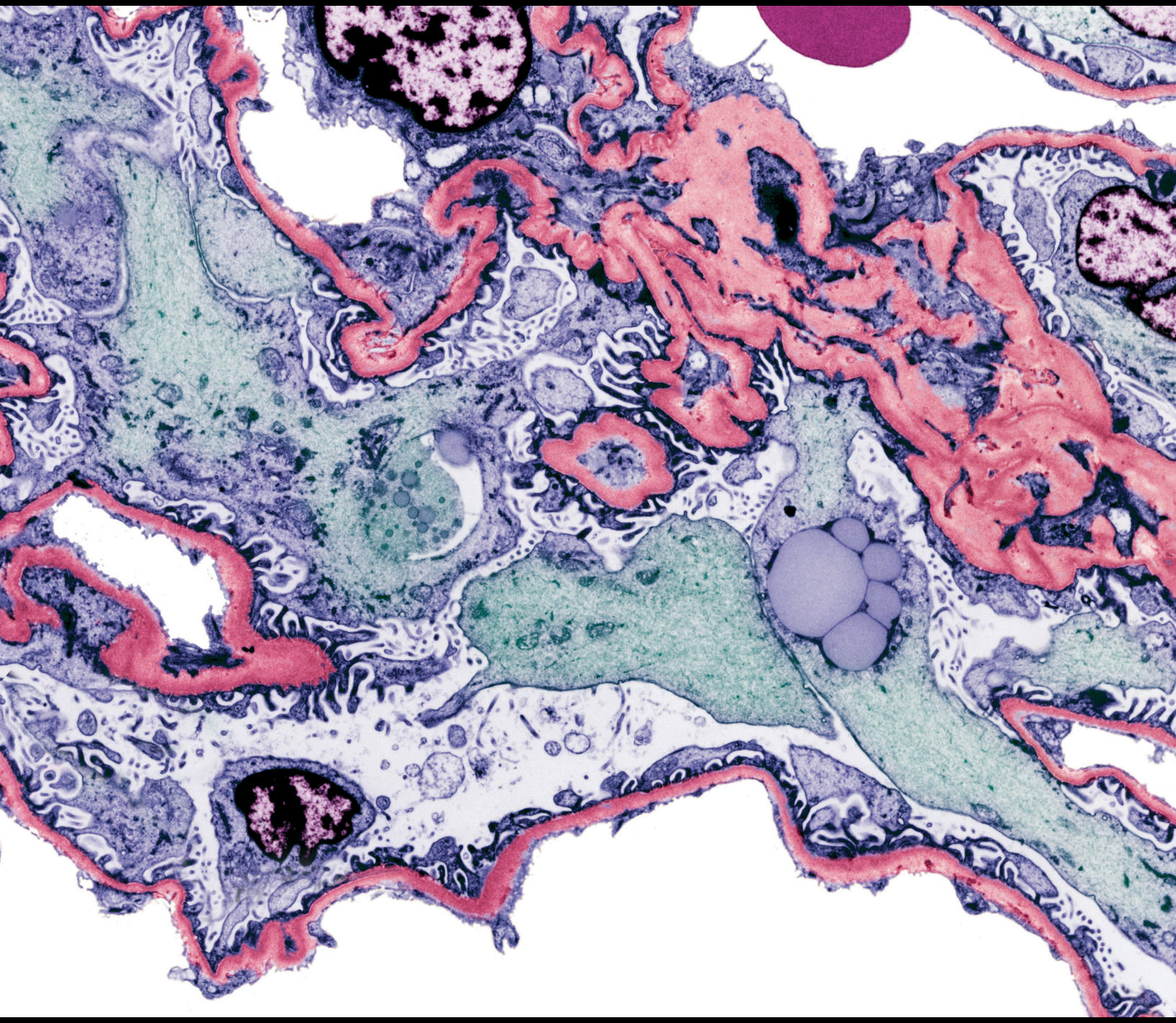
Autoimmune Diseases

Environmental Triggers and Autoimmunity

Guest Editors: Aristo Vojdani and K. Michael Pollard



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Editorial Board

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 798029, 2 pages
http://dx.doi.org/10.1155/2014/798029

Editorial

Environmental Triggers and Autoimmunity

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Received 9 October 2014; Accepted 9 October 2014; Published 24 December 2014

Currently, studies have shown that genetic predisposition accounts for approximately thirty percent of all autoimmune diseases. The rest, 70 percent, are due to environmental factors, including toxic chemicals, dietary components, gut dysbiosis, and infections (Figure 1).

Autoimmune disorder symptoms are initially nonspecific and include malaise and fatigue, low-grade fevers, aches, and pains. Due to this vagueness, patients are frequently diagnosed with an autoimmune disease after they become weak and unable to function normally, making the onset of the disease difficult to pinpoint and the possible triggers uncertain.

In this issue, we present a series of papers that review, discuss, and elaborate on various environmental triggers of autoimmunity.

A. Vojdani presents an extensive review of potential triggers of autoimmunity. The author discusses the loss of immune homeostasis and explains the mechanism of autoimmunity as related to infectious triggers by molecular mimicry, epitope spreading, and bystander activation. He describes the effects of dietary components, focusing particularly on recent studies with sodium chloride to explain the effects of this commonly used mineral on the immune system, in particular TH17, leading to an increased risk of autoimmunity. Milk and wheat components are reviewed, as are gluten sensitivity, celiac disease, and oral pathogens in their role in the induction of autoimmune diseases.

Over the last few years, studies have amplified our previous knowledge of the gut and demonstrated its wide-ranging importance and its potentials for triggering autoimmunity when dysbiosis occurs as a result of environmental factors (Figure 2). A. W. Campbell's review brings up essential facts about some of these environmental factors affecting not only the gut but also the mucosal immunity and describes gut microbiota links to autoimmune diseases. The author discusses the importance of early detection of autoimmunity via antibody testing to bring about a better outcome for patients by removing offending triggers.

A very interesting research article is presented in this issue by I. Burazor and A. Vojdani, discussing the strong link between poor dental health and cardiovascular disease due to several bacteria and then studying the potential association between these pathogens, the antibodies produced against them, and elevation of markers for inflammation in patients with acute myocardial atherothrombosis (AMA).

Decay-accelerating factor 1 (DAF1) or CD55 is a 70 KDa member of proteins which regulates complement system on the cell surfaces and protects cells from complement attack. In their review, C. B. Toomey et al. discussed the relationship between DAF1 and the complement system in the regulation of environmentally induced autoimmunity. They propose a hypothesis to explain how DAF expression may impact T cell differentiation via interaction with CD97 leading to T regulatory cells, increased production of IL-10, and immune tolerance. Further understanding of this novel mechanism by which DAF can regulate mercury-induced autoimmunity may lead to new strategies for regulation of DAF in various autoimmunities induced by environmental toxicants.

In a research study, D. M. Cauvi et al. show that the effect of DAF on autoimmunity is complex and may require

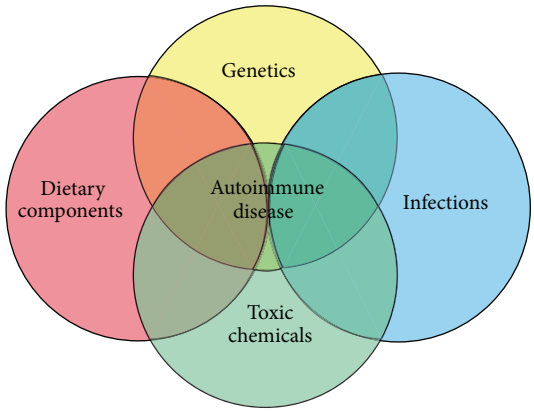

Figure 1: Factors that contribute to autoimmune disease.

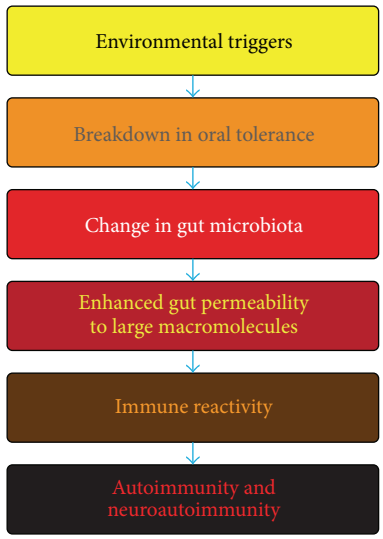

Figure 2: Mechanism for the induction of autoimmunity and neuroautoimmunity by environmental triggers.

multiple genetic elements such as a tandem repeat sequence (CTTTT)n or (TTTTC)n. This association between the absence of tandem repeats and the severity of autoimmunity may be due to linkage of tandem repeats with other predisposing variants that promote DAF1 expression.

Bisphenol A (BPA) may be a potential link to autoimmune diseases. It is ubiquitous in consumer products: more than 90% of Americans were found to have detectable levels of BPA in their urine. Studies have shown that BPA is an endocrine disruptor that can affect perinatal, childhood, and adult health. D. Kharrazian's review of BPA includes a general assessment of this highly prevalent chemical in our environment. He then describes eleven different pathophysiological and immunological mechanisms where BPA exposure may lead to autoimmunity.

In their article, J. Ong et al. sought to clarify the role of Hg through fish consumption and its relationship to increased autoimmune disease via testing for ANA and specific autoantibodies in blood in the Cheyenne River Sioux Tribe lands (CRST) community. The interactions of gender with blood Hg and arsenic proximity were significant, suggesting that complex interactions underlie autoimmunity.

A central issue in immunology is how, at different developmental stages, the fate of B-lymphocytes is determined and how B cell receptors (BCR) distinguish between signals that induce immune response versus immune tolerance. The alteration in BCR signaling by low levels of exposure to mercury for the pathogenesis of autoimmune disease is discussed by R. F. Gill et al. Their report showed that Hg^{2+} has little upstream effects on BHC tyrosine kinase, but SYK tyrosine kinase and B cell scaffolding protein BLNK are augmented by low levels of mercury, suggesting that low levels of mercury may interfere with central tolerance and may be a mechanism connecting mercury intoxication to autoimmune disease.

