects in γ-radiation induced apoptosis in RA PBMC

A unique gene expression profile found in the PBMCs of patients with autoimmune disease suggest that these lymphocytes may be defective in apoptosis [1, 179, 180]. To test this hypothesis, we performed viability studies on lymphocytes from RA and control individuals with a panel of agents that induce apoptosis, dexamethasone,

staurosporine, UV radiation, and γ radiation. PBMCs from control and RA individuals

were cultured in the presence or absence of agents that induce apoptosis. Cells were

harvested at the indicated time points, stained for surface markers (CD8, CD4, and

CD19), and incubated with the cell viability dye, 7-amino actinomycin D (7-AAD).

Viability was determined using flow cytometry by calculating the ratio of viable cells (7-

AAD negative) between the treated and untreated groups.

Each apoptosis-inducing agent caused apoptosis in lymphocytes (Fig. 1). We

found no difference in the level of apoptosis between RA and control lymphocytes after

treatment with dexamethasone, staurosporine or UV radiation. In contrast, lymphocytes

from RA patients were resistant to γ-radiation induced apoptosis relative to controls.

This defect in apoptosis was restricted to CD4+ and CD8+ T lymphocytes ($p<0.002$ and

$P <0.001$, respectively) (Fig. 20 A). A scatter plot of CD4+ T cell viability after γ-

radiation in different individuals demonstrated a highly homogeneous γ-radiation induced

apoptosis response in the control population, while there was substantial variability

among lymphocytes from RA patients (Fig. 20 B). Quantitative differences were

observed in the response to γ-radiation by memory and naive T cells. Naïve T cells

exhibited greater cell death than memory T cells (Figure 20 C). Both populations of

lymphocytes from individuals with RA were more resistant to apoptosis induced by γ-

radiation than were lymphocytes from controls. These results reveal a significant defect

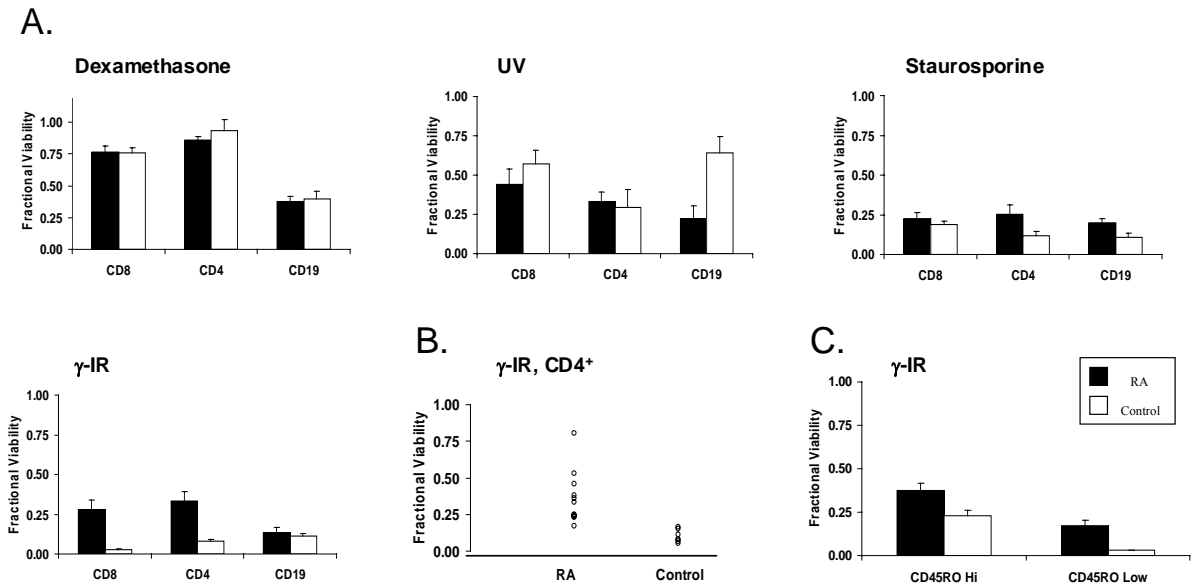in γ-radiation induced apoptosis in T lymphocytes from RA patients.

Fig. 20. Defects in γ-radiation mediated apoptosis in RA PBMCs. **A.** PBMCs isolated from control (n=6) or RA patients (n=9), were left untreated or challenged with one of the following apoptosis inducing agents: Dexamethasone (5μM), UV (100 J/m2), Staurosporine (1μM), or γ-radiation (10 gy). Cell viability was determined by flow cytometry after either three days (dexamethasone, UV, and γ-radiation) or one day (staurosporine). **B.** A representative scatter plot showing the viability of CD4+ lymphocytes from controls (n=6) and RA patients (n=12) three days after γ-radiation (10 gray). Data were collected and analyzed as described in A. **C.** Fractional viability of activated/memory (CD3+ CD45RO+) and naïve (CD3+ CD45RO-) T lymphocytes for controls (n=6) and RA patients (n=12) three days after γ-radiation (10 gray). Data were collected and analyzed as described in A.

Under-expression of TP53 RNA and p53 protein in RA PBMCs

Since γ-radiation induced apoptosis is dependent on functional p53 in thymocytes and peripheral lymphocytes[198, 199], low baseline *TP53* mRNA and p53 protein levels could contribute to defective apoptosis in RA. Results from previous microarray studies[1] revealed that *TP53* transcript levels were lower in RA patients than control individuals

(Fig. 21 A). *TP53* mRNA levels were relatively heterogeneous in control individuals but uniformly higher than in RA. Real-time RT-PCR analysis confirmed that *TP53* message levels were lower in individuals with RA than controls (Fig. 21 B). These results reveal that *TP53* mRNA was consistently under-expressed in the RA population relative to the control population.

To further characterize p53 in the RA population, we measured baseline p53 levels by western blotting. PBMCs from control individuals and RA patients were isolated, whole cell extracts prepared, electrophoresed, and immunoblotted with specific anti-p53 mAb. Luminescent intensities were measured using a Fluor-S-Max imaging system. A representative blot with a human cell line (HCT116), control individuals, and RA patients clearly demonstrated a marked reduction in p53 levels in RA patient PBMC in the resting state (Fig. 21 C). Compiled luminescence data revealed that baseline p53 levels were significantly lower (>10-fold) in the PBMC of individuals with RA compared to control individuals ($p<0.001$) (Fig. 21 D).

p53 levels after γ-radiation

In addition to measuring basal levels of p53 in the RA population, we wanted to examine p53 protein levels after γ-radiation. Under normal circumstances, p53 levels are relatively low in the resting state due to its rapid turnover through the ubiquitin-proteasome pathway[146]. In response to DNA damaging agents (i.e. γ-radiation), the N-terminal domain of p53 is phosphorylated, blocking protein turnover[200, 201]. As a result, levels of p53 increase markedly after γ-radiation. p53 acts as a transcription factor to

induce damage response target genes such as *CDKN1A* (p21)[154], *GADD45A*[161], *NOXA1*[164], and *BBC3* (PUMA)[165].

A.

TP53 mRNA Levels: Microarray

B.

TP53 mRNA Levels: Real-Time PCR

C.

D.

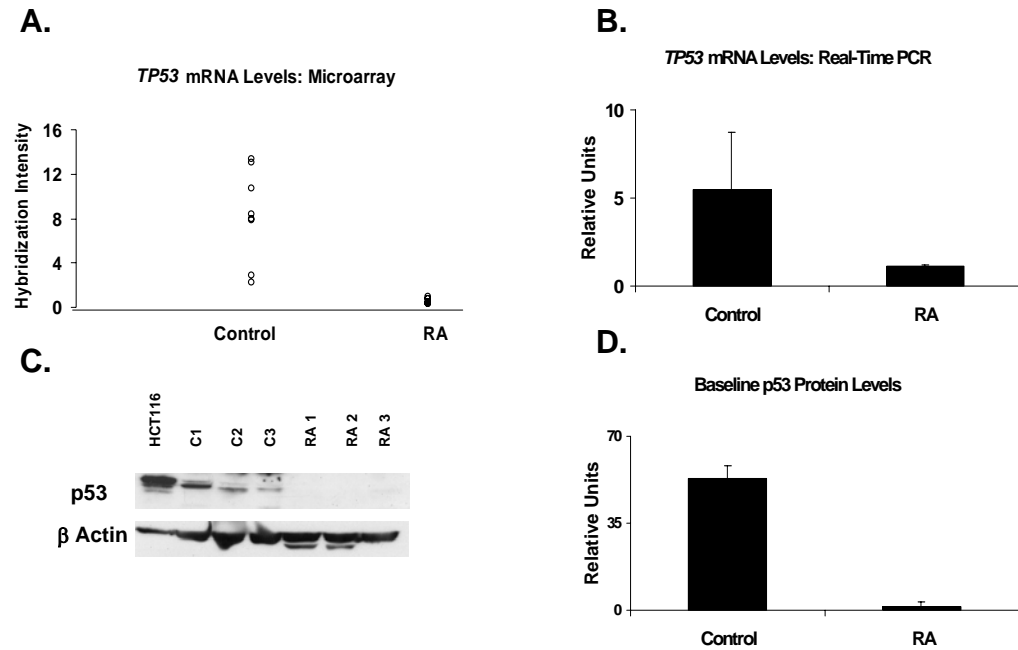**Baseline p53 Protein Levels**

Fig. 21. Baseline *TP53* transcript and p53 protein levels in RA PBMCs. **A.** A scatter plot displaying microarray results for *TP53* transcript levels in control (n=9) and RA PBMC (n=9) [1]. **B.** Analysis of *TP53* transcript levels in control (n=4) and RA PBMC (n=5). Real-time PCR was performed using *TP53* and *GAPDH* specific primers. Relative quantities of both *TP53* and *GAPDH* were determined by comparing threshold cycles to a standard curve constructed with serial dilutions of cDNA from a cell line standard. *TP53* was normalized to *GAPDH* and expressed as relative units. **C.** A representative immunoblot comparing baseline p53 levels in a cell line control (untreated HCT116), control (C1-3), and RA (RA 1-3) PBMC. β actin was included as a loading control. **D.** Compiled luminescence data for all individuals (control, n=9, RA, n=10) expressed in relative units. Background intensity was subtracted from all samples. Corrected intensities were normalized relative to the HCT116 cell line to permit comparisons among blots.

We measured increases in steady state levels of p53 in response to γ-radiation. We also measured p21 protein levels in the lysates, as an indicator of p53 transcriptional

activity. PBMCs from control individuals and RA patients were challenged with 10 gy γ-radiation or untreated. Twenty-four hours after challenge, whole cell extracts were prepared and immunoblotted for p53, p21, and β-actin. The high intensity of β-actin on the immunoblots made obtaining reproducible exposures between blots difficult, and thus it served as a control for equal protein loading within a blot rather than as a loading control between blots.   Instead, the HCT116 cell line was included as a positive control for p53-responsiveness to γ-radiation[202] and to serve as an indicator for exposure time and normalization in later quantitative analyses.

As expected, levels of p53 in control individuals (n=5) increased markedly in response to γ-radiation (Fig. 22 A). Increased levels of p21 confirmed downstream p53 transcriptional activity in response to γ-radiation.   The p53 dependent γ-radiation response in individuals with RA (n=10) differed from the control population. While some RA patients demonstrated increases in p53 protein after radiation, the most striking difference was that approximately half of the patients failed to increase p53 steady state levels after γ-radiation (Fig. 22). Based upon these results, RA patients were organized into two distinct groups. The γ-radiation non-responder (GNR) RA PBMC group failed to increase p53 levels in response to radiation (Fig. 22 B). The γ-radiation partial-responder (GPR) RA PBMC group displayed a modest increase in p53 levels, albeit to lower levels than controls (Fig. 22 C). In addition to the failure to increase levels of p53, GNR lymphocytes did not increase p21 levels after γ-radiation (Fig. 22 B). In contrast, GPR lymphocytes had increased p53 and p21 levels in response to γ-radiation, albeit to lower levels than controls (Fig. 22 C).

Two distinct defects in the p53 damage response in RA patients

To compare p53 levels in the different patient populations, we determined luminescent intensities from immunoblots (Fig. 23: control individuals (n=10), RA GNRs (n=7), and RA GPRs (n=5)) using the Fluor-S-Max imaging system. We examined protein levels for the untreated and irradiated conditions in control, GPR, and GNR groups. To accomplish this, we normalized luminescent intensities using the untreated HCT116 cell line present on all blots to make comparisons among blots. These results revealed that p53 levels in untreated and γ-irradiated cells from both the RA GNR and
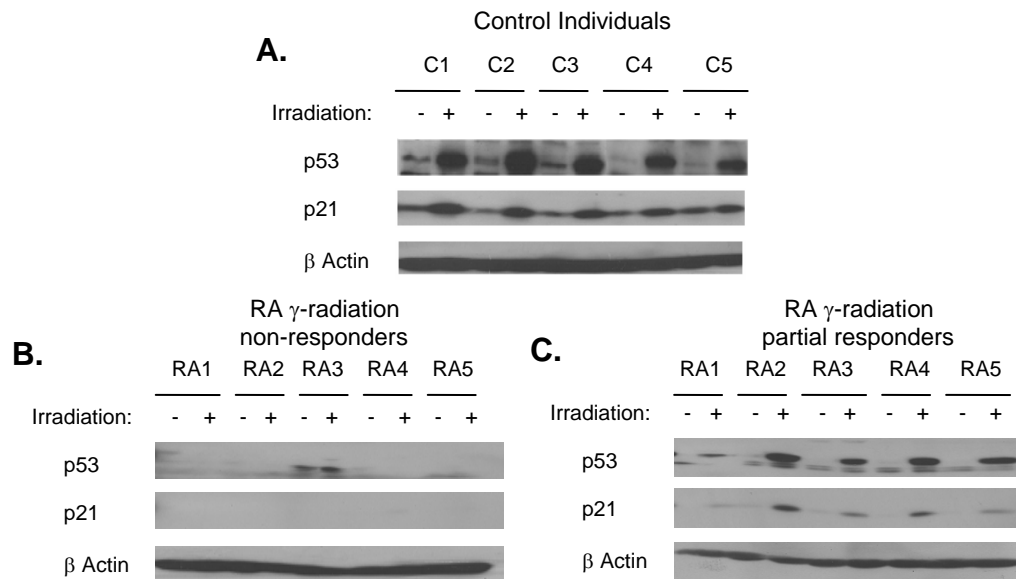

Fig. 22. Defects in p53 protein induction after γ-radiation in RA PBMC. PBMCs [control (n=5), RA (n=10)] were untreated (-) or treated (+) with 10 gy γ-radiation. Twenty-four hours later, whole cell extracts were prepared, electrophoresed, and immunoblotted for p53, p21, and β-actin. Due to high levels of β-actin in the samples, it was difficult to obtain equivalent exposures between immunoblots. Instead, in these figures β-actin serves to indicate equal protein loading of samples within a blot. RA PBMC were segregated into RA γ-radiation partial responders (GPRs, n=5) or RA γ-radiation non-responders (GNRs, n=7) based upon steady state p53 protein levels before and after radiation. Representative exposures are shown for each group: A) Control (C 1-5, n=5), B) RA GNRs (RA 1-5, n=5), and C) RA GPRs (RA 1-5, n=5).

GPR groups were significantly lower than in controls (p<0.001 for both groups) (Fig. 23 A). We believe that these lower p53 levels arise from lower *TP53* transcript levels in the RA patient population.

We also calculated γ-radiation induced fold induction of p53 and p21 for each individual in the control (n=10), GPR (n=5), and GNR (n=7) groups. Fold induction was calculated as the ratio of the luminescent intensity of protein bands between the γ-irradiated group and the untreated group. Fold induction was averaged for all individuals within the control, GPR, and GNR groups. Both control individuals and RA GPRs demonstrated increased induction of p53 in response to γ-radiation of comparable magnitude (Fig. 23 B). This implies that, despite low baseline p53 levels, the signaling events needed to increase p53 levels after γ-radiation are intact in GPRs. In contrast, the GNR group showed undetectable induction of p53 after γ-radiation, suggesting that additional defects are present in the p53-dependent damage response pathway in this group of RA patients. To a large degree, induction of p21 after γ-radiation mirrored the p53 results for the control, GPR, and GNR groups.

Defective induction of effectors of apoptosis in RA lymphocytes

p53 that accumulates after γ-radiation acts as a transcription factor to induce pro-apoptotic target genes[203, 204]. Since we observed defects in γ-radiation induced apoptosis, we wanted to determine if this was accompanied by defective induction of pro-apoptotic target genes. This was accomplished by immunoblotting whole cell lysates from representative untreated and γ-irradiated previously analyzed controls (n=2), RA GPRs (n=2), and RA GNRs (n=2). Protein extracts were analyzed for p53 levels to confirm
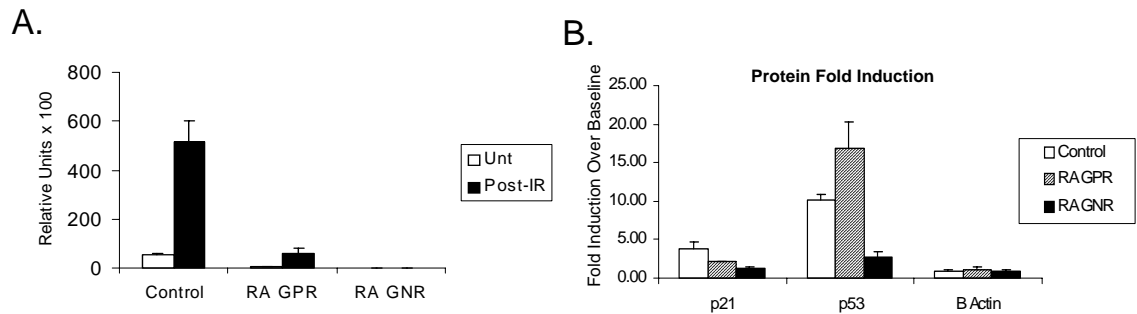
Fig. 23. Quantitative analysis of radiation response in control, GPR, and GNR groups. Luminescent intensities were compiled to permit quantitative comparisons among the groups previously analyzed as in Fig. 4.   A) Untreated and post-radiation p53 levels were determined for control (n=12), RA GPR (n=5), and RA GNR (n=7) groups. Untreated HCT116 cell lysates present on all blots were used for normalization, to correct for exposure time, and allow inter-blot comparisons. B) Average protein fold induction in response to γ-radiation was calculated for p53, p21, and β-actin among the different groups. Fold induction is defined as the ratio of γ-irradiated luminescent intensity versus the untreated luminescent intensity.

their partial responder/non-responder status. Extracts were also analyzed for "p53-upregulated mediator of apoptosis" (PUMA)[165, 166], poly (ADP-ribose) polymerase-1 (PARP-1) cleavage, a biochemical marker for apoptosis[205], PCNA as a measure of cellular proliferative status, and β-actin as a control. PCNA and β-actin levels were relatively consistent among samples and thus also served as protein loading controls (Fig. 24).

GNR RA patients, and to a more limited extent, GPR RA patients, demonstrated marked differences when compared to control individuals. Control individual lymphocytes exhibited increased p53 levels in response to γ-radiation with corresponding increases in PUMA and PARP-1 cleavage compared to untreated samples (Fig. 24). In

contrast, lymphocytes from GNR patients exhibited negligible increases in p53 levels and

PUMA after γ-radiation. GNR patients had lower levels of both full length and cleavage

forms of PARP-1 and did not display increased PARP-1 cleavage after γ-radiation. The

RA GPR patients exhibited variability in p53 levels and downstream effector function

after γ-radiation. One patient had low p53 levels, as well as negligible PUMA induction

and PARP-1 cleavage. The other RA patient had a somewhat higher response. These

results provide further evidence that downstream p53 apoptotic effector function is

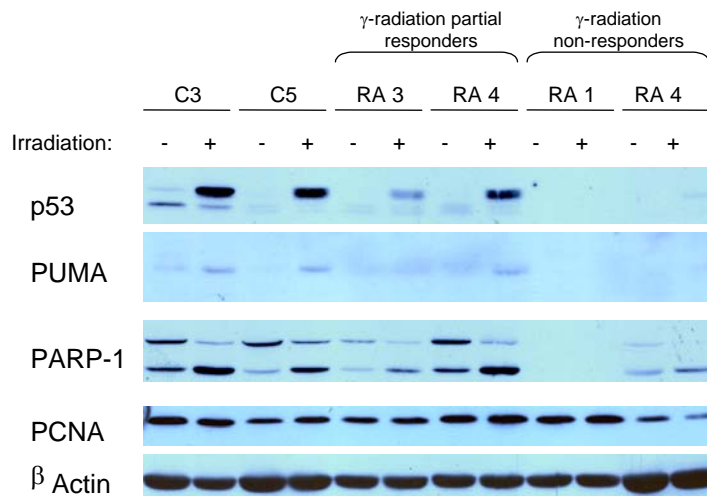compromised in lymphocytes of individuals with RA.

Fig. 24. Defective induction of p53 downstream effectors in RA PBMC. Whole cell extracts were from representative controls (n=2), GPRs (n=2), and GNRs (n=2) (see Fig. 4). Untreated (-) and γ-irradiated (+) samples were immunblotted twenty-four hours after challenge.   Lysates were probed for PARP-1, PCNA, PUMA, and β-actin.

Decreased Chk2 expression correlates with RA GNR status

Our quantitative analysis of relative protein levels and protein induction revealed that RA patients contain lower levels of p53 both before and after γ-radiation. However, the GNR group appeared to contain additional defects in their p53-dependent damage response that prevented them from increasing p53 levels after radiation. To explore potential causes for this additional defect, we examined Chk2, an upstream kinase that can phosphorylate p53[147] and prevent its ubiquitin-mediated degradation[150]. Extracts from previously identified RA GNR (n=5), RA GPR (n=5), and control individuals (n=5) were reanalyzed for Chk2. All control PBMCs contained comparable levels of Chk2 (Fig. 25 A). Levels of Chk2 in control lymphocytes decreased after γ-radiation, an observation that has been made in several other cell lines and is attributable to p53 activity[206]. RA GNR PBMC had uniformly low levels of Chk2 (Fig. 25 B), while RA GPR PBMC contained levels of Chk2 equivalent to control PBMC (Fig. 25 C).

To determine if low Chk2 levels in RA patients correlated with low transcript levels of *CHK2*, we analyzed PBMC RNA derived from previously unexamined RA patients and controls (Fig. 25 D). A portion of RA patients had *CHK2* transcript levels similar to controls and a portion had very low CHK2 transcript levels. Semi-quantitative RT-PCR analysis was used to more accurately determine relative levels of transcript in a representative control sample and RA sample with very low *CHK2* transcript abundance. This confirmed that *CHK2* transcript levels were substantially lower in a subset of RA patients compared to controls (Fig. 25 D). We believe that under-expression of *CHK2* mRNA may account, at least in part, for the GNR/GPR γ-radiation response status in RA lymphocytes.

100

Many p53-regulated genes are differentially expressed in PBMC from individuals with RA

Above studies focused on p53 protein, *TP53* transcript levels, and a few well-characterized downstream transcriptional targets of p53. We also wanted to determine whether additional known p53-regulated genes were differentially expressed in RA compared to control PBMCs. To do so, we compared results from our microarray analysis of differential gene expression between control and RA PBMC[1] to microarray analysis of differential gene expression between cell lines that have or do not have functional p53[207, 208]. Genes identified in these studies include both known direct transcriptional targets of p53 (p21, PUMA) as well as genes that may be direct transcriptional targets of p53 or may be differentially expressed as a result of secondary effects of the presence or absence of p53. We examined our microarray data to determine expression levels of genes identified in cell line studies with altered expression profiles in the presence or absence of p53 (Table VII). All genes that were over-expressed in p53 positive cell lines were under-expressed in RA PBMC. Conversely, all genes that were under-expressed p53 positive cell lines were over-expressed in RA PBMC. These results further support our hypothesis that defects in p53 expression and induction in PBMC of individuals with RA may account for a significant portion of the unique gene expression profile observed in these affected individuals.
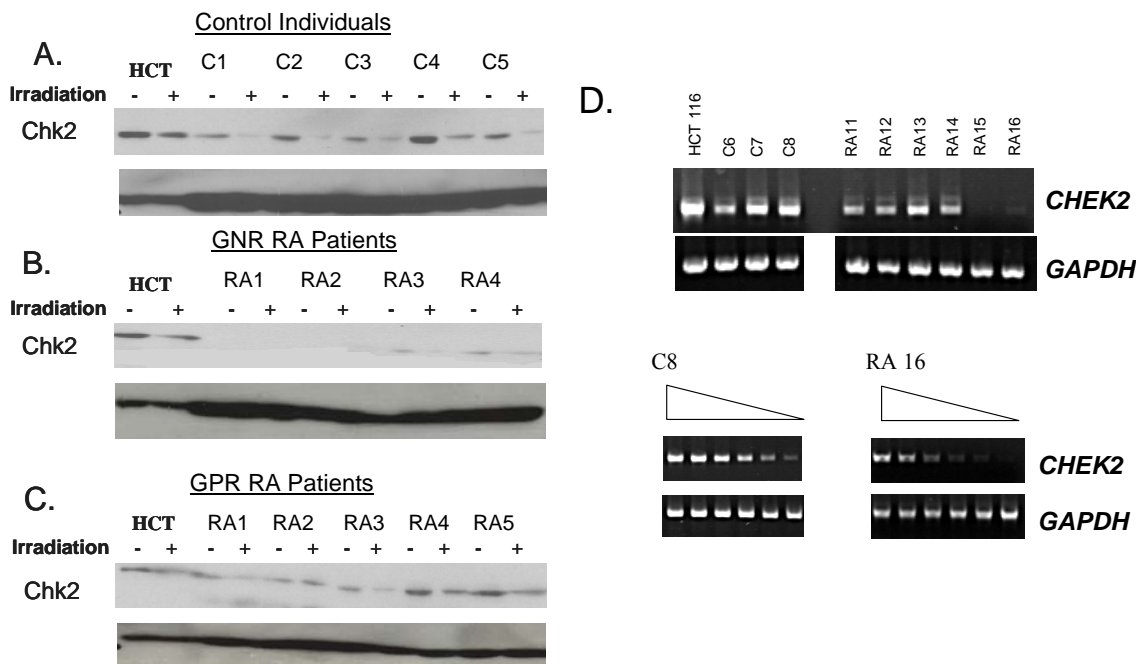
Fig. 25. <u>RA PBMCs have low levels of Chk2 protein and *Chk2* transcript</u>. Whole cell extracts from the same patient lysates (Untreated -, γ-irradiated +) in Fig. 4 were immunoblotted for Chk2 protein. A) Immunoblots of controls (n=5). B) Immunoblots of RA GNR PBMC (n=4). C) Immunoblots of RA GPR PBMC who stabilized p53 (n=5).   D) RT-PCR was performed on cDNA prepared from total RNA for previously unexamined control (n=3) and RA (n=6) PBMC. *CHK2* and *GAPDH* specific primers were used for amplification. Top panel shows amplification results after thirty cycles. Thirty-five cycle amplifications were performed on serial dilutions of cDNA for semi-quantitative PCR. Results for control (C8) and RA PBMC with low *CHK2* transcript levels (RA16) are shown in the paired lower figures.