J. C. Pfau et al. review the link between asbestos exposure and autoimmunity. The authors review rheumatoid arthritis, systemic sclerosis, and systemic lupus erythematosus, among others, and their association with asbestos and give a review of their hypotheses regarding the discordant and inconsistent results. They also discuss the most compelling evidence for a link between asbestos exposure and autoimmunity.

Trichloroethylene (TCE) is an industrial solvent known for being neurotoxic, hepatotoxic, nephrotoxic, and immunotoxic. It is also carcinogenic. To give insight into how TCE may cause possible immune related issues, K. M. Gilbert et al. provide us with a very interesting study on exposure to TCE in autoimmune prone female mice exposed during gestation or early life. The exposures were lower than acceptable human occupational exposures, yet they still resulted in changes in peripheral $CD4^+$ T cell in those mice exposed in early life.

The review article by H. A. N. El-Fawal describes the challenges and the need for neuroantibody biomarkers in neurodegenerative diseases (ND). There is a very interesting discussion of the neurotoxicity of nanoparticles (NPs). In support of body burden, the author explains that immune response to an exposure may not adhere to the old dogma of dose response and that environmental agents may affect multiple organ systems, including the brain.

The rapid rise of autoimmune disease (AD) globally has led some to label the situation as epidemic. We have presented in this special issue a sampling of the many possible environmental triggers of AD, but we hope that the readers of Autoimmune Diseases will also take away from this collection one very important fact: detection of predictive biomarkers in the early stages of autoimmune disorders can be used to identify, halt, and even reverse autoimmune disease. We sound the warning bell, but we also offer readers the hope of a solution.

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 340875, 12 pages
http://dx.doi.org/10.1155/2014/340875

Review Article

Neuroantibody Biomarkers: Links and Challenges in Environmental Neurodegeneration and Autoimmunity

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Received 6 January 2014; Revised 18 May 2014; Accepted 19 May 2014; Published 23 June 2014

Academic Editor: K. Michael Pollard

The majority of neurodegenerative (ND) and autoimmune diseases (AID) remain idiopathic. The contribution of environmental chemicals to the development of these disorders has become of great interest in recent years. A convergence of mechanism between of ND and AID development has also emerged. In the case of ND, including neurotoxicity, the focus of this review, work over the last two decade in the realm of biomarker development, indicates that the immune response provides a venue whereby humoral immunity, in the form of autoantibodies to nervous system specific proteins, or neuroantibodies (NAb), may provide, once validated, a sensitive high throughput surrogate biomarker of effect with the potential of predicting outcome in absence of overt neurotoxicity/neurodegeneration. In addition, NAb may prove to be a contributor to the progression of the nervous system pathology, as well as biomarker of stage and therapeutic efficacy. There is a compelling need for biomarkers of effect in light of the introduction of new chemicals, such as nanoengineered material, where potential neurotoxicity remains to be defined. Furthermore, the convergence of mechanisms associated with ND and AID draws attention to the neglected arena of angiogenesis in defining the link between environment, ND, and AID.

1. Introduction

Identification of etiological factors that precipitate autoimmune (AID) and neurodegenerative diseases (ND) continues to be a challenge. According to the World Health Organization (WHO), 1 in 6 individuals, worldwide, suffer from a neurological disorder, mostly idiopathic, while the prevalence of AID varies according to the organ/system affected. WHO has prioritized investigations of the link between the environmental factors (e.g., chemicals) and both these disease entities [1, 2]. This is paralleled by the recently formulated strategic plan of National Institute of Environmental Health Sciences (NIEHS) in the study of environmental links to both ND and AID [3, 4]. The debilitating impact, as well as social and economic burden, particularly in children and the elderly, is compelling reason to develop biomarkers that can translate to the clinical setting in order to diagnose, evaluate sequelae, and provide a means of measuring successful intervention and to the identification of etiological factors and defining the mechanisms involved in ND and AID. It has become evident in recent years that there is a convergence of mechanisms involved in the pathogenesis of many ND and AID. Central to both is the increased angiogenesis and autoinflammatory sequelae to tissue damage. Also relevant to both is the involvement of integrins and Th17 lymphocytes. Additionally, the involvement of oxidative stress and necrotic-apoptotic events with exposure of autoantigens and the ensuing inflammation strengthens the proposition that the immune system may be a major effector of neurodegeneration. What is acknowledged in ND and AID is the loss and/or alterations in structural proteins, organ/cell-specific or common antigens, and an autoimmune, often humoral, signature. Indeed, because many of these proteins are sequestered intracellular proteins, the presence of immune effectors at the site of injury results in a humoral immune response (i.e., immunoglobulins (Ig)) directed against these autoantigens has been demonstrated in response to environmental chemical exposures. Work in our laboratory over the course of two decades has demonstrated that autoantibodies to NS proteins (neuroantibodies) may

provide biomarkers of injury and may possibly be pathogenic [5].

It should be noted that in the context of this review and neurotoxicity, the discussion focuses on the effects of known environmental and occupational chemicals known to directly cause nervous system damage and not the recently described autoimmune/inflammatory syndrome induced by adjuvants (ASIA). Yehuda Shoenfeld's [6] group coined the term ASIA, also known as Shoenfeld's syndrome, as an umbrella to describe the clinical conditions of siliconosis, Gulf War syndrome, macrophagic myofasciitis syndrome, sick building syndrome, and postvaccination phenomena which share similar signs or symptoms, some of which are neurological and may be associated with demyelination and the presence of autoantibodies to an adjuvant material. The premise in ASIA is that the adjuvant may set in motion biological and immunological events that, in susceptible individuals, ultimately lead to the development of autoimmune disease, whereas in neurotoxicity we are often dealing with chemicals that directly induce neuronal death, apoptotic and necrotic, glial dysfunction, and aberrant neurotransmission [5].

This review seeks to address a major challenge in the identification and diagnosis of neurotoxicity and, by extension, ND, which is the development and validation of biomarkers of nervous system insult. However, in so much that these suggested biomarkers rely on an immune response to autoantigen (i.e., an autoimmune response), a possible epiphenomenon secondary to insults, whether acute or chronic, it raises the question as to whether this response may prove to be pathogenic, contributing to progression of the neuropathology. It should be noted that the study of neurotoxicity provides a useful paradigm for the development and testing of potential biomarkers of nervous system insult, since the level of injury can be controlled by dose, in the case of preclinical models, and the etiological factor(s) with target selectivity (neuronal versus demyelination) can be defined based on the agents used.

2. The Generation of Neuroantibodies as Biomarkers

Since proteins, many of them intracellular, are invariably lost during the neurodegenerative process and/or neurotoxic insult, several studies have advocated for the detection of proteins in cerebrospinal fluid (CSF) and blood serum or plasma as biomarkers of neurodegeneration. This has been reviewed elsewhere [7, 8]. This approach assumes that the clinician is aware of the precipitating event (e.g., stroke, traumatic brain injury, toxic exposure). However, the reality is that functional deficits are often slow in development and indeed, in the case of Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), may take years before a diagnosis is made based on overt morphological and behavioral alterations, when intervention may be of limited benefit. Unfortunately, this approach has its limitations, most notably the short half-life of many of these proposed proteins in the periphery and/or their specificity for NS as shown in Table 1 [9-13]. In contrast, because many of these proteins are sequestered intracellular proteins, the presence of immune effectors *in situ* or following translocation to peripheral lymphoid tissue (i.e., cervical lymph nodes and spleen) results in a humoral immune response directed against these autoantigens. With the development of immunological memory (IgG) or chronic degeneration (IgM), these immunoglobulins are likely to persist. Thus, capitalizing on the immune response, work in the Neurotoxicology Laboratory has advocated and demonstrated that these autoantibodies (neuroantibodies or NAb) provide a stable signature of nervous system (NS) injury [5]. This hypothesis is summarized in Figure 1.

Table 1: Serum/plasma half-life of some proteins proposed as biomarkers of neurodegeneration.

Protein	Half-life	Reference
Neuron-specific enolase (NSE)	48 hr	[9]
S100β	20–120 minutes	[10, 11]
Glial fibrillary acidic protein (GFAP)	10–17 hr	[12]
Myelin basic protein (MBP)	4 hr (plasma) 12 minutes (serum)	[13]

However, once again, the question may be raised, if NAb are validated as biomarkers of effect, what advantage do they provide for the clinician and when would they be applied? In the context of exposure to known neurotoxicants, particularly occupational exposures, exposure monitoring, individual and environmental, is common as required by the Occupational Safety and Health Administration (OSHA) in the United States and the European Agency for Safety and Health at Work (EU-OSHA). This requires periodic monitoring of workers and their rotation within the industry based on exposure levels (e.g., blood Pb). It is conceivable that a validated high-throughput biomarker of effect may be a useful adjunct to routine exposure monitoring in occupational settings. This is particularly relevant with the increased evidence linking environment and ND, as well as gene-environmental interactions. In addition, many countries now require the determination of blood Pb levels in school aged children. Environmental and occupational chemicals, solvents, polychlorinated biphenyls (PCB), methyl mercury, lead, and pesticides are known to be neurotoxic in adults and developmental neurotoxicants in children, yet diagnosis of effects is often delayed until overt behavioral manifestations occur, despite knowledge of exposure histories. The availability of a validated biomarkers of insult would prove beneficial to detect early effects in vulnerable populations. Today, monitoring of blood cholesterol, hemoglobin A1C (HbA1c), and prostate-specific antigen (PSA), once validated, have become routine tests to evaluate risk of cardiovascular disease, diabetes, and prostate cancer, respectively. It is conceivable that a validated biomarker of nervous system insult, such as NAb, may come to enjoy such a status. Indeed, the need for routine, economical, relatively noninvasive blood-based biomarkers, with early testing, in absence of overt signs, has been advocated in the case of AD, as a result of a roundtable convened in 2012 by the Alzheimer's Association and the Alzheimer's Drug Discovery Foundation [14]. In this