Table VII. Expression levels of p53-regulated genes

p53-inducible genes		p53-repressible genes	
fold reduced		fold increased	
Gene Title	RA	Gene Title	RA
transglutaminase	-11.1	adenosine deaminase	3.8
E-cadherin	-6.3	DAG kinase	4.1
CDKN1B, p27	-5.0	fibronectin 1	4.8
caspase 6	-5.3	IL-8	5.4
myosin 1B	-5.6	EGF receptor	3.6
epoxide hydrolase	-8.3	cyclin A2	3.4
RAD52 homolog	-4.8	stathmin	2.9
Ubiquinone	-4.5	NF-IL3	3.1
APAF1	-4.3	RAD51 homolog	2.9
GADD45A	-3.2	carboxypeptidase	3.5
PIG11	-2.6	CDC28 PK2	2.4
c-fos	-1.8	COP9	1.9
endoglin	-2.5	cyclin E1	2.5
BTG family, 2	-2.1	CDC6 homolog	1.9
tyrosinase	-2.1	galectin 3	2.5
		SGK	2.1

Compiled data from previous microarray experiments[1]. p53-responsive genes[207, 208] were selected from our microarray data. Fold induction values represent the ratio of the average expression level of the RA population over the average expression intensity of controls. Under-expressed fold induction values are shown as the negative inverse of the ratio.

Discussion

In this study, we found uniform defects in the p53 damage response pathway in lymphocytes from patients with RA. T lymphocytes from patients diagnosed with RA are significantly more resistant to γ-radiation induced cell death than control individuals. In contrast, lymphocytes from RA patients do not display defects in p53-independent modes of apoptosis. Both p53 protein and *TP53* mRNA baseline levels are substantially reduced in RA PBMCs, providing a possible explanation for defects in γ-radiation

induced apoptosis. p21 (a cyclin dependent kinase inhibitor) levels are also substantially lower in RA patients. Furthermore, half the RA patients fail to exhibit increased steady state levels of p53 after γ-radiation. We have classified the RA patients that exhibit relatively normal increases in p53 protein in response to γ-radiation "GPRs", while patients that do not demonstrate increased p53 protein in response to γ-radiation are termed "GNRs". These GNR RA patients also contain negligible levels of Chk2, an upstream kinase that phosphorylates p53[147] and may prevent ubiquitin-mediated degradation of p53[209] after DNA damage.

Defects in lymphocyte apoptosis are hypothesized to contribute to development of autoimmunity. Some of the best support for this theory comes from observations of *lpr* or *gld* mutations (mutations in FAS or FASL, respectively) on the MRL murine background[182]. These mice develop autoantibodies and succumb to fatal glomerulonephritis. With the exception of the rare Autoimmune Lymphoproliferative Syndrome (ALPS)[183], efforts to identify defects in FAS or FASL in more common human autoimmune disorders have been relatively unsuccessful[210]. Our results clearly demonstrate that there are uniform defects in apoptosis in lymphocytes from individuals with RA, but that these defects are present in the p53 damage response pathway.

Other investigators have addressed the role of p53 in RA. The majority of these studies have focused on the synovium. It has been proposed that high levels of oxidative stress in rheumatoid synovium may cause somatic mutations in the *TP53* gene[211]. Presumably mutant synovial cell p53 allows pathological proliferation of synovial cells that may lead to joint destruction and other clinical manifestations of RA. Alternatively, it has been proposed that the cytokine, macrophage migratory inhibitory factor (MIF),

may cause decreased cellular p53 levels[212, 213]. MIF lowers endogenous p53 levels both *in vivo* and *in vitro*, and high levels of MIF could contribute to synovial proliferation and pannus formation. Although these results specifically address the impact of MIF on the synovium, serum levels of MIF are also elevated in RA patients compared to controls[214]. Elevated MIF levels may contribute to the under-expression of p53 in the PBMC of RA patients. However, our results clearly show that T lymphocytes, representing 80% of our PBMC preparations, are defective in p53-mediated apoptosis and T lymphocytes are not known to respond to MIF.

There is also evidence that p53 maintains tolerance in lymphocytes by regulating cell cycle progression. Human T lymphocytes from peripheral blood or intestinal lamina propria show an inverse relationship between p53 levels and the rate of progression through the cell cycle[215]. Cell cycle delays mediated by elevated levels of p53 in lamina propria T lymphocytes may be a mechanism that maintains tolerance against environmental antigens. Preliminary studies by Morand et al., using an antigen-induced arthritis model on a p53 -/- background, reveal that T lymphocytes proliferate more readily and produce more IFN-γ in the absence of p53[216]. Similar results in models of collagen-induced arthritis[217], suggest that inflammatory responses may be exacerbated in the absence of p53.

Lymphocytes from healthy individuals with the HLA-DRB1∗04 allele[218] and rheumatoid arthritis patients[117] show signs of inappropriate aging as measured by telomeric shortening. T lymphocytes from RA patients also proliferate less readily (compared to controls) in response to stimulation with anti-CD3 or recall antigens. Similar observations have been made in Trp53 -/- mice[219]. These studies revealed no

defects in lymphocyte development. Rather, lymphocytes from p53 -/- mice exhibited signs of accelerated aging and unresponsiveness to TCR stimuli. Recent studies also demonstrate that p53 is a necessary element of cellular senescence[220-222]. Therefore, we speculate that chronic under-expression of *TP53* mRNA and p53 protein and perhaps *CHK2* mRNA and Chk2 protein may explain accelerated aging and lack of lymphocyte responsiveness to TCR signals found in RA patients.

We found that approximately half the RA patients failed to demonstrate increased steady-state levels of p53 in response to γ-radiation, and that this correlated with depressed levels of Chk2. *CHK2* mRNA levels are almost absent in this subset of RA patients. Chk2 is believed to be an upstream regulator of p53 stability in response to certain types of DNA damage[201, 209], although there is now debate in the field about its absolute necessity for p53 stabilization[151, 223]. Studies in Chk2 -/- mice reveal no abnormalities in lymphocyte development, however nothing more is known about the role of Chk2 in the immune system.

Defects in downstream p53 target genes may also play a role in promoting systemic autoimmunity. Microarray results and our studies presented here demonstrate that many p53 downstream gene targets are dysregulated in a fashion consistent with p53 dysfunction. For example, p21, a downstream cyclin dependent kinase inhibitor and transcriptional target of p53, is also under-expressed in RA patients. Although controversial[224], studies have documented increased autoantibody production, glomerulonephritis, and mortality in p21 -/- female mice[225]. T lymphocytes from these mice are hyperproliferative when cultured with IL-2 after activation compared to wild type littermates. Additional studies examining GADD45A, another downstream target of

p53 effector function, link this gene to systemic autoimmunity and abnormalities in T lymphocyte function[226]. p21 -/- GADD45A -/- mice exhibit aggressive autoimmunity comparable to *lpr*/MRL mice. These observations, combined with our results in the RA patient population, indicate that defects in the expression of molecules in the DNA damage response pathway might play a role in autoimmune pathogenesis. These proteins may also represent new targets for therapeutic approaches. It might be possible to design therapies to either correct defects in the p53 damage response pathway or to inhibit downstream effectors that are normally inhibited by damage response proteins such as p53, p21 or GADD45A.

CHAPTER V

OVERVIEW

The underlying disease mechanisms for most autoimmune disorders remain elusive. In large part, this difficulty arises from the fact that there is considerable heterogeneity in the symptoms and presentation of individual autoimmune disease, and additional heterogeneity among these disorders. We initiated this set of experiments with the ultimate goal of identifying gene expression patterns that either characterized an individual disease or were shared among disorders.

To accomplish these ends, we utilized microarrays to obtain the gene expression profiles from control individuals and patients with a range of autoimmune disorders. Gene expression profiling allowed us to screen over 4,000 variables in a single experiment. Since gene expression more closely reflects cellular biochemistry than is possible by gross clinical measurements, patterns revealed through this type of analysis might reflect cellular liabilities. Potential liabilities can be reconfirmed by measuring protein and transcript levels. However, to determine if the defects have a biological impact, the ultimate test of a functional liability requires examination of the known biochemistry of the target(s) of interest. In this set of studies we have attempted to address all of these issues through our identification of novel defects in the lymphocytes of patients with RA.

In the first chapter of the dissertation, we compared the gene expression profiles of control individuals with patients suffering from a range of autoimmune disorders (RA, SLE, IDDM, and MS). Initial unsupervised clustering revealed that gene expression profiles from control individuals were distinct from profiles of patients with autoimmune disorders. Furthermore, the gene expression profiles from the autoimmune patients did not segregate into disease specific branches, suggesting that the gene expression profiles of autoimmune patients were relatively similar. Closer examination of the clustered differentially expressed genes confirmed that patients with autoimmune disease shared a common gene expression pattern that we refer to as the autoimmune signature. While the over-expressed cluster of autoimmune genes was comprised of genes with relatively heterogeneous functions, we found that many of under-expressed autoimmune genes encode proteins that regulate cell cycle progression and apoptosis.

The identification of an autoimmune gene expression signature promises to have direct clinical applicability. Early diagnosis and treatment of many autoimmune disorders can prevent irreversible end-organ damage. For example, as mentioned in the introduction much of the erosive joint damage in RA occurs within the first 5-10 years of disease. One study shows the majority of damage takes place as early as two years after symptomatic onset of disease. Early intervention with DMARDs has proven effective at slowing or preventing the damage. However, early diagnosis is difficult due to the range of symptoms present in patients. It may take from six months to one year to establish a definitive diagnosis. Our ability to detect an unambiguous gene expression pattern in the peripheral blood of patients with a range of autoimmune disorders promises to have applicability as a rapid diagnostic tool in the clinic.

In the second chapter of the dissertation, we wanted to determine the origin of the autoimmune signature. Since autoimmune disorders are believed to arise from an immune response against self, we considered the hypothesis that the autoimmune signature reflected an immune response. To address this hypothesis, we modeled an *in vivo* immune response by immunizing healthy individuals with inactivated influenza vaccine. Ultimately, we found that there was no overlap between our autoimmune signature and the genes differentially expressed during an immune response.

In addition to the immune response, we also considered the possibility that disease duration might contribute to the autoimmune signature. In this sense, our initial selection of autoimmune patients was biased, since many of the patients had established disease. To address this possibility, we compared expression patterns in established RA patients (average disease duration: 10 ± 3 years) to early RA patients (average disease duration: 1 year ± 0.2 years). While the early RA patients did contain unique clusters of differentially expressed genes, they also contained the autoimmune signature in its entirety. This suggests that our autoimmune signature does not reflect disease duration.

Both epidemiologic and genetic linkage studies support an underlying genetic component to autoimmunity. In light of this evidence, we hypothesized that our autoimmune signature reflected family resemblance between parent and offspring. To test this hypothesis, we examined gene expression profiles in unaffected first degree relatives and compared them to profiles from control individuals and patients with established autoimmune disease. Initial hierarchical clustering comparisons revealed that the unaffected family members were distinct from control individuals, and instead more closely resembled autoimmune patients. Closer examination of the gene expression

110

profiles in unaffected family members revealed that a majority of the differentially expressed genes were indeed autoimmune signature genes. Studies conducted in parent-offspring pairs revealed that the expression levels of the autoimmune genes displayed a high degree of family resemblance. These data support the notion that our observed autoimmune signature reflects a heritable trait or traits.

In the third chapter of this dissertation, we attempted to determine if the autoimmune signature is associated with a functional liability. As mentioned previously, numerous under-expressed genes in the autoimmune signature encode proteins involved in basic cellular processes such as cell cycle progression and apoptosis. Closer examination of the signature revealed that approximately 10% of the differentially expressed genes have been previously shown to be primary or secondary targets of p53. Additionally, *TP53*, the gene that encodes p53, is one of the most consistently under-expressed genes in the autoimmune signature.