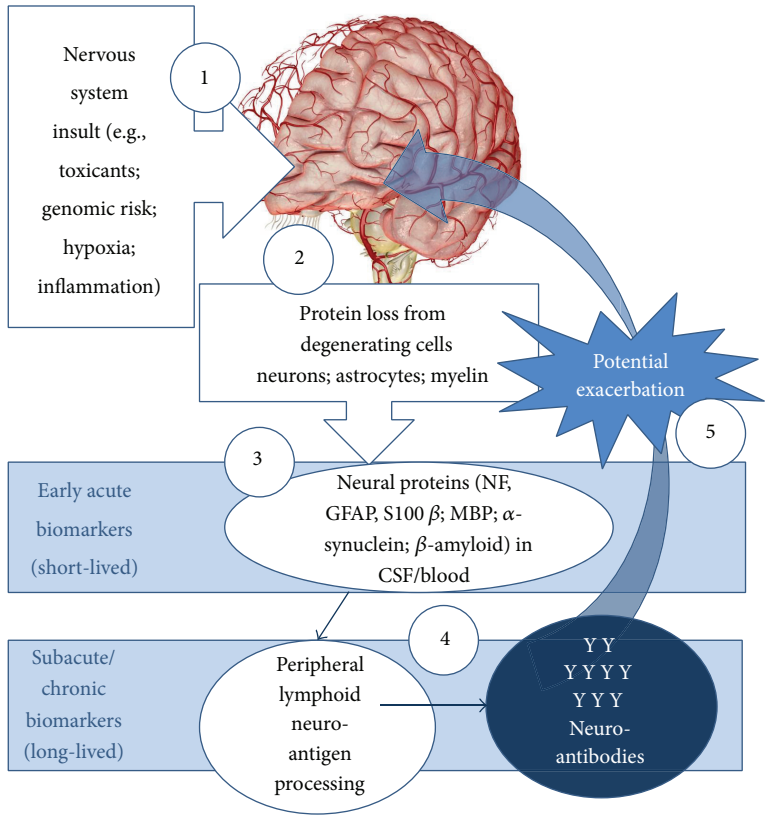

FIGURE 1: (1, 2) In the presence of toxicant-induced neurodegeneration, alterations in intracellular structural proteins and proteolysis, antigens (e.g., neuronal neurofilaments, α-synuclein, β-amyloid) are released into (3) the CSF and blood where (4) they are processed by antigen-presenting cells (APC) in the lymphoid tissue to induce humoral autoimmune responses. This autoimmune response, manifested in the form of autoantibodies, provides an accessible biomarker of neurotoxic effects [5]. These antibodies may propagate neurodegenerative changes through complement activation and direct targeting of the neural and vascular architecture, particularly in the presence of increased vascular permeability. Modified from [5].

context, it should be noted that the threshold (i.e., quantity of autoantigen) to produce an immune response is significantly less than the magnitude of neuronal loss to detect overt clinical manifestations (e.g., 60–80% nigrostriatal 3 dopaminergic neuronal loss in PD, [15]).

The parallels between neurodegeneration and neurotoxicities, including the generation of NAb, have recently been reviewed [5] and will not be detailed here. This has included preclinical and clinical studies of acute and chronic exposures to heavy metals (inorganic lead, methyl mercury, trimethyl tin) organic compounds (insecticides, solvents, and organophosphorus compounds) and in hemodialysis patients [16–24]. Rather, this review will use examples to highlight the utility of NAb and future challenges in the sphere of neurotoxicity, ND, and AID.

3. Approach

For the toxicologist, experimental and clinical, pursuing the development and use of biomarkers, it is of great benefit to bear in mind two guiding principles. The first of these is the association between the biomarker and the associated cellular substrate in terms of relevance. For the neurotoxicologist, even if evaluating an environmental exposure of unknown neurotoxic potential, proteins unique to the NS should be identified. While many investigations in the last two decades have measured blood cytokine levels with interest in a particular system insult, because of investigator specialty, the relevance of these cytokines can only be revealing if tied into that system of interest by a measure unique to that system. It also becomes incumbent on the investigator that she/he recognizes that, for environmental exposures, multiple organs may be targeted by toxic compounds. For examples, PCB or lead may target the liver, the kidney, reproductive system(s), and immune and nervous systems. The detection of biomarkers against the liver, the kidney, or reproductive system(s) does not minimize or preclude the relevance and toxicity of having detected biomarkers that indicate NS involvement, only because other organ systems are involved.

The second principle to bear in mind, particularly with the increased interest in gene-environment, environment-ND, and environment-AID associations, is that preclinical and prospective clinical toxicology studies provide a model paradigm to develop and validate emerging biomarkers for genetic/genomic risk, ND, and AID. While strides have been

Table 2: Protein antigens, their cellular source, and function.

Cell	Protein	Function
Neuron	Neurofilament (NF) Triplet*	Neuronal intermediate filaments (IF)
	NF-L (light; NF-68)	Mechanical stability of soma, dendrites, and axon
	NF-M (medium; NF-160)	Mechanical stability of soma, dendrites, and axon
	NF-H (heavy; NF-200)	Together with NF-L and NF-M, mechanical stability of the axon
Astrocyte	Glial fibrillary acidic protein (GFAP)	IF of mature astrocytes biomarker of reactive gliosis
	Vimentin	IF transiently expressed during development
Myelinating cells	Myelin basic protein (MBP)	Compaction protein of myelin in CNS and PNS
Oligodendroglia	Myelin oligodendrocyte protein (MOG)	CNS myelin
Schwann Cell	Peripheral myelin protein-22 (PMP-22)	PNS myelin

*Individual proteins differ in their immunogenicity.

made in developing biomarkers of ND, if one looks at the AD, PD, or ALS literature, these biomarkers are, more often than not, developed in patients with overt ND, robbing them of their predictive value as to disease outcome. However, with a "training set" of environmental chemicals, if a biomarker is developed that indicates the likelihood of neurodegenerative changes *prior* to emergence of frank deficits and pathology, such a biomarker would likely prove of benefit in defining idiopathic ND, as well.

A useful approach in the development and validation of NAb has been predicated on three tiers.

(1) Do NAb indicate neuropathology, regardless of a specific etiological factor (toxicity, physical trauma, ND)? This recognizes that there are common protein substrates (autoantigens) found in all neurons (e.g., neurofilaments (NF)), astrocytes (e.g., GFAP), and elaborated by myelinating cells (e.g., myelin basic protein (MBP)).

(2) Can NAb that identifies a unique cellular target (e.g., cholinergic versus catecholaminergic neurons)? This recognizes that there are neurotransmitter proteins, particularly enzymes (e.g., acetylcholinesterase (AChE), tyrosine hydroxylase), neuron-specific structural proteins (e.g., DARP-32 in dopaminergic neuron), or protein aggregates (α-synuclein, β-amyloid). It also recognizes that different neurotoxicants and neurodegenerative conditions target different populations of neural cells.

(3) Are NAb pathogenic? This recognizes that autoantibodies which may be epiphenomena, secondary to injury, have frequently been shown to have agonistic or antagonistic activity, bind (and penetrate) cells, as well as activate complement and other immune effectors.

Whenever possible, it is prudent to look for concordance between experimental and clinical studies. The study and figures below demonstrate the tiered approach using organophosphate-induced delayed polyneuropathy (OPIDP) as an example.

4. Application

In this proof of concept approach, the Neurotoxicology Laboratory has chosen the detection of NAb against proteins representing the cellular heterogeneity of the nervous system, while being common to all neurons, regardless of specialization based on neurotransmitters. Some of these protein antigens and the cellular substrates they represent are summarized in Table 2.

Organophosphorus compounds represent a large class of chemical agents that include insecticides and nerve agents, as well as chemicals that are used as lubricants, fuel, and industrial additives. The acute toxicity of the insecticides and nerve agents due to severe AChE and pseudoesterase inhibition has long been recognized. Chronic low level exposure may induce what is known as an intermediate syndrome, whereas acute single exposures of lubricant, fuel, and industrial additives may induce a central-peripheral neuropathy known as OPIDP. This is believed to be independent of anti-AChE activity, although some AChE inhibitors may also precipitate OPIDP. We have previously published result of NAb utility in OPIDP induced by phenyl saligenin phosphate (PSP) and amelioration with calcium channel blockade in the hen, the Environmental Protection Agency's (EPA) mandated model [23]. These results have been confirmed in humans by AbouDonia and colleagues [25].