Based on this information, we hypothesized that p53 dependent pathways would be defective in PBMCs from patients with autoimmune disorders. To test this, we utilized γ-radiation to examine the p53-dependent damage response in PBMCs from patients with RA. Initial baseline *TP53* transcript and p53 protein measurements confirmed that p53 levels were lower in RA PBMCs compared to control individuals. We found that T lymphocytes from RA patients were resistant to γ-radiation induced apoptosis compared to control lymphocytes. In order to mediate apoptosis, p53 protein levels increase after DNA damage, thus allowing it to act as a transcription factor for genes that can initiate cell cycle arrest or apoptosis. Examination of PBMC p53 protein levels after γ-radiation challenge revealed that post-IR p53 protein levels were

universally lower in RA lymphocytes compared to controls. We also found that the downstream effectors of p53 activity, such as p21 and PUMA, were also lower in the RA patient population, providing a plausible explanation for the decreases in apoptosis. However, the most striking observation was that approximately half of RA patient did not demonstrate any appreciable increase in p53 protein levels, and correspondingly did not display any downstream effector function.

p53 protein increases in response to DNA damage requires the function of upstream damage response signal transducers. Since approximately half of the RA patients did not display significant increases in p53 protein, we examined Chk2, a protein kinase shown to phosphorylate and block p53 turnover in some cell culture and animal models. We found that the patients who did not display increases in post-IR p53 protein levels also did not have detectable levels of Chk2 protein when compared to controls and responsive RA patients.

Based on these results, we conclude that observations based on our initial microarray analysis of autoimmune patients have revealed functional defects in RA PBMCs. Our functional analysis has uncovered at least two defects in the p53-dependent damage response pathway in RA patients: one defect associated with low p53 transcript and protein levels, and a second defect, present in a subset of the RA patients, associated with the inability to increase p53 protein levels and the absence of Chk2 protein. In the course of these studies, we have also documented abnormal PARP protein levels in some of the RA patients, suggesting that additional defects may be additionally be present in other non-p53 dependent DNA repair pathways[227]. Polymorphisms in the PARP-1 promoter have been associated with RA[228]. Perhaps these RA associated polymorphism

might explain the low level expression of PARP-1 in some of the RA patients. Regardless, further studies will have to be conducted in RA lymphocytes to gain a greater appreciation of the extent of defective DNA damage and repair responses in the RA population.

Our identification of these functional defects raises several important questions that have yet to be answered. One of the most interesting questions posed by the studies is whether defects in the cellular DNA damage response can contribute to the development of autoimmunity, or simply reflects an endpoint of autoimmunity. Relatively little is known about the role of these pathways in the immune response. However, there is evidence that p53 could play an immunomodulatory role. For example, while p53 -/- mice do not develop spontaneous autoimmunity, both the AIA and CIA murine models of autoimmunity are aggravated when performed on a p53 -/- background. What role p53 plays in regulating the immune system is currently unknown, but given p53's central role in mediating cell cycle arrest and apoptosis, numerous possibilities exist.

The cause of decreased p53 protein and transcript levels in the PBMC of RA patients is also unknown. It is possible that there are RA-associated alterations in the promoter region of *TP53* could alter the expression level of p53. Alternatively, abnormalities in p53 levels may reflect the autoimmune environment rather than a cell intrinsic defect. The identification of pro-inflammatory cytokines such as MIF, which are capable of altering p53 functional activity and RNA/protein levels, could an alternate explanation for our observations. Regardless, further experiments will have to be conducted to clarify our observations.

CHAPTER VI

REVIEW: IMPLICATIONS OF MICROARRAY SIGNATURES IN THE CONTEXT OF RHEUMATOID ARTHRITIS

Introduction: Challenges to the Classical View of Autoimmunity

Basic concepts in immunology have shaped current theories regarding mechanisms responsible for autoimmunity. One of the fundamental principles arising from decades of research is that the immune system is able to discriminate self from non-self. Due to the random nature of VDJ recombination during the generation of a polyclonal lymphocyte repertoire, the receptors generated may be viable, nonfunctional, or auto-reactive. However, despite positive and negative selection during thymocyte development, auto-reactive T lymphocytes persist in periphery. Mechanisms such as molecular mimicry have been proposed to result in cross reactivity against self-antigen, culminating in the activation of auto-reactive lymphocytes in an antigen specific manner. The end result of such a process would be organ specific autoimmunity. While there is evidence in animal models to support this notion, findings from patients with rheumatoid arthritis (RA) raise questions regarding the role of a single, dominant antigen driven immune response as an inciting cause for autoimmunity.

Examination of synovial T lymphocyte TCR (T cell receptor) sequences for evidence of antigen driven clonal expansion in RA instead reveal oligoclonal T lymphocyte expansion[229-231] with no common expanded TCR sequence identified among RA patients[108].   Oligoclonal T lymphocyte expansions are not restricted solely to

synovium of patients with RA, but are also present in the periphery[116] in both RA patients and their unaffected siblings[232]. These findings appear related to elevated levels of lymphocyte proliferation in the RA population. This is supported by an increased number of RA T lymphocytes in the S-G2/M phase of the cell cycle[116] age inappropriate telomere shortening[116,118], and decreased TCR Recombination Excision Circles (TRECs) positive CD4$^+$ T lymphocytes when compared to age matched control lymphocytes[117]. These findings appear to be independent of disease duration or severity, but rather reflect a genetic predisposition. In particular, HLA-DR4 positivity was identified as a key genetic determinant for telomere shortening[218].

In addition to evidence of increased proliferation in the periphery, functional changes are present in lymphocytes from patients with RA that suggest immunosenescence. RA patients have increased levels of CD28 ^{null} T lymphocytes (a marker of T lymphocyte immunosenescence)[233] which display abnormal function such as the presence of killer immunoglobulin-like receptor (KIR) surface markers, deregulated production of IFN-γ[234-236] and resistance to apoptosis[237,238]. Additionally, CD4$^+$ lymphocytes from RA patients proliferate less readily *ex vivo* when stimulated with plate bound anti-CD3 for extended periods of time (up to 6-7 weeks).

These collective observations have lead to an alternate model to explain origins of RA based upon the concept of homeostatic proliferation (Fig 26). In this model, lymphopenia followed by repeated rounds of compensatory homeostatic expansion ultimately gives rise to immunosenescence. Immunosenescence results in a deregulated immune system, which could ultimately promote an autoimmune state[119,239]. Evidence from model systems lends support to aspects of this model. Lymphopenia in model

systems, such as thymectomy in the newborn mice, give rise to autoimmunity[240]. Several prior studies focusing on homeostatic lymphocyte expansion indicate that T cell proliferation in the setting of lymphopenia is dependent upon competition for self-antigen/MHC complexes resulting in expansion of autoreative clones[241-244]. In support of these findings, lymphopenia in the NOD mouse is accompanied by homeostatic expansion of autoreactive CD4 T lymphocytes[245].
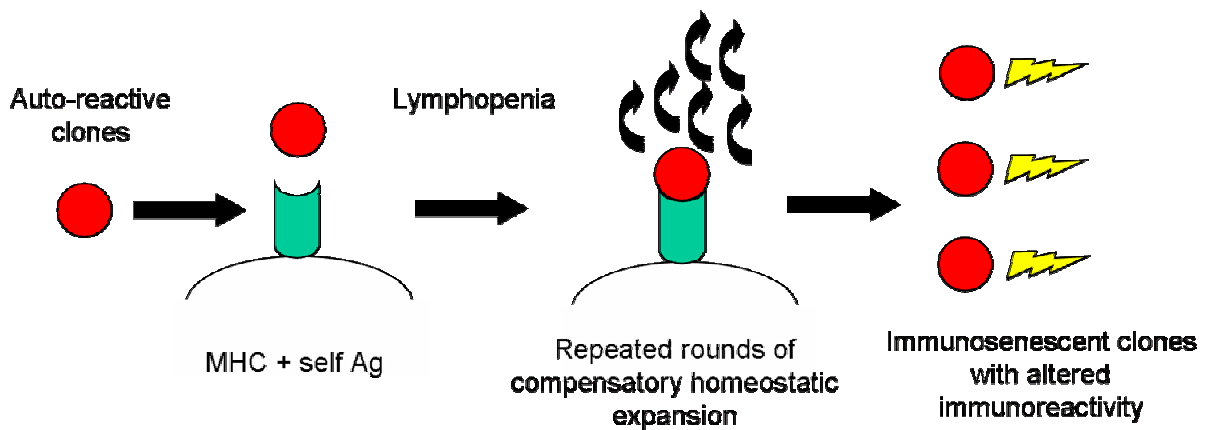

Fig. 26. <u>Homeostatic Expansion in RA</u>. Under conditions of lymphopenia, autoreactive clones undergo compensatory homeostatic expansion. This process is dependent upon self-antigen in the context of MHC. Repeated rounds of proliferation could ultimately lead to a state of immunosenescence. The expanded populations of auto-reactive immunosenescent lymphocytes are functionally abnormal, and may promote autoimmunity.

Parallels Between Cellular And Gene Expression Profile Studies

Several intriguing parallels exist between the extensive body of cellular data gathered in the RA patient population and recent gene expression profile studies in patients with autoimmune disease. As mentioned previously, one paradigm regarding the origin of autoimmunity suggests that after an initial break in the mechanisms of tolerance,

the immune system mounts an antigen driven immune response against self. If this theory is true, there should be some degree of resemblance between a model *in vivo* immune response and ongoing autoimmune disease.

In an effort to test this hypothesis, gene expression profiles from patients with a range of autoimmune disorders (RA, SLE, IDDM, and SLE) were compared to those of healthy control individuals before and at various time points after immunization with inactivated influenza vaccine. These studies revealed no overlap between gene expression profiles from healthy control individuals (either before or after immunization) relative to patients with autoimmune disease[1]. Interestingly, patients with autoimmune disease had relatively similar profiles and shared a common set of under-expressed genes, dubbed "autoimmune signature genes". Many of these downregulated genes encode proteins involved in apoptosis and cell cycle regulation, such as p53, BRCA1, GADD45A, and p21[1].

Like observations of T lymphocyte oligoclonality, decreased TREC levels, and telomere erosion in RA, the observed autoimmune gene expression signature appears to be independent of disease severity or duration. The initial autoimmune signature was derived from patients with a range of disease severity, some with well-controlled rheumatoid arthritis, while others suffered from flares in the disease. Thus, it seems unlikely that the gene expression signatures observed could be explained by disease activity. In an effort to determine if the previously identified autoimmune signature was a reflection of disease activity, signatures from "early" RA patients (patients diagnosed with RA for less than two years duration) were compared to profiles from patients with established RA (average duration of ten years). Both early and established RA patients

117

contained the autoimmune signature in its entirety, discounting the notion that disease duration accounted for the gene expression pattern[190].

While disease duration and severity do not account for the cellular abnormalities seen in RA, studies in unaffected individuals indicate that there might be a genetic predisposition for these phenomena. Similarly, studies indicate a possible genetic contribution to the autoimmune signature. This notion is supported by comparisons of gene expression profiles from autoimmune patients with profiles from unaffected first-degree relatives of autoimmune patients. These studies revealed that the autoimmune signatures were present in unaffected individuals, thus suggesting a heritable component[246]. Further work will be necessary to determine the precise genetic determinants necessary for conferring the observed gene expression pattern.

Numerous under-expressed genes in the autoimmune signature encode proteins involved in basic cellular processes such as cell cycle progression and apoptosis. In particular, gene expression profiles revealed that genes such as p53, BRCA1, p21, and GADD45 are under-expressed in the autoimmune signature[1]. Confirmatory studies demonstrate defects in p53-dependent pathways in lymphocytes from patients with RA. Levels of p53 and p21 proteins at baseline were lower in the RA patient population compared to control subjects[247]. T lymphocytes from individuals with RA were resistant to γ-irradiation induced apoptosis, a process known to be dependent upon p53, but were not resistant to apoptosis induced by p53-independent mechanisms (such as staurosporine, UV radiation, and dexamethasone). Additionally, in response to γ-irradiation challenge, downstream targets of normally induced by p53 were either absent or only weakly induced[247].

118

Role for the Autoimmune Signature in the Context of Homeostatic Expansion

The number of parallels between the cellular findings and microarray results in patients with RA indicates that these results are inter-related. We propose that the autoimmune signature acts as a catalyst to drive autoimmunity by contributing to homeostatic proliferation and alterations in lymphocyte function. In modification of the proposed model of homeostatic expansion, absence of cell cycle regulation would contribute to the abnormal proliferation observed in the periphery of patients with RA. Furthermore, loss of the damage response pathway components, such as p53, results in alterations in lymphocyte effector functions, such as cytokine secretion and apoptosis (Fig. 27).

Several lines of evidence support the notion that decreased levels of these proliferation control and damage response genes contribute to autoimmunity. Studies in an antigen-induced arthritis model on a p53 -/- background reveal that T lymphocytes proliferate more readily and produce more IFN-γ in the absence of p53(216). Similar results in models of collagen-induced arthritis, suggest that inflammatory responses may be exacerbated in the absence of p53[217]. Lymphocytes from p53 -/- mice exhibit signs of accelerated aging and unresponsiveness to TCR stimuli, a finding highly reminiscent of the immunosenescence found in the lymphocytes of RA patients[219]. Additionally, resistance to FAS mediated apoptosis has been reported in lymphocytes from patients with RA. Studies show that p53 controls cell surface trafficking of FAS[170], thus serving as a possible explanation for this set of observations.
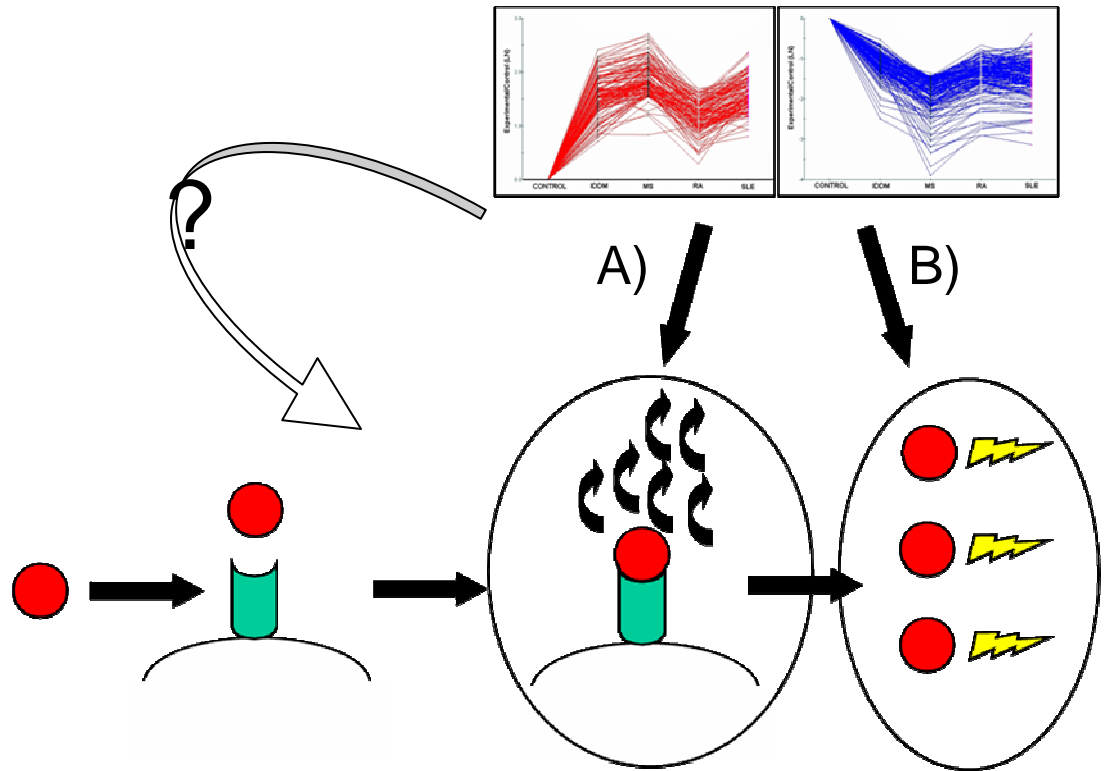
Fig. 27. <u>Gene Expression Signature Gives Rise to Perturbations in the Periphery</u>. Under-expressed damage response and cell cycle regulation genes may promote more a) aggressive proliferation in the periphery and b) alterations in lymphocyte effector function, such as abnormal cytokine secretion and decreased apoptosis, which could favor autoimmunity. Additionally, given the correct combination of deficits in these pathways, an initiating lymphopenic environment could be generated.

gene products, such as p21 and GADD45, contribute to systemic autoimmunity. Studies

have documented increased autoantibody production, glomerulonephritis, and mortality

in p21 -/- female mice[225]. T lymphocytes from these mice are hyperproliferative

compared to wild type littermates. Additional studies examining GADD45A, another

downstream target of p53 effector function, link this gene to systemic autoimmunity and

abnormalities in T lymphocyte function[226]. p21 -/- GADD45A -/- mice exhibit aggressive autoimmunity comparable to that in the *lpr*/MRL mice.

Some of the observed deficits associated with the signature may also provide an alternate explanation for initial lymphopenia that triggers homeostatic expansion. It has been proposed that age-associated thymic involution contributes to the lymphopenia found in patients with RA[239]. The correct combination of damage response deficits could also result in a genetically predetermined lymphopenia. For example, another damage response gene, BRCA1, is also highly under-expressed in RA lymphocytes[248]. In murine models, BRCA1 -/- thymocytes are completely deleted, *in vivo*, by p53 dependent apoptosis because absence of BRCA1 results in loss of chromosome integrity, which stimulates p53-dependent apoptosis. Mice that are BRCA1 -/-, p53 -/- have a reconstituted immune system but remain lymphopenic throughout life[249]. Thus, reduced levels of both BRCA1 and p53 could cause smoldering lymphopenia and stimulate homeostatic expansion of lymphocytes.

Conclusion

Loss of tolerance to specific self-antigens is generally believed to cause autoimmunity. However, data gathered over the past decade are inconsistent with this notion. Instead, studies in patients with RA have revealed multiple systemic abnormalities in their lymphocytes, including oligoclonal expansions, telomere erosions, and decreased TREC levels; findings consistent with increased general lymphocyte proliferation in the periphery. Additionally, as a result of multiple rounds of replication, T lymphocytes from RA patients appear to be immunosenescent. Rather than heralding inactivity, these immunosenescent lymphocytes are

resistant to apoptosis, secrete cytokines, and express a new set of surface markers. In general, these observations in RA patients appear to be independent of disease activity or duration, but do appear to have a heritable component.

Although the most thorough examination of lymphocyte abnormalities has been performed in RA, other autoimmune disorders display evidence of similar perturbations. For example, lymphocytes from patients with SLE[120] and type I diabetes[121] also suffer from age inappropriate telomere loss. Although initial observation of patients with multiple sclerosis have not confirmed telomere shortening, decreased TREC levels were found in MS lymphocytes compared to age matched control individuals[122]. Growing evidence supports the notion that defects in lymphocyte homeostasis, as assessed by proliferation studies, TREC levels, and telomere erosion, are associated with autoimmunity and may contribute to the development of these diseases.

In order to explain these findings, autoimmunity arising from homeostatic expansion has been forwarded as an alternate model. According to this model, lymphopenia, possibly secondary to age-associated thymic involution or infection, is accompanied by compensatory lymphocyte homeostatic expansion. Homeostatic expansion is dependent on self-antigen presented in the context of MHC, thus resulting in expansion of potentially autoreactive clones. Repeated rounds of replication may be accompanied by the development of immunosenescence, further altering lymphocyte function and possibly contributing to the development of autoimmunity.

Several parallels exist between the human data supporting homeostatic expansion and recent microarray studies. Initial examination of gene expression profiles from autoimmune patients reveal that there was no resemblance with an *in vivo* model immune

122

response, a finding inconsistent with classical views of autoimmunity. The gene expression patterns observed in the autoimmune patients do not appear to be related to disease activity or duration, but did appear to be heritable. Additionally, many of the genes under-expressed in the autoimmune signature are associated with apoptosis and cell cycle regulation. We hypothesize that many of the cellular abnormalities observed in human RA may be a result of deficits in cell cycle regulation and damage response pathways that may further exacerbate the process of pathologic homeostatic expansion.

REFERENCES

1.   Maas K, Chan S, Parker J, et al. Cutting edge: molecular portrait of human autoimmune disease. J Immunol 2002; 169:5-9.

2.   Courtenay JS, Dallman MJ, Dayan AD, Martin A, Mosedale B. Immunisation against heterologous type II collagen induces arthritis in mice. Nature 1980; 283:666-8.

3.   Segal BM. Experimental autoimmune encephalomyelitis: cytokines, effector T cells, and antigen-presenting cells in a prototypical Th1-mediated autoimmune disease. Curr Allergy Asthma Rep 2003; 3:86-93.

4.   Wooley PH, Luthra HS, Stuart JM, David CS. Type II collagen-induced arthritis in mice. I. Major histocompatibility complex (I region) linkage and antibody correlates. J Exp Med 1981; 154:688-700.

5.   Holmdahl R, Bockermann R, Backlund J, Yamada H. The molecular pathogenesis of collagen-induced arthritis in mice--a model for rheumatoid arthritis. Ageing Res Rev 2002; 1:135-47.

6.   Yoshino S, Cleland LG, Mayrhofer G. Treatment of collagen-induced arthritis in rats with a monoclonal antibody against the alpha/beta T cell antigen receptor. Arthritis Rheum 1991; 34:1039-47.

7.   Chiocchia G, Boissier MC, Fournier C. Therapy against murine collagen-induced arthritis with T cell receptor V beta-specific antibodies. Eur J Immunol 1991; 21:2899-905.

8.   Wooley PH, Luthra HS, Lafuse WP, Huse A, Stuart JM, David CS. Type II collagen-induced arthritis in mice. III. Suppression of arthritis by using monoclonal and polyclonal anti-Ia antisera. J Immunol 1985; 134:2366-74.

9.   Terato K, Hasty KA, Cremer MA, Stuart JM, Townes AS, Kang AH. Collagen-induced arthritis in mice. Localization of an arthritogenic determinant to a fragment of the type II collagen molecule. J Exp Med 1985; 162:637-46.

10.   Jacobson DL, Gange SJ, Rose NR, Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. Clin Immunol Immunopathol 1997; 84:223-43.

11.   Marrack P, Kappler J, Kotzin BL. Autoimmune disease: why and where it occurs. Nat Med 2001; 7:899-905.

12. Lahita RG. Systemic Lupus Erythematosus. In: Lahita RG, ed. Textbook of the Autoimmune Diseases. Philadelphia: Lipinncott Williams & Wilkins, 2000:573-594.

13. Fessel WJ. Systemic lupus erythematosus in the community. Incidence, prevalence, outcome, and first symptoms; the high prevalence in black women. Arch Intern Med 1974; 134:1027-35.

14. Cush JJ, Kavanaugh AF, Olsen NJ, Stein CM, Kazi S, Saag KG. Rheumtology: Diagnosis and Therapeutics. In: Pine JW, ed. Baltimore: Williams & Wilkins, 1999:559.

15. Appel GB, Silva FG, Pirani CL, Meltzer JI, Estes D. Renal involvement in systemic lupud erythematosus (SLE): a study of 56 patients emphasizing histologic classification. Medicine (Baltimore) 1978; 57:371-410.

16. Koffler D, Agnello V, Carr RI, Kunkel HG. Variable patterns of immunoglobulin and complement deposition in the kidneys of patients with systemic lupus erythematosus. Am J Pathol 1969; 56:305-16.

17. Beutner EH, Blaszczyk M, Jablonska S, et al. Preliminary, dermatologic first step criteria for lupus erythematosus and second step criteria for systemic lupus erythematosus. Int J Dermatol 1993; 32:645-51.

18. Blaszczyk M, Jablonska S, Chorzelski TP, Jarzabek-Chorzelska M, Beutner EH, Kumar V. Clinical relevance of immunologic findings in cutaneous lupus erythematosus. Clin Dermatol 1992; 10:399-406.

19. Duquette P, Pleines J, Girard M, Charest L, Senecal-Quevillon M, Masse C. The increased susceptibility of women to multiple sclerosis. Can J Neurol Sci 1992; 19:466-71.

20. Kurtzke JF. A reassessment of the distribution of multiple sclerosis. Acta Neurol Scand 1975; 51:137-57.

21. Coyle P. Multiple Sclerosis. In: Lahita RG, ed. Textbook of the Autoimmune Diseases. Philadelphia: Lippincott Williams & Wilkins, 2000:595-610.

22. Ferguson B, Matyszak MK, Esiri MM, Perry VH. Axonal damage in acute multiple sclerosis lesions. Brain 1997; 120 (Pt 3):393-9.

23. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. N Engl J Med 1998; 338:278-85.

24. Herold K, Sarne D. Autoimmune Endocrine Disorders. In: Lahita RG, ed. Textbook of the Autoimmune Diseases. Philadelphia: Lippincott Williams & Wilkins, 2000:391-397.

25.   Karvonen M, Viik-Kajander M, Moltchanova E, Libman I, LaPorte R, Tuomilehto J. Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. Diabetes Care 2000; 23:1516-26.

26.   Karvonen M, Tuomilehto J, Libman I, LaPorte R. A review of the recent epidemiological data on the worldwide incidence of type 1 (insulin-dependent) diabetes mellitus. World Health Organization DIAMOND Project Group. Diabetologia 1993; 36:883-92.

27.   Palmer JP, Asplin CM, Clemons P, et al. Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science 1983; 222:1337-9.

28.   Baekkeskov S, Aanstoot HJ, Christgau S, et al. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 1990; 347:151-6.