In a study confirming our 2008 [23] findings, NAb, IgG (avian IgY), against NF, GFAP, and MBP were detected in hens as early as 7 days following a single dose of PSP (Figure 2). Titers of anti-NF, but not GFAP or MBP, NAb significantly correlated with changes in gait considered indicative of ataxia in OPIDP, decrease in stride length, and increase in width (Table 3). The lack of associations with GFAP and MBP is consistent with the primary targeting of neurons in OPIDP, although the earlier study did show associations with anti-MBP, secondary myelin involvement, and scored clinical ataxia [23, 26]. In addition, IgG against AChE was detected in sera of these hens (Figure 3). In a preliminary study, Ig fraction separated by dialysis, when incubated with the biventer cervicis nerve-muscle preparation [26] increased the magnitude of twitch responses of fast muscle fibers to electrical stimulation, as well as the response of slow muscle

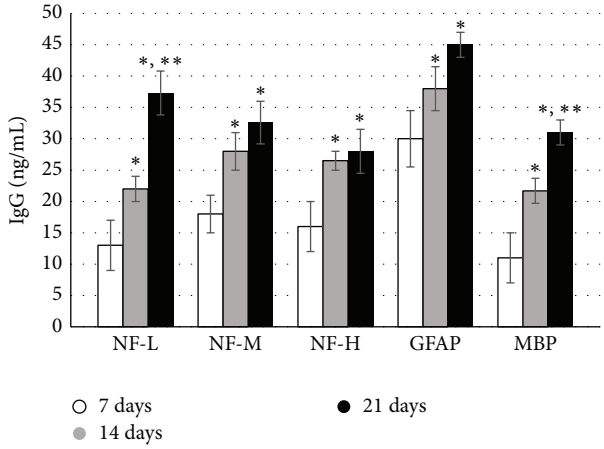

Figure 2: Serum titers of IgG against neurotypic (NF) and gliotypic (GFAP and MBP) proteins in hens ($n = 7$) administered a single dose of OPIDP-inducing phenyl saligenin phosphate (2.5 mg/kg, im). Serum was collected at 7, 14, and 21 days. Mean levels of IgG (±S.E.) were significantly ($^*P < 0.05$) higher at 14 and 21 days compared to 7 days. With the exception of anti-NF-L and anti-MBP titers, there were no statistical differences between 14 and 21 days, suggesting peaking of the anti-NF-M, anti-NF-H, and anti-GFAP titers against these antigens. There was no detectable titer of antibodies against these antigens in control hens or in sera of hens prior to PSP administration.

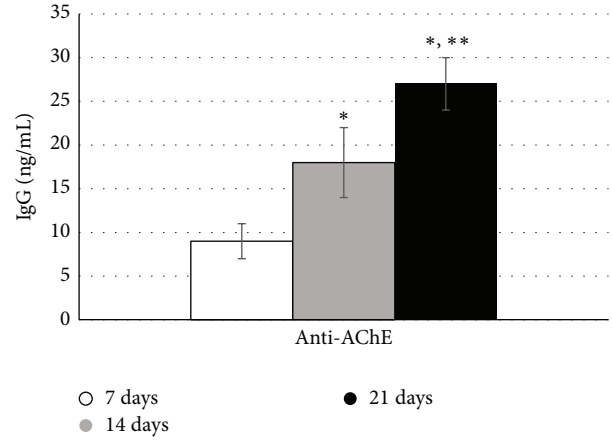

Figure 3: Serum titers of IgG against AChE, the enzyme responsible for acetylcholine hydrolysis, in hens ($n = 7$) administered a single dose of OPIDP-inducing phenyl saligenin phosphate (PSP, 2.5 mg/kg, im). Serum was collected at 7, 14, and 21 days. Mean levels of IgG (±S.E.) were significantly ($^*P < 0.05$) higher at 14 and 21 days compared to 7 days and at 21 days compared to 14 days ($^{**}P < 0.05$). Although OPIDP development is believed to be independent of AChE inhibition, some organophosphates may induce acute inhibition and phosphorylation of the enzyme.

Table 3: Pearson's correlation coefficients for antineurofilament IgG titers and changes in gait length and width of hens monitored for 21 days*.

IgG	Stride length		Stride width	
	r	P	r	P
Anti-NF-L	−0.45	0.04	0.52	0.01
Anti-NF-M	−0.47	0.04	0.48	0.03
Anti-NF-H	−0.33	0.10	0.42	0.05
Anti-GFAP	−0.11	0.63	0.21	0.36
Anti-MBP	−0.23	0.31	0.34	0.13

*Total gait measurements; $n = 21$ measured gaits.

fibers to exogenous acetylcholine (Figure 4). This indicates that serum elements may have had inhibitory activity on catabolic enzyme or agonistic activity. This was further confirmed, in part, by measuring muscle homogenate AChE activity in the absence and presence of dialysis fractions of pooled sera with detectable anti-AChE antibodies (Figure 5).

It is important to insert a word of caution regarding the use of autoantibody detection in the context of toxicological studies, in general, and for neurotoxicology, in particular. Classical toxicology (and pharmacology) is quite often wed to dose, where an increase in dose beyond threshold is predicted to result in an increase in response and by extension deficits. While, in our experience, this is frequently the case, it quite often may not hold true when measuring an immune response. Several factors are likely to account for this. In brief, the following should be recognized.

(1) Traditional measurements of internal dose, exposure biomarkers, to an environmental agent often reflect cross-sectional measurements (single time point), ignoring the kinetics and accumulation of the agent and where it may be sequestered (e.g., blood lead). They do not necessarily reflect past exposures or biological response. Borrowing from the virology and vaccine literature [27], in developing and validating NAb as biomarkers of neurotoxicity, the question is that given a particular exposure are these autoantibodies present and do they exceed background or the "natural repertoire" of antibodies. In the latter case, determination of antibody class and subclass is also likely to be useful.

(2) The immune response, cellular and humoral, is a dynamic processes, but not infinite. Classical studies of immunoglobulin responses to vaccines recognize fluctuations and peaking of immunoglobulin production to a given antigen. It is likely that autoantibody levels peak even in the presence of continued exposure or accumulation of toxic chemicals.

(3) Antibodies changes in affinity and avidity with repeated exposure to antigen and the development of immunological memory. It is useful in preclinical studies to harvest lymphoid tissue for cell isolation and *in vitro* challenge. It should also be recognized that antigens have multiple epitopes which impacts on polyclonal titer levels, as well as the exposure of new epitopes that may not be exposed in the native protein(s).

(4) In the presence of high levels of circulating released antigens, including autoantigens, due to progressive

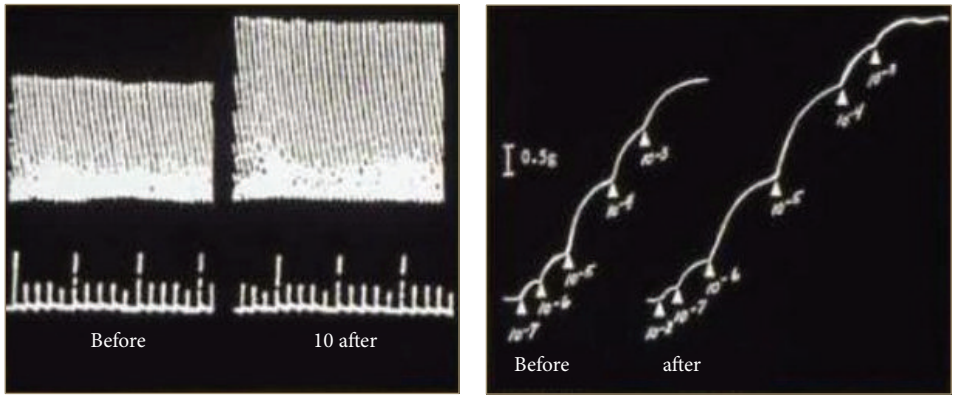

Figure 4: Representative original polygraph tracings of chicken biventer cervicis nerve-muscle preparation (in Krebs-Henseleit solution aerated with 95% O_2/5% CO_2) in response to electrical stimulation of fast twitch fibers and contraction to exogenous acetylcholine (slow muscle fibers) in the presence (after) and absence (before) of pooled immunoglobulin fraction (21 days after PSP administration). This suggests that these serum immunoglobulins may alter neuromuscular function.

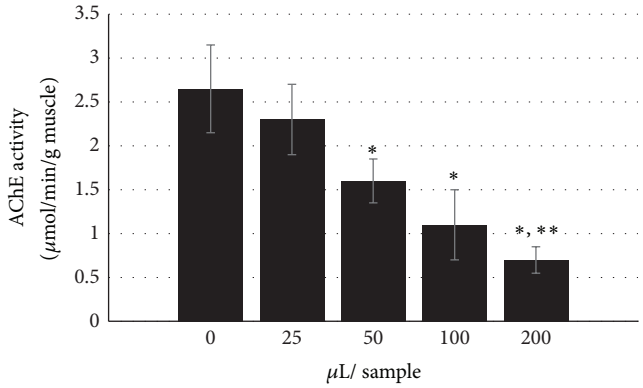

Figure 5: Activity of skeletal muscle homogenate AChE in the in the presence of different volumes of pooled serum Ig fraction from hens ($n = 7$) at 21 days following a single exposure to OPIDP-inducing phenyl saligenin phosphate (PSP, 2.5 mg/kg, im). This confirms that serum elements, possibly the detected anti-AChE, may interfere with neuromuscular function as observed in the isolated muscle preparations (see Figure 4).

and significant insult, antigen-antibody complexes are formed which gives misleading low titer levels in traditional assays (i.e., ELISA and microarray), since the Ig is already bound in the serum. It may also suggest that these autoantibodies are bound to cellular targets *in vivo*. In our own work, we have noted these decreases followed by rebound in chronic progressive neuropathologies.

It should be noted that in the context of environment-AID and environment-NAb interactions many of these issues remain to be addressed.

5. Challenges

5.1. Neurotoxicity of Nanoparticles. Linking environmental exposures to ND and AID is a challenge, particularly with the increase in both disorders. Ironically, the development of biomarkers of effects for the vast number of industrial and pharmaceutical chemicals remains a work in progress, yet with technological progress we continue to introduce new materials. The emergence of nanotechnology, which takes advantage of the unique physicochemical properties of submicron-sized nanomaterials, has profoundly impacted every aspect of daily life in the 21st century. In biomedical fields, the demand for nanotechnology and its applications is rapidly growing. Evidence for rapid growth of nanotechnology is reflected by the increase in the annual budget for the National Nanotechnology Initiative from $650 million in 2005 to $1.7 billion in 2014 [27] and by its annual growth rate of more than 17% [28, 29]. The National Science Foundation estimated that in the near future half of all pharmaceutical industry products will have some association with nanotechnology [30]. In spite of public concerns over their potential health impacts [31–35], comparatively little effort has been devoted to understanding the safety profiles of these nanomaterials. From a toxicology perspective, nanoparticles (NPs) possess important characteristic features, which differ from the features of their native parent materials and are greatly influenced by the formulation of NPs. Hence, there is a clear need to better understand the potential adverse health effects associated with emerging nanotechnologies, their biocompatibility, and potential toxicity.