29.   Riley WJ, Maclaren NK, Krischer J, et al. A prospective study of the development of diabetes in relatives of patients with insulin-dependent diabetes. N Engl J Med 1990; 323:1167-72.

30.   Hagopian WA, Sanjeevi CB, Kockum I, et al. Glutamate decarboxylase-, insulin-, and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. J Clin Invest 1995; 95:1505-11.

31.   Serreze DV, Chapman HD, Varnum DS, et al. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice. J Exp Med 1996; 184:2049-53.

32.   Noorchashm H, Noorchashm N, Kern J, Rostami SY, Barker CF, Naji A. B-cells are required for the initiation of insulitis and sialitis in nonobese diabetic mice. Diabetes 1997; 46:941-6.

33.   Miller BJ, Appel MC, O'Neil JJ, Wicker LS. Both the Lyt-2+ and L3T4+ T cell subsets are required for the transfer of diabetes in nonobese diabetic mice. J Immunol 1988; 140:52-8.

34.   Weyand CM, and Goronzy, J.J. Rheumatoid Arthritis. In: Lahita RG, ed. Textbook of the Autoimmune Diseases. Philadelphia: Lippincott Williams & Wilkins, 2000:573-594.

35.   Gibofsky A, Visvanathan K, Kerwar S, Zabriskie J. Rheumatic Fever. In: Lahita RG, ed. Textbook of the Autoimmune Diseases. Philadelphia: Lippincott Williams & Wilkins, 2000:679-692.

36.   Dale JB, Beachey EH. Multiple, heart-cross-reactive epitopes of streptococcal M proteins. J Exp Med 1985; 161:113-22.

126

37.   Sargent SJ, Beachey EH, Corbett CE, Dale JB. Sequence of protective epitopes of streptococcal M proteins shared with cardiac sarcolemmal membranes. J Immunol 1987; 139:1285-90.

38.   Goldstein I, Caravano R. Determination of anti group A streptococcal polysaccharide antibodies in human sera by an hemagglutination technique. Proc Soc Exp Biol Med 1967; 124:1209-12.

39.   Cromartie WJ, Craddock JG, Schwab JH, Anderle SK, Yang CH. Arthritis in rats after systemic injection of streptococcal cells or cell walls. J Exp Med 1977; 146:1585-602.

40.   Vyse TJ, Todd JA. Genetic analysis of autoimmune disease. Cell 1996; 85:311-8.

41.   Seldin MF, Amos CI, Ward R, Gregersen PK. The genetics revolution and the assault on rheumatoid arthritis. Arthritis Rheum 1999; 42:1071-9.

42.   Hochberg MC. The application of genetic epidemiology to systemic lupus erythematosus. J Rheumatol 1987; 14:867-9.

43.   Ebers GC, Kukay K, Bulman DE, et al. A full genome search in multiple sclerosis. Nat Genet 1996; 13:472-6.

44.   Sawcer S, Jones HB, Feakes R, et al. A genome screen in multiple sclerosis reveals susceptibility loci on chromosome 6p21 and 17q22. Nat Genet 1996; 13:464-8.

45.   Haines JL, Ter-Minassian M, Bazyk A, et al. A complete genomic screen for multiple sclerosis underscores a role for the major histocompatability complex. The Multiple Sclerosis Genetics Group. Nat Genet 1996; 13:469-71.

46.   Davies JL, Kawaguchi Y, Bennett ST, et al. A genome-wide search for human type 1 diabetes susceptibility genes. Nature 1994; 371:130-6.

47.   Hashimoto L, Habita C, Beressi JP, et al. Genetic mapping of a susceptibility locus for insulin-dependent diabetes mellitus on chromosome 11q. Nature 1994; 371:161-4.

48.   Rowe RE, Wapelhorst B, Bell GI, Risch N, Spielman RS, Concannon P. Linkage and association between insulin-dependent diabetes mellitus (IDDM) susceptibility and markers near the glucokinase gene on chromosome 7. Nat Genet 1995; 10:240-2.

49.   Gaffney PM, Ortmann WA, Selby SA, et al. Genome screening in human systemic lupus erythematosus: results from a second Minnesota cohort and combined analyses of 187 sib-pair families. Am J Hum Genet 2000; 66:547-56.

50.   Moser KL, Neas BR, Salmon JE, et al. Genome scan of human systemic lupus erythematosus: evidence for linkage on chromosome 1q in African-American pedigrees. Proc Natl Acad Sci U S A 1998; 95:14869-74.

51.   Shai R, Quismorio FP, Jr., Li L, et al. Genome-wide screen for systemic lupus erythematosus susceptibility genes in multiplex families. Hum Mol Genet 1999; 8:639-44.

52.   Lindqvist AK, Steinsson K, Johanneson B, et al. A susceptibility locus for human systemic lupus erythematosus (hSLE1) on chromosome 2q. J Autoimmun 2000; 14:169-78.

53.   Jawaheer D, Seldin MF, Amos CI, et al. A genomewide screen in multiplex rheumatoid arthritis families suggests genetic overlap with other autoimmune diseases. Am J Hum Genet 2001; 68:927-36.

54.   Cornelis F, Faure S, Martinez M, et al. New susceptibility locus for rheumatoid arthritis suggested by a genome-wide linkage study. Proc Natl Acad Sci U S A 1998; 95:10746-50.

55.   Becker KG, Simon RM, Bailey-Wilson JE, et al. Clustering of non-major histocompatibility complex susceptibility candidate loci in human autoimmune diseases. Proc Natl Acad Sci U S A 1998; 95:9979-84.

56.   Heinzlef O, Alamowitch S, Sazdovitch V, et al. Autoimmune diseases in families of French patients with multiple sclerosis. Acta Neurol Scand 2000; 101:36-40.

57.   Shamim EA, Miller FW. Familial autoimmunity and the idiopathic inflammatory myopathies. Curr Rheumatol Rep 2000; 2:201-11.

58.   Lin JP, Cash JM, Doyle SZ, et al. Familial clustering of rheumatoid arthritis with other autoimmune diseases. Hum Genet 1998; 103:475-82.

59.   Morel L, Rudofsky UH, Longmate JA, Schiffenbauer J, Wakeland EK. Polygenic control of susceptibility to murine systemic lupus erythematosus. Immunity 1994; 1:219-29.

60.   Wakeland EK, Liu K, Graham RR, Behrens TW. Delineating the genetic basis of systemic lupus erythematosus. Immunity 2001; 15:397-408.

61.   Mohan C, Alas E, Morel L, Yang P, Wakeland EK. Genetic dissection of SLE pathogenesis. Sle1 on murine chromosome 1 leads to a selective loss of tolerance to H2A/H2B/DNA subnucleosomes. J Clin Invest 1998; 101:1362-72.

62.   Mohan C, Morel L, Yang P, Wakeland EK. Genetic dissection of systemic lupus erythematosus pathogenesis: Sle2 on murine chromosome 4 leads to B cell hyperactivity. J Immunol 1997; 159:454-65.

63. Mohan C, Yu Y, Morel L, Yang P, Wakeland EK. Genetic dissection of Sle pathogenesis: Sle3 on murine chromosome 7 impacts T cell activation, differentiation, and cell death. J Immunol 1999; 162:6492-502.

64. Morel L, Croker BP, Blenman KR, et al. Genetic reconstitution of systemic lupus erythematosus immunopathology with polycongenic murine strains. Proc Natl Acad Sci U S A 2000; 97:6670-5.

65. Ritchlin C, Dwyer E, Bucala R, Winchester R. Sustained and distinctive patterns of gene activation in synovial fibroblasts and whole synovial tissue obtained from inflammatory synovitis. Scand J Immunol 1994; 40:292-8.

66. Scott DL, Grindulis KA, Struthers GR, Coulton BL, Popert AJ, Bacon PA. Progression of radiological changes in rheumatoid arthritis. Ann Rheum Dis 1984; 43:8-17.

67. Sharp JT, Wolfe F, Mitchell DM, Bloch DA. The progression of erosion and joint space narrowing scores in rheumatoid arthritis during the first twenty-five years of disease. Arthritis Rheum 1991; 34:660-8.

68. Kaarela K, Kautiainen H. Continuous progression of radiological destruction in seropositive rheumatoid arthritis. J Rheumatol 1997; 24:1285-7.

69. Fuchs HA, Kaye JJ, Callahan LF, Nance EP, Pincus T. Evidence of significant radiographic damage in rheumatoid arthritis within the first 2 years of disease. J Rheumatol 1989; 16:585-91.

70. Fuchs HA, Pincus T. Radiographic damage in rheumatoid arthritis: description by nonlinear models. J Rheumatol 1992; 19:1655-8.

71. Larsen A, Thoen J. Hand radiography of 200 patients with rheumatoid arthritis repeated after an interval of one year. Scand J Rheumatol 1987; 16:395-401.

72. Pincus T, Callahan LF, Sale WG, Brooks AL, Payne LE, Vaughn WK. Severe functional declines, work disability, and increased mortality in seventy-five rheumatoid arthritis patients studied over nine years. Arthritis Rheum 1984; 27:864-72.

73. Wolfe F, Cathey MA. The assessment and prediction of functional disability in rheumatoid arthritis. J Rheumatol 1991; 18:1298-306.

74. Yelin E, Meenan R, Nevitt M, Epstein W. Work disability in rheumatoid arthritis: effects of disease, social, and work factors. Ann Intern Med 1980; 93:551-6.

75. Makisara GL, Makisara P. Prognosis of functional capacity and work capacity in rheumatoid arthritis. Clin Rheumatol 1982; 1:117-25.

76.   Pincus T, Brooks RH, Callahan LF. Prediction of long-term mortality in patients with rheumatoid arthritis according to simple questionnaire and joint count measures. Ann Intern Med 1994; 120:26-34.

77.   Pincus T, Callahan LF, Vaughn WK. Questionnaire, walking time and button test measures of functional capacity as predictive markers for mortality in rheumatoid arthritis. J Rheumatol 1987; 14:240-51.

78.   Mitchell DM, Spitz PW, Young DY, Bloch DA, McShane DJ, Fries JF. Survival, prognosis, and causes of death in rheumatoid arthritis. Arthritis Rheum 1986; 29:706-14.

79.   Uddin J, Kraus AS, Kelly HG. Survivorship and death in rheumatoid arthritis. Arthritis Rheum 1970; 13:125-30.

80.   Allebeck P. Increased mortality in rheumatoid arthritis. Scand J Rheumatol 1982; 11:81-6.

81.   Vandenbroucke JP, Hazevoet HM, Cats A. Survival and cause of death in rheumatoid arthritis: a 25-year prospective followup. J Rheumatol 1984; 11:158-61.

82.   Koota K, Isomaki H, Mutru O. Death rate and causes of death in RA patients during a period of five years. Scand J Rheumatol 1977; 6:241-4.

83.   Mutru O, Laakso M, Isomaki H, Koota K. Ten year mortality and causes of death in patients with rheumatoid arthritis. Br Med J (Clin Res Ed) 1985; 290:1797-9.

84.   Pincus T, Sokka T. How can the risk of long-term consequences of rheumatoid arthritis be reduced? Best Pract Res Clin Rheumatol 2001; 15:139-70.

85.   Baecklund E, Ekbom A, Sparen P, Feltelius N, Klareskog L. Disease activity and risk of lymphoma in patients with rheumatoid arthritis: nested case-control study. Bmj 1998; 317:180-1.

86.   Pincus T, Callahan LF. Rheumatology function tests: grip strength, walking time, button test and questionnaires document and predict longterm morbidity and mortality in rheumatoid arthritis. J Rheumatol 1992; 19:1051-7.

87.   Pincus T, Callahan LF. Taking mortality in rheumatoid arthritis seriously--predictive markers, socioeconomic status and comorbidity. J Rheumatol 1986; 13:841-5.

88.   Fries JF. Reevaluating the therapeutic approach to rheumatoid arthritis: the "sawtooth" strategy. J Rheumatol Suppl 1990; 22:12-5.

89.   Wilske KR, Healey LA. Remodeling the pyramid--a concept whose time has come. J Rheumatol 1989; 16:565-7.

90. Olsen NJ, Stein CM. New drugs for rheumatoid arthritis. N Engl J Med 2004; 350:2167-79.

91. Pincus T, Sokka T. The Locomotor System: Current treatment of rheumatoid arthritis. In: Rankel R, Bope E, eds. Conn's Current Therapies. Philadelphia: W.B. Saunders Company, 2004:1021-1029.

92. Mottonen T, Paimela L, Ahonen J, Helve T, Hannonen P, Leirisalo-Repo M. Outcome in patients with early rheumatoid arthritis treated according to the "sawtooth" strategy. Arthritis Rheum 1996; 39:996-1005.

93. Rau R, Herborn G, Sander O, al. e. Long-term treatment with the fully human anti-TNF-antibody D2E7 shows radiographic disease progression in rheumatoid arthritis (abstract 1978) [abstract]. Arthritis and Rheumatism 1999; 42:S400.

94. Abu-Shakra M, Toker R, Flusser D, et al. Clinical and radiographic outcomes of rheumatoid arthritis patients not treated with disease-modifying drugs. Arthritis Rheum 1998; 41:1190-5.

95. Sokka T, Mottonen T, Hannonen P. Disease-modifying anti-rheumatic drug use according to the 'sawtooth' treatment strategy improves the functional outcome in rheumatoid arthritis: results of a long-term follow-up study with review of the literature. Rheumatology (Oxford) 2000; 39:34-42.

96. Krause D, Schleusser B, Herborn G, Rau R. Response to methotrexate treatment is associated with reduced mortality in patients with severe rheumatoid arthritis. Arthritis Rheum 2000; 43:14-21.

97. Lehtinen K, Isomaki H. Intramuscular gold therapy is associated with long survival in patients with rheumatoid arthritis. J Rheumatol 1991; 18:524-9.

98. Wolfe F, Hawley D, Pincus T. Methotrexate alters the course of rheumatoid arthritis (RA): increased survival of methotrexate treated RA patients: a 25-year study of 1842 patients (ACR Plenary Session) [abstract]. Arthritis and Rheumatism 1998; 41:S188.

99. Cush J, Kavanaugh A. Biologic interventions in rheumatoid arthrtis. Rhem Dis Clin North Am 1995; 21:797-816.

100. Finck B, Martin R, Fleishmann R, al. e. A phase III trial of etanercept vs methotrexate (MTX) in early rheumatoid arthritis (Enberl ERA Trial) (abstract 280) [abstract]. Arthritis and Rheumatism 1999; 42:S117.

101. Lipsky R, St Clair W, Furst D, al. e. 54-week clinical and radiographic results from the ATTRACT trial: a phase III study of infliximab (Remicade) in patients with active RA (abstract 1980) [abstract]. Arthritis and Rheumatism 1999; 42:S401.

102.	Kang YM, Zhang X, Wagner UG, et al. CD8 T cells are required for the formation of ectopic germinal centers in rheumatoid synovitis. J Exp Med 2002; 195:1325-36.

103.	Weyand CM, Goronzy JJ. Ectopic germinal center formation in rheumatoid synovitis. Ann N Y Acad Sci 2003; 987:140-9.

104.	Travaglio-Encinoza A, Chaouni I, Dersimonian H, et al. T cell receptor distribution in rheumatoid synovial follicles. J Rheumatol 1995; 22:394-9.

105.	Rowley MJ, Stockman A, Brand CA, et al. The effect of HLA-DRB1 disease susceptibility markers on the expression of RA. Scand J Rheumatol 1997; 26:448-55.

106.	Hasstedt SJ, Clegg DO, Ingles L, Ward RH. HLA-linked rheumatoid arthritis. Am J Hum Genet 1994; 55:738-46.

107.	Striebich CC, Falta MT, Wang Y, Bill J, Kotzin BL. Selective accumulation of related CD4+ T cell clones in the synovial fluid of patients with rheumatoid arthritis. J Immunol 1998; 161:4428-36.

108.	Fang Q, Sun YY, El-Gabalawy H, et al. Synovial T cell receptor heterogeneity in early arthritis. Pathobiology 1999; 67:59-74.

109.	Cooper SM, Roessner KD, Naito-Hoopes M, Howard DB, Gaur LK, Budd RC. Increased usage of V beta 2 and V beta 6 in rheumatoid synovial fluid T cells. Arthritis Rheum 1994; 37:1627-36.

110.	Li Y, Sun GR, Tumang JR, Crow MK, Friedman SM. Characterization of oligoclonal synovial T-cells in rheumatoid arthritis. Ann N Y Acad Sci 1995; 756:192-4.

111.	Emery P, Panayi GS, Nouri AM. Interleukin-2 reverses deficient cell-mediated immune responses in rheumatoid arthritis. Clin Exp Immunol 1984; 57:123-9.

112.	Emery P, Panayi GS, Welsh KI, Cole BC. Relationship of HLA-DR4 to defective cellular immunity in rheumatoid arthritis using PPD, and mycoplasma and lectin mitogens. J Rheumatol 1985; 12:859-64.

113.	Malone DG, Wahl SM, Tsokos M, Cattell H, Decker JL, Wilder RL. Immune function in severe, active rheumatoid arthritis. A relationship between peripheral blood mononuclear cell proliferation to soluble antigens and synovial tissue immunohistologic characteristics. J Clin Invest 1984; 74:1173-85.

114.	Cope AP, Liblau RS, Yang XD, et al. Chronic tumor necrosis factor alters T cell responses by attenuating T cell receptor signaling. J Exp Med 1997; 185:1573-84.

115. Isomaki P, Panesar M, Annenkov A, et al. Prolonged exposure of T cells to TNF down-regulates TCR zeta and expression of the TCR/CD3 complex at the cell surface. J Immunol 2001; 166:5495-507.

116. Wagner UG, Koetz K, Weyand CM, Goronzy JJ. Perturbation of the T cell repertoire in rheumatoid arthritis. Proc Natl Acad Sci U S A 1998; 95:14447-52.

117. Koetz K, Bryl E, Spickschen K, O'Fallon WM, Goronzy JJ, Weyand CM. T cell homeostasis in patients with rheumatoid arthritis. Proc Natl Acad Sci U S A 2000; 97:9203-8.

118. Ponchel F, Morgan AW, Bingham SJ, et al. Dysregulated lymphocyte proliferation and differentiation in patients with rheumatoid arthritis. Blood 2002; 100:4550-6.

119. Weyand CM, Fulbright JW, Goronzy JJ. Immunosenescence, autoimmunity, and rheumatoid arthritis. Exp Gerontol 2003; 38:833-41.

120. Honda M, Mengesha E, Albano S, et al. Telomere shortening and decreased replicative potential, contrasted by continued proliferation of telomerase-positive CD8+CD28(lo) T cells in patients with systemic lupus erythematosus. Clin Immunol 2001; 99:211-221.

121. Jeanclos E, Krolewski A, Skurnick J, et al. Shortened telomere length in white blood cells of patients with IDDM. Diabetes 1998; 47:482-6.

122. Hug A, Korporal M, Schroder I, et al. Thymic export function and T cell homeostasis in patients with relapsing remitting multiple sclerosis. J Immunol 2003; 171:432-7.

123. Schulze A, Downward J. Navigating gene expression using microarrays--a technology review. Nat Cell Biol 2001; 3:E190-5.

124. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000; 25:169-93.

125. Al-Taher A, Bashein A, Nolan T, Hollingsworth M, Brady G. Global cDNA amplification combined with real-time RT-PCR: accurate quantification of multiple human potassium channel genes at the single cell level. Yeast 2000; 17:201-10.

126. Lockhart DJ, Winzeler EA. Genomics, gene expression and DNA arrays. Nature 2000; 405:827-36.

127. Luo L, Salunga RC, Guo H, et al. Gene expression profiles of laser-captured adjacent neuronal subtypes. Nat Med 1999; 5:117-22.

128. Brady G. Expression profiling of single mammalian cells--small is beautiful. Yeast 2000; 17:211-7.

129. Golub TR, Slonim DK, Tamayo P, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999; 286:531-7.

130. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 2000; 403:503-11.

131. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. Nature 2000; 406:747-52.

132. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 1998; 95:14863-8.

133. Heyer LJ, Kruglyak S, Yooseph S. Exploring expression data: identification and analysis of coexpressed genes. Genome Res 1999; 9:1106-15.

134. Sherlock G. Analysis of large-scale gene expression data. Curr Opin Immunol 2000; 12:201-5.

135. Tamayo P, Slonim D, Mesirov J, et al. Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. Proc Natl Acad Sci U S A 1999; 96:2907-12.

136. Nyberg KA, Michelson RJ, Putnam CW, Weinert TA. Toward maintaining the genome: DNA damage and replication checkpoints. Annu Rev Genet 2002; 36:617-56.

137. Carney JP, Maser RS, Olivares H, et al. The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. Cell 1998; 93:477-86.

138. Paull TT, Gellert M. Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex. Genes Dev 1999; 13:1276-88.

139. Stewart GS, Maser RS, Stankovic T, et al. The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. Cell 1999; 99:577-87.

140. Zhao S, Weng YC, Yuan SS, et al. Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. Nature 2000; 405:473-7.

141. Bartek J, Falck J, Lukas J. CHK2 kinase--a busy messenger. Nat Rev Mol Cell Biol 2001; 2:877-86.

142. Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K, Elledge SJ. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc Natl Acad Sci U S A 2000; 97:10389-94.

143. Melchionna R, Chen XB, Blasina A, McGowan CH. Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. Nat Cell Biol 2000; 2:762-5.

144. Ahn JY, Schwarz JK, Piwnica-Worms H, Canman CE. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. Cancer Res 2000; 60:5934-6.

145. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. Nature 1997; 387:299-303.

146. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature 1997; 387:296-9.

147. Shieh SY, Ahn J, Tamai K, Taya Y, Prives C. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. Genes Dev 2000; 14:289-300.

148. Kussie PH, Gorina S, Marechal V, et al. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science 1996; 274:948-53.

149. Stewart ZA, Pietenpol JA. p53 Signaling and cell cycle checkpoints. Chem Res Toxicol 2001; 14:243-63.

150. Chehab NH, Malikzay A, Stavridi ES, Halazonetis TD. Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proc Natl Acad Sci U S A 1999; 96:13777-82.

151. Ahn J, Urist M, Prives C. Questioning the role of checkpoint kinase 2 in the p53 DNA damage response. J Biol Chem 2003; 278:20480-9.

152. Jallepalli PV, Lengauer C, Vogelstein B, Bunz F. The Chk2 tumor suppressor is not required for p53 responses in human cancer cells. J Biol Chem 2003; 278:20475-9.

153. Kern SE, Kinzler KW, Bruskin A, et al. Identification of p53 as a sequence-specific DNA-binding protein. Science 1991; 252:1708-11.

154. el-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993; 75:817-25.

155. Deng C, Zhang P, Harper JW, Elledge SJ, Leder P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell 1995; 82:675-84.

156. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature 1993; 366:701-4.

157. Dulic V, Kaufmann WK, Wilson SJ, et al. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell 1994; 76:1013-23.

158. Gu Y, Turck CW, Morgan DO. Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. Nature 1993; 366:707-10.

159. Mailand N, Falck J, Lukas C, et al. Rapid destruction of human Cdc25A in response to DNA damage. Science 2000; 288:1425-9.

160. Smith ML, Chen IT, Zhan Q, et al. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 1994; 266:1376-80.

161. Kastan MB, Zhan Q, el-Deiry WS, et al. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 1992; 71:587-97.

162. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995; 80:293-9.

163. Shibue T, Takeda K, Oda E, et al. Integral role of Noxa in p53-mediated apoptotic response. Genes Dev 2003; 17:2233-8.

164. Oda E, Ohki R, Murasawa H, et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 2000; 288:1053-8.

165. Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell 2001; 7:673-82.

166. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 2001; 7:683-94.

167. Wu GS, Burns TF, McDonald ER, 3rd, et al. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet 1997; 17:141-3.

168. Friesen C, Herr I, Krammer PH, Debatin KM. Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat Med 1996; 2:574-7.

169. Muller M, Strand S, Hug H, et al. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. J Clin Invest 1997; 99:403-13.

170. Bennett M, Macdonald K, Chan SW, Luzio JP, Simari R, Weissberg P. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. Science 1998; 282:290-3.

171. Kukreja A, Maclaren NK. Current cases in which epitope mimicry is considered as a component cause of autoimmune disease: immune-mediated (type 1) diabetes. Cell Mol Life Sci 2000; 57:534-41.

172. Ufret-Vincenty RL, Quigley L, Tresser N, et al. In vivo survival of viral antigen-specific T cells that induce experimental autoimmune encephalomyelitis. J Exp Med 1998; 188:1725-38.

173. Quayle AJ, Wilson KB, Li SG, et al. Peptide recognition, T cell receptor usage and HLA restriction elements of human heat-shock protein (hsp) 60 and mycobacterial 65-kDa hsp-reactive T cell clones from rheumatoid synovial fluid. Eur J Immunol 1992; 22:1315-22.

174. Kotzin BL. Systemic lupus erythematosus. Cell 1996; 85:303-6.

175. Chen Y, *et al.* J. Biomedical Optics 1997; 2:364-374.

176. Rosen A, Casciola-Rosen L. Clearing the way to mechanisms of autoimmunity. Nat Med 2001; 7:664-5.

177. Baechler EC, Batliwalla FM, Karypis G, et al. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. Proc Natl Acad Sci U S A 2003; 100:2610-5.

178. Bennett L, Palucka AK, Arce E, et al. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. J Exp Med 2003; 197:711-23.

179. Bomprezzi R, Ringner M, Kim S, et al. Gene expression profile in multiple sclerosis patients and healthy controls: identifying pathways relevant to disease. Hum Mol Genet 2003; 12:2191-9.

180. Ramanathan M, Weinstock-Guttman B, Nguyen LT, et al. In vivo gene expression revealed by cDNA arrays: the pattern in relapsing-remitting multiple sclerosis patients compared with normal subjects. J Neuroimmunol 2001; 116:213-9.