Oberdörster and his collaborators [36–38] and Elder et al. [39] have demonstrated and reviewed studies that inhaled and possibly ingested or topically applied ultrafine particles can be translocated to the brain and nervous system. This translocation is likely to be via the olfactory bulbs and/or the systemic circulation. In the latter case this would involve movement across the blood-brain barrier (BBB), a key strategy for drug delivery using NP [40, 41]. Because of their size, which falls within the same range as viruses, it is possible that systemically distributed NP may also gain access to the nervous system at the neuromuscular junction (NMJ), similar to the poliovirus [42]. Transport whether from the olfactory nerves, the NMJ, or within the brain would

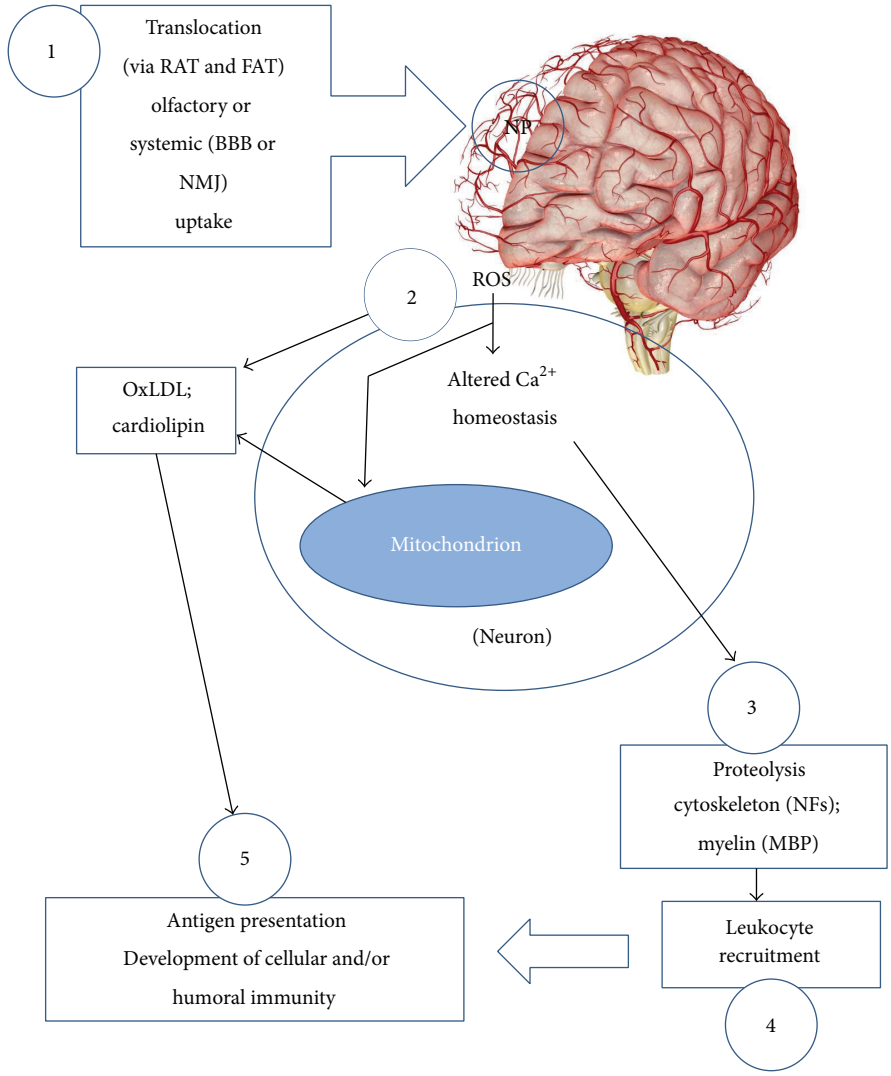

Figure 6: Mechanisms by which NP produce neurotoxicity and resulting autoantibody generation. (1) Translocation of NP introduced via the olfactory bulbs (or neuromuscular junction: NMJ) to the nervous system via retrograde (RAT) and fast anterograde (FAT) transport; (2) translocated NP may induce lipid peroxidation (e.g., LDL) and oxidative stress, as well as Ca^{2+} overload and displacement from ER/mitochondria; (3) cytoskeletal proteolysis, as a result of Ca^{2+} overload, which also results in mitochondrial derangement (e.g., cardiolipin dissociation). These events result in exposure of autoantigens; (4) recruitment of immune effectors, including microglia, *in situ*, and peripheral leukocytes; (5) antigen processing, presentation, and antibody production in lymphoid tissue.

likely rely on retrograde axonal transport (RAT) and fast anterograde axonal transport (FAT). Assessment of possible neurodegenerative changes or neurotoxic potential of NP is relatively new. Combustion-derived NPs are capable of being translocated to the brain. One example, manganese oxide, generated during arc welding, may be an occupational contributor to PD in susceptible individuals [43].

In several reviews and opinion papers [44, 45], it has been suggested that NP may precipitate autoimmune responses in exposed individuals by acting as haptens, exposing cytoskeletal elements, or promoting degeneration as a consequence of calcium overload. Based on published evidence, we have hypothesized that NPs and agents not normally considered neurotoxic may induce NS insult if generated as NPs (Figure 6).

In a preliminary study using arc-spark generated nickel NPs (≤40 nm; Ni-NP), a metal not typically associated with neurotoxicity, mice were assessed for the generation of NAb against NF, GFAP and MBP following chronic inhalation exposure of 6 weeks. The mice used included wild type C57BL/6 and their $APO^{-/-}$ (knockout counterpart). The choice of this model for neurotoxicity studies of NP is to provide a model of a human population susceptible to neurodegeneration. $ApoE^{-/-}$ mice have been shown to be susceptible to excitotoxicity in models of AD [44, 45], hyperphosphorylation of *tau* [46, 47], sensitivity to synaptic derangement [48, 49], and an increased susceptibility to oxidative damage [50]. These are believed to mimic changes associated with aging and increased susceptibility to nervous system damage following trauma and stroke. In addition,

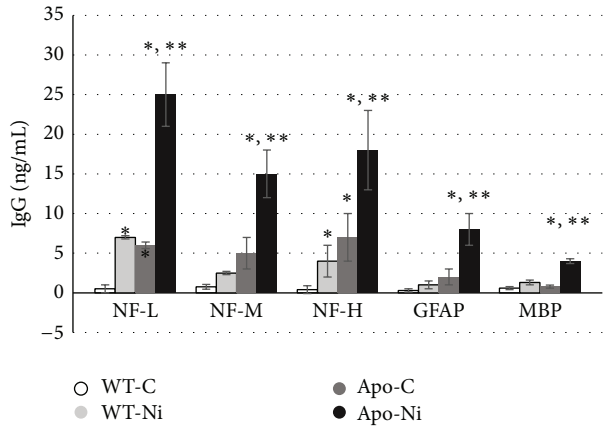

Figure 7: Inhalation exposure of wild-type C57BL/6 (WT) and ApoE knock out male mice to arc-spark generated Ni-NP (≤40 nm; 5 h/wk for 6 wks) resulted in detectable levels of IgG against neurotypic (NF) and gliotypic (GFAP and MBP) proteins. Levels of IgG were significantly ($P < 0.05$) higher in ApoE$^{-/-}$ mice exposed to Ni-NP compared to WT controls, WT exposed to Ni-NP (∗) or ApoE$^{-/-}$ control mice (∗∗) (inhalation exposures conducted by L. C. Chen and P. Gillespie at New York University Institute of Environmental Medicine).

although there are apparently no differences in lymphocyte populations, ApoE$^{-/-}$ mice tend to respond stronger to antigen challenge and may be predisposed to autoimmunity [51], including the development of autoantibodies to nervous system antigens [52].

Titer levels, both IgM and IgG, were detected in sera of mice. Levels of NAb and IgG are shown in Figure 7. While wild type mice exposed to Ni-NP had detectable levels of NAb against all antigens, the more susceptible ApoE null mice had significantly higher titers. In addition titers of anticardiolipin (ACA) and antioxidized LDL (oxLDL) were also detected (not shown).

OxLDL has been shown to accumulate in astrocytes following cerebral infarcts and stimulates IL6 release from astrocytes in culture [53], while ACA have been shown to reduce viability of neuronal cultures [54] and damage to cerebral white and gray matter and inhibit astrocyte function [55, 56].

With the increased development and use of NP in industry and as therapeutic delivery systems, the safety of nanoengineered particles and anthropogenic NP (i.e., ultrafine particle pollutants) should be a priority. Utilizing NAb detection, particularly with inhalation exposure, would provide a cost-effective option in determining neurotoxicity.

5.2. Angiogenesis, Neurodegeneration, and Autoimmunity. Angiogenesis, the elaboration of neovasculature, has emerged as playing a central role on both ND and AID. Despite this and the significant role played by vascularization in neurodevelopment, maintenance of the nervous system, and inflammatory autoimmunity, it remains a neglected area of research in the field of toxicology, outside the sphere of carcinogenesis.