181. Cohen PL, Eisenberg RA. The lpr and gld genes in systemic autoimmunity: life and death in the Fas lane. Immunol Today 1992; 13:427-8.

182. Nagata S, Suda T. Fas and Fas ligand: lpr and gld mutations. Immunol Today 1995; 16:39-43.

183. Fisher GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell 1995; 81:935-46.

184. Drappa J, Vaishnaw AK, Sullivan KE, Chu JL, Elkon KB. Fas gene mutations in the Canale-Smith syndrome, an inherited lymphoproliferative disorder associated with autoimmunity. N Engl J Med 1996; 335:1643-9.

185. Huang QR, Danis V, Lassere M, Edmonds J, Manolios N. Evaluation of a new Apo-1/Fas promoter polymorphism in rheumatoid arthritis and systemic lupus erythematosus patients. Rheumatology (Oxford) 1999; 38:645-51.

186. Bertolo F, De Vita S, Dolcetti R, et al. Lack of Fas and Fas-L mutations in patients with lymphoproliferative disorders associated with Sjogren's syndrome and type II mixed cryoglobulinemia. Clin Exp Rheumatol 1999; 17:339-42.

187. Lorenz HM, Grunke M, Hieronymus T, et al. In vitro apoptosis and expression of apoptosis-related molecules in lymphocytes from patients with systemic lupus erythematosus and other autoimmune diseases. Arthritis Rheum 1997; 40:306-17.

188. Brown MA, Kennedy LG, MacGregor AJ, et al. Susceptibility to ankylosing spondylitis in twins: the role of genes, HLA, and the environment. Arthritis Rheum 1997; 40:1823-8.

189. Krichevsky AM, Metzer E, Rosen H. Translational control of specific genes during differentiation of HL-60 cells. J Biol Chem 1999; 274:14295-305.

190. Olsen N, Sokka, T., Seehorn, C. L., Kraft, B., Maas, K., Moore, J., and Aune, T. Gene Expression Signature for Recent Onset Rheumatoid Arthritis in Peripheral Blood Mononuclear Cells. Ann Rheum Dis 2004.

191. Cavalieri D, Townsend JP, Hartl DL. Manifold anomalies in gene expression in a vineyard isolate of Saccharomyces cerevisiae revealed by DNA microarray analysis. Proc Natl Acad Sci U S A 2000; 97:12369-74.

192. Sandberg R, Yasuda R, Pankratz DG, et al. Regional and strain-specific gene expression mapping in the adult mouse brain. Proc Natl Acad Sci U S A 2000; 97:11038-43.

193. Primig M, Williams RM, Winzeler EA, et al. The core meiotic transcriptome in budding yeasts. Nat Genet 2000; 26:415-23.

194. Brem RB, Yvert G, Clinton R, Kruglyak L. Genetic dissection of transcriptional regulation in budding yeast. Science 2002; 296:752-5.

195. Schadt EE, Monks SA, Drake TA, et al. Genetics of gene expression surveyed in maize, mouse and man. Nature 2003; 422:297-302.

196. Watts JA, Morley M, Burdick JT, et al. Gene expression phenotype in heterozygous carriers of ataxia telangiectasia. Am J Hum Genet 2002; 71:791-800.

197.   Prahalad S, Shear ES, Thompson SD, Giannini EH, Glass DN. Increased prevalence of familial autoimmunity in simplex and multiplex families with juvenile rheumatoid arthritis. Arthritis Rheum 2002; 46:1851-6.

198.   Clarke AR, Purdie CA, Harrison DJ, et al. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 1993; 362:849-52.

199.   Seki H, Kanegane H, Iwai K, et al. Ionizing radiation induces apoptotic cell death in human TcR-gamma/delta+ T and natural killer cells without detectable p53 protein. Eur J Immunol 1994; 24:2914-7.

200.   Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. Cancer Res 1991; 51:6304-11.

201.   Hirao A, Kong YY, Matsuoka S, et al. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science 2000; 287:1824-7.

202.   Kennedy AS, Harrison GH, Mansfield CM, Zhou XJ, Xu JF, Balcer-Kubiczek EK. Survival of colorectal cancer cell lines treated with paclitaxel, radiation, and 5-FU: effect of TP53 or hMLH1 deficiency. Int J Cancer 2000; 90:175-85.

203.   Wu X, Deng Y. Bax and BH3-domain-only proteins in p53-mediated apoptosis. Front Biosci 2002; 7:d151-6.

204.   Yu J, Zhang L. No PUMA, no death: implications for p53-dependent apoptosis. Cancer Cell 2003; 4:248-9.

205.   Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 1994; 371:346-7.

206.   Matsui T, Katsuno Y, Inoue T, et al. Negative regulation of Chk2 expression by p53 is dependent on the CCAAT-binding transcription factor NF-Y. J Biol Chem 2004.

207.   Zhao R, Gish K, Murphy M, et al. Analysis of p53-regulated gene expression patterns using oligonucleotide arrays. Genes Dev 2000; 14:981-93.

208.   Kannan K, Amariglio N, Rechavi G, et al. DNA microarrays identification of primary and secondary target genes regulated by p53. Oncogene 2001; 20:2225-34.

209.   Chehab NH, Malikzay A, Appel M, Halazonetis TD. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev 2000; 14:278-88.

210.   McNally J, Yoo DH, Drappa J, et al. Fas ligand expression and function in systemic lupus erythematosus. J Immunol 1997; 159:4628-36.

211.   Yamanishi Y, Boyle DL, Rosengren S, Green DR, Zvaifler NJ, Firestein GS. Regional analysis of p53 mutations in rheumatoid arthritis synovium. Proc Natl Acad Sci U S A 2002; 99:10025-30.

212.   Hudson JD, Shoaibi MA, Maestro R, Carnero A, Hannon GJ, Beach DH. A proinflammatory cytokine inhibits p53 tumor suppressor activity. J Exp Med 1999; 190:1375-82.

213.   Leech M, Lacey D, Xue JR, et al. Regulation of p53 by macrophage migration inhibitory factor in inflammatory arthritis. Arthritis Rheum 2003; 48:1881-9.

214.   Leech M, Metz C, Hall P, et al. Macrophage migration inhibitory factor in rheumatoid arthritis: evidence of proinflammatory function and regulation by glucocorticoids. Arthritis Rheum 1999; 42:1601-8.

215.   Sturm A, Itoh J, Jacobberger JW, Fiocchi C. p53 negatively regulates intestinal immunity by delaying mucosal T cell cycling. J Clin Invest 2002; 109:1481-92.

216.   Michelle Leech JRX, Georgia Poulos, Pamela Hall, Eric Morand. p53 Modulates the Systemic Immune Response and Arthritis Severity in Antigen-Induced Arthritis. Orlando, Florida: American College of Rheumatology, 2003.

217.   Yamanishi Y, Boyle DL, Pinkoski MJ, et al. Regulation of joint destruction and inflammation by p53 in collagen-induced arthritis. Am J Pathol 2002; 160:123-30.

218.   Schonland SO, Lopez C, Widmann T, et al. Premature telomeric loss in rheumatoid arthritis is genetically determined and involves both myeloid and lymphoid cell lineages. Proc Natl Acad Sci U S A 2003; 100:13471-6.

219.   Ohkusu-Tsukada K, Tsukada T, Isobe K. Accelerated development and aging of the immune system in p53-deficient mice. J Immunol 1999; 163:1966-72.

220.   Sharpless NE, DePinho RA. p53: good cop/bad cop. Cell 2002; 110:9-12.

221.   Campisi J. Cancer and ageing: rival demons? Nat Rev Cancer 2003; 3:339-49.

222.   d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, et al. A DNA damage checkpoint response in telomere-initiated senescence. Nature 2003; 426:194-8.

223.   Keramaris E, Hirao A, Slack RS, Mak TW, Park DS. Ataxia telangiectasia-mutated protein can regulate p53 and neuronal death independent of Chk2 in response to DNA damage. J Biol Chem 2003; 278:37782-9.

224.   Lawson BR, Kono DH, Theofilopoulos AN. Deletion of p21 (WAF-1/Cip1) does not induce systemic autoimmunity in female BXSB mice. J Immunol 2002; 168:5928-32.

225. Balomenos D, Martin-Caballero J, Garcia MI, et al. The cell cycle inhibitor p21 controls T-cell proliferation and sex-linked lupus development. Nat Med 2000; 6:171-6.

226. Salvador JM, Hollander MC, Nguyen AT, et al. Mice lacking the p53-effector gene Gadd45a develop a lupus-like syndrome. Immunity 2002; 16:499-508.

227. Bernstein C, Bernstein H, Payne CM, Garewal H. DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis. Mutat Res 2002; 511:145-78.

228. Pascual M, Lopez-Nevot MA, Caliz R, et al. A poly(ADP-ribose) polymerase haplotype spanning the promoter region confers susceptibility to rheumatoid arthritis. Arthritis Rheum 2003; 48:638-41.

229. Goronzy JJ, Bartz-Bazzanella P, Hu W, Jendro MC, Walser-Kuntz DR, Weyand CM. Dominant clonotypes in the repertoire of peripheral CD4+ T cells in rheumatoid arthritis. J Clin Invest 1994;94:2068-76.

230. Schmidt D, Goronzy JJ, Weyand CM. CD4+ CD7- CD28- T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. J Clin Invest 1996;97:2027-37.

231. Fitzgerald JE, Ricalton NS, Meyer AC, West SG, Kaplan H, Behrendt C, et al. Analysis of clonal CD8+ T cell expansions in normal individuals and patients with rheumatoid arthritis. J Immunol 1995;154(7):3538-47.

232. Waase I, Kayser C, Carlson PJ, Goronzy JJ, Weyand CM. Oligoclonal T cell proliferation in patients with rheumatoid arthritis and their unaffected siblings. Arthritis Rheum 1996;39:904-13.

233. Effros RB. Loss of CD28 expression on T lymphocytes: a marker of replicative senescence. Dev Comp Immunol 1997;21:471-8.

234. Namekawa T, Snyder MR, Yen JH, Goehring BE, Leibson PJ, Weyand CM, et al. Killer cell activating receptors function as costimulatory molecules on CD4+CD28null T cells clonally expanded in rheumatoid arthritis. J Immunol 2000;165:1138-45.

235. Warrington KJ, Takemura S, Goronzy JJ, Weyand CM. CD4+,CD28- T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. Arthritis Rheum 2001;44:13-20.

236. Yen JH, Moore BE, Nakajima T, Scholl D, Schaid DJ, Weyand CM, et al. Major histocompatibility complex class I-recognizing receptors are disease risk genes in rheumatoid arthritis. J Exp Med 2001;193:1159-67.

141

237. Vallejo AN, Schirmer M, Weyand CM, Goronzy JJ. Clonality and longevity of CD4+CD28null T cells are associated with defects in apoptotic pathways. J Immunol 2000;165:6301-7.

238. Schirmer M, Vallejo AN, Weyand CM, Goronzy JJ. Resistance to apoptosis and elevated expression of Bcl-2 in clonally expanded CD4+CD28- T cells from rheumatoid arthritis patients. J Immunol 1998;161:1018-25.

239. Goronzy JJ, Weyand CM. Aging, autoimmunity and arthritis: T-cell senescence and contraction of T-cell repertoire diversity - catalysts of autoimmunity and chronic inflammation. Arthritis Res Ther 2003;5:225-34.

240. Gleeson PA, Toh BH, van Driel IR. Organ-specific autoimmunity induced by lymphopenia. Immunol Rev 1996;149:97-125.

241. Moses CT, Thorstenson KM, Jameson SC, Khoruts A. Competition for self ligands restrains homeostatic proliferation of naive CD4 T cells. Proc Natl Acad Sci U S A 2003;100:1185-90.

242. Prlic M, Jameson SC. Homeostatic expansion versus antigen-driven proliferation: common ends by different means? Microbes Infect 2002;4:531-7.

243. Kieper WC, Jameson SC. Homeostatic expansion and phenotypic conversion of naive T cells in response to self peptide/MHC ligands. Proc Natl Acad Sci U S A 1999;96:13306-11.

244. Marleau AM, Sarvetnick N. T cell homeostasis in tolerance and immunity. J Leukoc Biol 2005;78:575-84.

245. King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. Cell 2004;117:265-77.

246. Maas K, Chen H, Shyr Y, Olsen NJ, Aune T. Shared gene expression profiles in individuals with autoimmune disease and unaffected first-degree relatives of individuals with autoimmune disease. Hum Mol Genet 2005;14:1305-14.

247. Maas K, Westfall M, Pietenpol J, Olsen NJ, Aune T. Reduced p53 in peripheral blood mononuclear cells from patients with rheumatoid arthritis is associated with loss of radiation-induced apoptosis. Arthritis Rheum 2005;52:1047-57.

248. Aune T. Decreased BRCA transcript levels in RA PBMCs compared to healthy control individuals. Personal Communication. Nashville; 2006.

249.   Mak TW, Hakem A, McPherson JP, Shehabeldin A, Zablocki E, Migon E, et al. Brcal required for T cell lineage development but not TCR loci rearrangement. In: Nat Immunol; 2000. p. 77-82.