Recently completed studies from the Neurotoxicology Laboratory and Pharmaceutical Research Institute have demonstrated that neurotoxic thyromimetic PCB induce angiogenesis through the $\alpha v\beta 3$ integrin receptor. Not only is this receptor responsible for mediating proangiogenic activity of thyroid hormone, but it is also targeted by vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The $\alpha v\beta 3$ integrin receptor also activates Th17 lymphocytes. The role of these cells in neuroinflammation and neuroimmunity has recently been reviewed by Vojdani et al. and Marwaha et al. [57, 58]. Th17, designated as such because of the production of IL-17, may be major effector of autoimmunity [59, 60], including the production of antibodies, where they provide B help [61, 62]. In addition, recent evidence implicates Th17 and ND such as PD, AD, and MS [63, 64]. Relevant to the toxicity of PCB is evidence that Th17 differentiation and activity may be mediated via $\alpha v\beta 3$ integrin receptors in experimental autoimmune encephalopathy [65] or via the aryl-hydrocarbon receptor (AHR) [66], of which several PCB congeners are agonists. In addition, IL-17 has been shown to induce VEGF release, thereby contributing to encephalopathy in SLE [67]. Furthermore, the participation of VEGF and angiogenesis in AID, reviewed by Shoenfeld's group [68], plays a significant role in SLE, RA, and MS. In ND, angiogenesis and hypervascularization mediated by VEGF and $\alpha v\beta 3$ integrin receptor-dependent process with ensuing hyperpermeability has been reported [69–77]. In addition, activation of the $\alpha v\beta 3$ receptor plays a role in recruitment of leukocytes in response to CCL2 (aka MCP-1) produced from astrocytes [78] and in response to ICAM and VCAM [79]. This is consistent with the localization of $\alpha v\beta 3$ integrin to T and B lymphocytes for interaction with the vitronectin matrix during migration and endothelial interactions [80]. The participation of $\alpha v\beta 3$ integrin receptor in angiogenesis and immune cell migration and activation in AID underlies its promising potential as a therapeutic target in RA [81] and experimental glomerulonephritis [82].

In the context of the NS, aside from angiogenesis, $\alpha v\beta 3$ is implicated in upregulation of glutamate receptor production and excitotoxicity [83, 84], major effector of neurotoxicity and ND [5]. It is relevant that some PCB increase extracellular glutamate availability by inhibiting its uptake into astrocytes [85]. $\alpha v\beta 3$ integrin is also implicated in reactive astrogliosis, a hallmark of neurotoxicity, and inhibition of neurite growth and process retraction [86], while $\alpha v\beta 3$ integrin receptors play a role in microglia-induced neuroinflammation models of AD, PD, MS, and ALS [87–89].

Taken together, hypervascularization in the nervous system, as demonstrated in the pathogenesis of ND, and increased permeability may provide opportunities for toxicant entry and NAb penetration into the CNS, thereby exacerbating neuropathology.

6. Conclusion

Emerging recognition that environmental agents may play a role in ND and AID and that many ND have an autoimmune and/or autoinflammatory component provides not only potential targets of intervention but also biomarkers

of effect. Defining these interactions and capitalizing on the humoral immune response, in the form of autoantibodies, provide accessible markers for predicting and diagnosing neurological outcome. As epiphenomena, secondary to initial insult, regardless of etiological factor(s), these NAbs may be indices of neurotoxicity and neurodegenerative processes, as well as a means of monitoring therapeutic efficacy. The potential contribution of NAb to the pathogenesis of ND and neurotoxicity, whether through complement activation, activation of phagocytes, or exacerbation of neuroinflammation, needs to be delineated. This may be a key to understanding disease progression and developing effective interventions in environmentally induced disease. Work over the last two decades in neurotoxicology has concentrated, for the most part, on the use of NAb in preclinical studies and remains to be validated in large cohorts of at-risk humans. In attempts at clinical translation, it should be recognized that immune responses in the presence of exposure may not adhere to the dogma of dose response. It is also important that one should not lose sight of the reality that environmental agents often demonstrate pleiotropic activity, impacting multiple systems. It should also be noted that cellular immune response remains unexplored in the context of neurotoxicology.

In tandem with the use NAb of as potential biomarkers, initial studies have begun to explore the pathogenicity of NAb generated in response to some environmental chemicals. It is also believed that development and validation of biomarkers that capitalize on the immune response may prove useful for determining the safety and/or potential toxicity of emerging technologies (i.e., nanoengineered materials).

Finally, for both the neurotoxicologist and immunotoxicologist, a long neglected area of research at the crossroads of ND and AID is angiogenesis and the effect of chemicals, directly and indirectly, in promoting increased vascular permeability and providing an avenue of disease exacerbation.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 152428, 12 pages
http://dx.doi.org/10.1155/2014/152428

Review Article

Autoimmunity and the Gut

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Received 31 December 2013; Accepted 11 February 2014; Published 13 May 2014

Academic Editor: Aristo Vojdani

Autoimmune diseases have increased dramatically worldwide since World War II. This is coincidental with the increased production and use of chemicals both in industrial countries and agriculture, as well as the ease of travel from region to region and continent to continent, making the transfer of a pathogen or pathogens from one part of the world to another much easier than ever before. In this review, triggers of autoimmunity are examined, principally environmental. The number of possible environmental triggers is vast and includes chemicals, bacteria, viruses, and molds. Examples of these triggers are given and include the mechanism of action and method by which they bring about autoimmunity.

1. Introduction

Autoimmune diseases have registered an alarming increase worldwide since the end of the Second World War. This pandemic includes more than 80 autoimmune disorders and increases in both the incidence and prevalence of autoimmune disorders such as Crohn's disease, rheumatoid arthritis, multiple sclerosis, and type I diabetes [1, 2]. In the United States, it is far more commonly found in women and is one of the top 10 leading causes of death in female children and women of all age groups. The National Institutes of Health (NIH) estimates that 23.5 million Americans have an autoimmune disease. In contrast, cancer affects 13 million Americans. Symptoms involve many medical specialties and can affect all body organs (http://www.aarda.org/autoimmune-information-statistics/).

Genetic predisposition, environmental factors (including infections), and gut dysbiosis play major roles in the development of autoimmune diseases (Figure 1). Autoimmunity develops over time, and preclinical autoimmunity precedes clinical disease by many years and can be detected in the peripheral blood in the form of circulating autoantibodies [3]. Initially, symptoms of autoimmune disorders are vague and include fatigue, low-grade fever, muscle and joint aches, and malaise. They usually progress and become debilitating with significant morbidity. Patients are often seen by physicians only after their disease process has become symptomatic, clouding the understanding of the early events leading to disease. The clinician familiar with triggers for autoimmunity can order the right combination of laboratory analyses necessary to elucidate the type and stage of the patient's autoimmune reaction. This in some cases may help the clinician initiate preventive therapies aimed at removing the offending triggers and thereby reverse the progression of the autoimmune disorder with the possibility of eliminating the autoimmune disease.

2. Genetics

There are genetic variants that predispose humans to multiple autoimmune diseases and, secondly, multiple genes predispose humans to each disease. The major histocompatibility complex (MHC) is central in mediating inflammatory responses to pathogens. The unique coding or noncoding genetic variations of HLA alleles determine the antigenic responses to self- or non-self-antigens [4]. One of the most common genetic associations with autoimmune disorders is the protein tyrosine phosphatase gene PTPN22 expressed in lymphocytes. The tryptophan allele within PTPN22 has been found in patients with many autoimmune disorders, including type 1 diabetes mellitus, rheumatoid arthritis (RA),

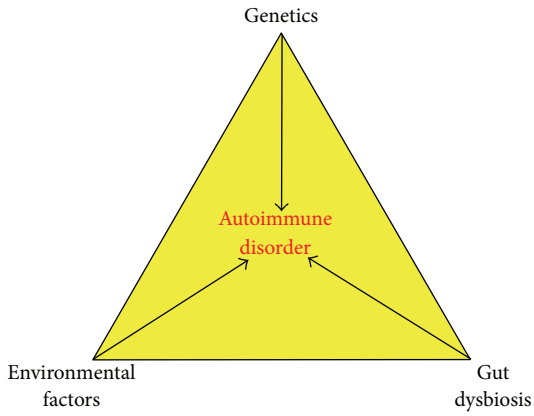

Figure 1: The triangle of autoimmune triggers. Gut dysbiosis and genetic and environmental factors play major roles in the development of autoimmune diseases.

systemic lupus erythematosus (SLE), and autoimmune thyroiditis [5, 6]. Cytokines and cytokine receptors are also associated with autoimmune disorders, as can be seen in IL-12/IL-23 pathway in inflammatory bowel disease (IBD), ankylosing spondylitis, and psoriasis [6]. Tumor necrosis factor (TNF) has been linked with autoimmune disorders, notably the TNF-inducible protein A20, which has been associated with RA, psoriasis, and SLE [7]. The importance of CD40 in the maintenance of effector T cell populations in autoimmune diseases has been described in recent studies. Patients with type 1 diabetes (T1D) have increased $CD4^{lo}CD40^{+}$ T cells in peripheral blood compared with T2D patients or healthy controls [8]. A polymorphism of CD40 that enhances CD40 signaling is common in patients of Mexican and South American descent; these two groups are known to have increased severity of SLE [9]. In celiac disease (CD), 95% of patients possess the HLA DQ gene; in RA, the HLA variants are DR genes.

There is familial clustering in some autoimmune diseases, suggesting common genetic, developmental, and environmental factors. This has been demonstrated in twin studies with higher disease concordance in monozygotic twins as compared to dizygotic twins. A large population-based survey revealed patients with multiple sclerosis (MS) or rheumatoid arthritis (RA) were more likely to have other autoimmune diseases [10, 11]. However, this concordance rate is only 10–40% for most autoimmune diseases, indicating environmental factors as playing a major role [12].

3. Environmental Factors

There are a host of environmental factors that trigger autoimmune disorders, including chemical toxicants, heavy metals, viruses, bacteria, emotional stress, and drugs. For example, adjuvants, such as aluminum hydroxide used in vaccines and medical silicones used in breast implants, can cause an autoimmune disorder known as Shoenfeld's syndrome [13]. A recent study published in the journal Apoptosis demonstrates that hepatitis B vaccine causes liver cell destruction in Hepa1-6 cells. This cell death is attributed to the use of the adjuvant aluminum hydroxide, increasingly identified as a contributing cause of autoimmune disease in immunized patients [14]. Studies show that hepatitis C is almost indistinguishable from autoimmune hepatitis based on biochemical and clinical features. Autoantibodies detected in patients with autoimmune hepatitis are also frequently found in patients with hepatitis C, and both groups of patients suffer from the same immune-mediated symptoms and diseases with chronic hepatitis C [15]. Indeed, 40–70% of patients suffering from hepatitis C also develop at least one extrahepatic inflammatory disorders, including arthritis, vasculitis, and sicca syndrome [16].

Women with silicone breast implants frequently fulfill the diagnostic criteria for autoimmune syndrome induced by adjuvants, known as autoimmune syndrome induced by adjuvants (ASIA). Although the exact mechanism is not known, medical silicones in breast implants are associated with systemic lupus erythematosus, rheumatoid arthritis, vasculitis, and progressive systemic sclerosis [17, 18].

Smoking is a known risk for RA and recent studies have demonstrated that cigarette smoking may induce citrullination of proteins in pulmonary alveolar cells. This is an important finding because antibodies to citrullinated peptides are highly specific for RA as are the HLA associations that are related to the development of these autoantibodies [19, 20].

Infectious agents, including bacteria, viruses, fungi, and parasites, are also known to trigger autoimmune disorders through several mechanisms: molecular mimicry, epitope spreading, standard activation, viral persistence, polyclonal activation, dysregulation of immune homeostasis, and autoinflammatory activation of innate immunity. It is important to note that an infection may not necessarily be the inducer but rather the total burden of infections from childhood on that trigger autoimmunity [21]. Moreover, an infection can amplify an autoimmune disease by either exacerbating an ongoing disorder, including a relapse, or by leading to chronic progressive disease [22].

An example of infectious agents associated with autoimmune disorders is the link between dysregulation of Epstein-Barr virus (EBV) with the occurrence of systemic autoimmune diseases (SADS), a group of connective tissue diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjogren's syndrome (SS), and mixed connective tissue disease (MCTD), with overlapping symptoms and antibody development. EBV is an omnipresent infectious virus, affecting approximately 95% of the world's population [23]. It is a DNA virus of the herpes family transmitted in saliva and initially infects epithelial cells in the oro- and nasopharynx. Afterwards, EBV enters the underlying tissues and infects B-cells [24]. In childhood, EBV causes a mild asymptomatic infection; in adolescents, it causes infectious mononucleosis (IM) in 30–70% of cases, and up to 20% of B-cells are infected with EBV [25]. After the first lytic infection, EBV persists in resting memory B-cells for the rest of the patient's life and can switch between an active lytic cycle and a latent state from which it occasionally reactivates, making it a continuous challenge to the patient's immune system [26].

Patients with SLE have an elevated viral load in the peripheral blood mononuclear cells (PBMCs) compared to healthy controls, anywhere from 10 to 40 times higher. The viral load is coupled with disease activity and unrelated to any immunosuppressive medication. A study found an elevated EBV DNA in the serum in 42% of patients compared with 3% of healthy controls [27–29]. Lastly, elevated levels of IgA antibodies to early antigen diffuse (EA/D) were found in 58% of SLE patients versus healthy controls and unrelated to immunosuppressive medication, demonstrating that the antibodies were not due to reactivation of EBV due to a suppressed immune system from medications [30].

In patients with RA, EBV DNA/RNA has been found in PBMCs in saliva, synovial fluid, and synovial membranes, as well as a 10-fold higher frequency of EBC-infected B-cells than in healthy controls [31–33]. This demonstrates widespread lytic EBV infection in RA patients that is also localized in the joints, signifying EBV-infected cells in the synovial inflammation that is characteristic of RA patients [34].

EBV infection has also been demonstrated in SS patients, with EBV-directed antibodies and increased viral load [35, 36]. Patients with SS also have a higher risk for EBV-associated lymphomas [37]. Elevated levels of antibodies to EBNA, VCA, and EA have been found in the serum of SS patients [38, 39]. One study showed IgG antibodies to EA/D in 36% of SS patients compared to 4.5% of healthy controls; these antibodies were not associated with immunosuppressive medication [40].

In conclusion, EBV infection is an example of one of the causal environmental factors in autoimmune disorders. As discussed above, EBV infection can lead to SADS as it can persist in the patient as a latent infection that can occasionally reactivate and cause flares as seen in chronic SADS and other autoimmune disorders.

4. Mucosal Immunity

The diet of humans has changed dramatically since the Second World War, especially in industrialized countries and in urban areas. For thousands of generations, humans ate food shortly after harvesting and when it was in season. Meat was occasionally consumed and much of it was caught in the wild. In the past 50 or so years, our foods have undergone a considerable transformation. We have developed new strains of grains, especially in wheat, rice, soy, and corn. In the United States, we use more genetically modified crops than the rest of the world combined. We use chemicals such as pesticides, fungicides, and insecticides for other crops such as fruits and vegetables; we inject dairy cows with hormones passing them on into dairy products; antibiotics, heavy metals, such as arsenic, and hormones are used in concentrated animal feeding operations (CAFO's) which include cattle, hogs, turkey, and chicken; we have chemical ingredients in our foods such as artificial preservatives, colorings, and flavorings; we use artificial sweeteners abundantly, especially in soft drinks; we consume more than twice the amount of salt that we should, leading to cardiovascular disorders and contributing to immune reactions leading to autoimmune disorders [41–44]. Our abundant use of plasticizers such as bisphenol A in food and beverage containers contributes to this overreaching environmental exposure to xenobiotics as well. The widespread use of antibiotics, antacids, proton pump inhibitors, histamine 2 blockers, and other drugs, many of which are available over the counter, adds to what we consume.

Parallel to these dietary changes, there has been a considerable increase in autoimmune diseases such as type 1 diabetes, Crohn's disease, and multiple sclerosis (MS), especially in developed industrialized countries, suggesting a link between diet and autoimmune problems. For example, it has been established that ingestion of gluten leads to gluten enteropathies and vitamin D deficiency has been epidemiologically correlated with a higher risk for autoimmune diseases [45]. Indeed, type 1 diabetes and MS are also linked to low vitamin D levels as are other autoimmune diseases [46].

There are a large number of bacteria in the oral cavity, approximately 10^{12}, which include the tongue, teeth, and periodontal tissues. In contrast, the stomach has only 10^3-10^4 bacteria and there are 10^8-10^9 in the terminal ileum. The greatest number of bacteria is in the large intestine. The majority of these bacteria, approximately 70%, cannot be cultivated by current laboratory microbiological methods [47]. The gut, with a surface area of approximately 200 square meters, is where we come into greatest contact with the outside world and it follows that the gut also has the largest collection of immune cells, consisting of 70% of all lymphoid tissues in the body [48, 49]. It serves to prevent the outgrowth of pathogenic organisms. Recent studies have discussed the human microbiome and its composition in the healthy gut [50, 51]. We carry approximately $1 \times 10^{(13)}$ microorganisms in our gut, more than 10 times the total number of cells in our bodies [52]. The two predominant bacterial phylotypes are *Bacteroidetes* and *Firmicutes* [53]. Interestingly, the number of genes of our intestinal microbiota is 150 times greater than the number of genes in the human genome (Figure 2) [54]. Diet can substantially effect the microbiota. For example, in a diet that is high in fat and protein, *Bacteroides spp*. enterotype predominate, whereas in a diet that is high in carbohydrates, *Prevotella spp.* enterotypes predominate [55].

Mucins are highly glycosylated macromolecules, forming the first barrier between the contents of the gut and epithelial cells. This barrier provides protection for the epithelial cells from direct contact with commensal bacteria and their elements (Figure 3). Changes in either the composition or amount of mucus may lead to inflammatory responses [56]. Secretory IgA is one of the main humoral defense mechanisms ensuring the proper functioning of the mucosal surface barrier. It prevents the adherence of bacteria to mucosal surfaces and the penetration of antigens into the internal environment of the host by specific and nonspecific mechanisms [57, 58]. However, in persons with selective IgA deficiency, the mucosal barrier is deficient and more permeable to immunogens and allergens. Dendritic cells are the main cells that present antigens to the adaptive arm of the mucosal immune system [59]. A mucosal immune

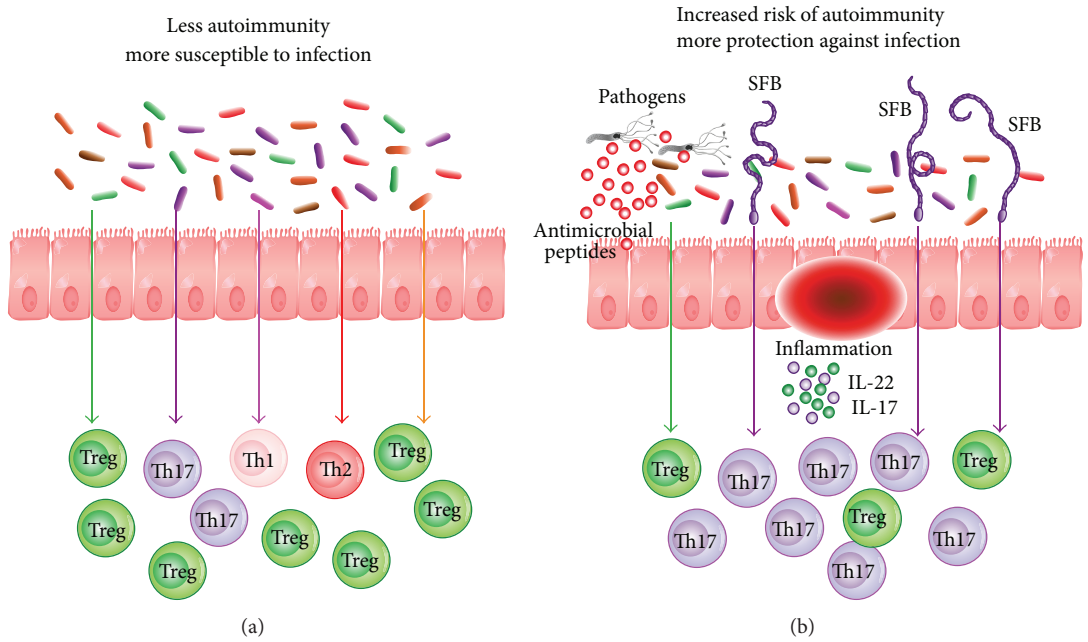

FIGURE 2: The composition of the intestinal microbiota is involved in the regulation of immune homeostasis. (a) Signals from different components of the microbiota (different colored arrows) regulate different branches of mucosal T cell response (corresponding color immune cells) in the lamina propria. (b) Changes in the composition of commensal bacteria, for example, the introduction of segmented filamentous bacteria (SFB), effect a change in the immune homeostasis, in this case, increasing the signals mediating induction of Th17 cells (purple arrows). This changes the immunological fitness of the individual. In the case of SFB, the increased production of Th17 cell effector cytokines, for example, IL-17 and IL-22, and the consecutive increase in antimicrobial peptide production from epithelial cells (red circles) increase the ability of the host to fight off intestinal infections. However, this increase in proinflammatory cytokines may also render the host more susceptible to chronic autoimmune inflammation. In this way, differences in the composition of the commensal bacteria in the gut may account for differences in individual response in the face of similar environmental challenges. (Adapted from: Ivanov I, Littman D. Segmented filamentous bacteria take the stage. Mucosal Immunology, 3(3):209-12, 2010.).

response, either one of tolerance or stimulation, depends on the partaking of different populations of dendritic cells responsible for the activation of regulatory T-cells subpopulations [60]. Activation of regulatory T-cells that inhibit the immune response and induce mucosal tolerance is dependent on the production of IL-10 and transforming growth factor-beta [61]. The maturation of dendritic cells is dependent on inducement by pathogenic organisms organisms and this then brings about the activation of effector T cells crucial for clearing infections and the prevention of subsequent infections with the same or related bacteria.

The epithelial cells of the gut have secretory, digestive, and absorptive functions and have receptors to facilitate their participation in immunological processes. The signaling pathways of these cells are highly regulated by pathways and molecules to provide a negative feedback system to avert uncontrolled inflammatory responses [62, 63]. Epithelial cells are the first point of contact for gut bacteria [64]. The epithelial layer of the gut is a major barrier between the host and the environment and is composed of a single layer of interconnected epithelial cells. This layer is reinforced by tight junctions in the paracellular spaces between the epithelial cells. These tight junctions of the epithelial layer of the gut act as a highly regulated entry that open and close depending on signals, such as cytokines and bacterial components from the lumen, lamina propria, and epithelium. Tight junctions are essential to the intestinal diffusion mechanisms [65]. The epithelial cells also make contact with the immune system of the gut and line the lamina propria of the small and large intestines and Peyer's patches which are organized lymphoid tissues. The Peyer's patches are critical for the direct antigen sampling from the gut and are where immune responses are induced and regulated. This is essential for gut health as too little or no bacterial exposure, as in germ-free conditions, can impair immune response, whereas excessive contact with bacteria may cause an increase in proinflammatory immune response. IgA and IgM derived from T-cell dependent and T-cell independent activation of B-cells and their differentiation into immunoglobulin secreting plasma cells are fundamental for the regulation of antigen penetration across the gut [66]. Immune regulation assists the gut to support microbiota and to ensure that effector immune responses are activated as a response to invading pathogens. Studies have shown the importance of Tregs in maintaining tolerance to the microbiota in the gut [67].

5. The Gut Microbiota

The gut microbiota can be influenced by several factors: the motility of the gastrointestinal tract (GIT); the intake of

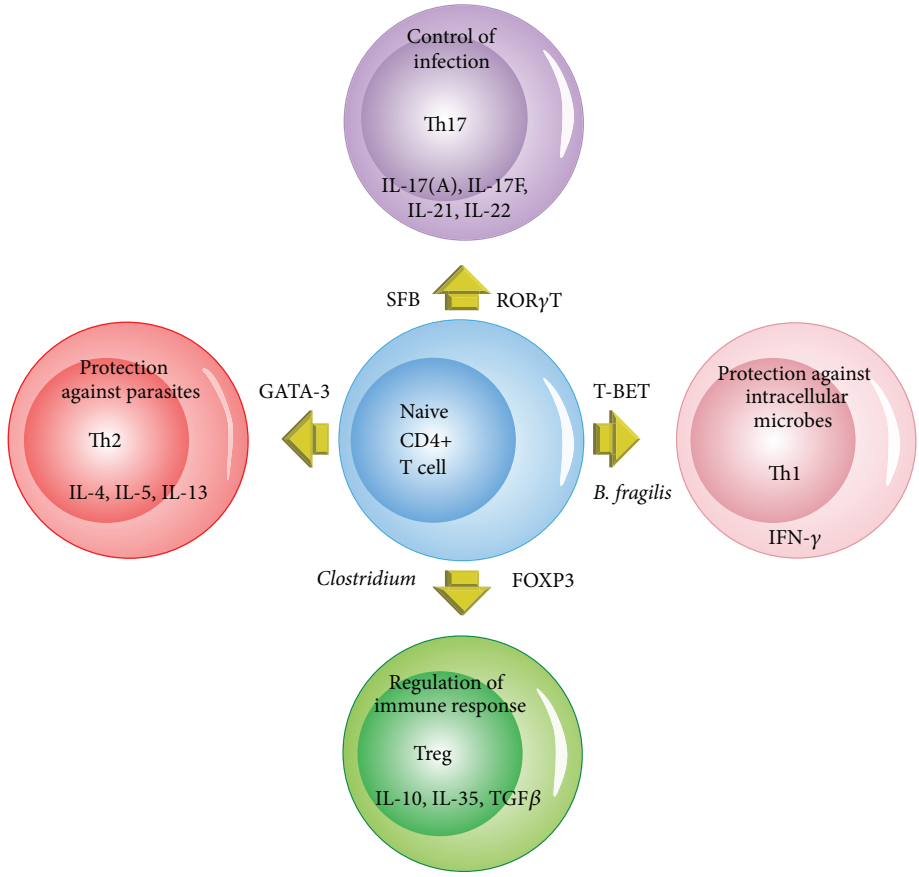

Figure 3: Commensal bacteria induce CD4+ T cell differentiation. Naïve CD4+ T cells can differentiate into four major cell types: Th1, Th2, Tregs, and Th17. The differentiation of each lineage requires the induction of a transcription factor that is unique to each lineage. Once differentiated, each lineage secretes a special set of cytokines. Th1 cells play an important role in eliminating intracellular pathogens while Th2 function to control parasitic infection. The primary role of Th17 is to control infection, while that of Tregs is to regulate immune response. The type of bacteria species that has been shown to induce a particular T cell differentiation pathway is also shown. (Adapted from Wu H, Wu E. The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes 3 : 1, 4-14, 2012.).

pharmaceutical medications, including antacids, antibiotics, and nonsteroidal anti-inflammatory drugs; smoking; the use of alcohol; the GIT transit time; mucosal blood flow; and renal clearance [68, 69]. These factors can lead to the uptake of antigens from the lumen, which play an important role in the pathogenesis of gastrointestinal disorders (Figure 6). The disproportionate uptake of these antigens, coupled with the suppression of immune responsiveness or the failure in immunological tolerance, can lead to immunological reactions both within the gut and in other organs and follow one of two pathways: physiologic transport and pathological transport. Physiologic transport consists of ligand-receptor uptake, antibody uptake, and lastly microfold or M cell transport. Pathological transport is either antigen-specific or -nonspecific. Antigen specific transport via the transcellular of paracellular pathways has the ability to bring about a specific disease.

Examples include celiac disease (CD), gliadin, and allergic gastroenteropathies with casein and beta-lactoglobulin. The antigen nonspecific transport occurs when the tight junction becomes more permeable due to environmental factors which activate inflammatory cascade via transcellular or paracellular pathways [70, 71]. Vojdani in his recent study concluded that "increased antigen uptake in the intestine precedes the onset of many immunologically mediated gastrointestinal diseases" [72]. CD is frequently associated with other autoimmune disorders, in particular type 1 diabetes (T1D) and thyroiditis. This suggests that CD shares some common pathogenic mechanisms with other autoimmune diseases [73]. Genetic studies in patients with CD and T1D have shown gut mucosal barrier dysfunction [74–76]. In CD, we now know that disease-specific autoantibodies are directed against the enzyme transglutaminase 2 (TG2) brought about by gluten-reactive T cells within the celiac lesions, giving rise to glutamic acid (deaminated glutamine) [77–79].

One of the easiest ways to affect human health is through nutrition and diet. This, in turn, is influenced to a significant degree by the gut microbiota. Going from a low fat, plant polysaccharide rich diet to a high fat, high sugar Western diet changed the microbiota in one day in GF mice. There were more members of the Firmicute classes Erysipelotrichi

and Bacilli (Enterococcus) and less Bacteroidetes associated with the Western diet. Another notable finding was that there was a significant increase in adiposity in humanized mice fed the Western diet as compared to those fed the low fat plant polysaccharide diet. These are important findings as they demonstrate that the gut microbiome can change over a very short period of time [80].

Recent studies have shown that the colonization of the small intestine in mice with a single commensal microbe, segmented filamentous bacterium (SFB), induced Th17 cells in the lamina propria. SFB are spore-forming Gram-positive bacteria related to the genus *Clostridium* and are found in many species as well as in humans. They are associated with reduced colonization and growth of pathogenic bacteria in the ileum where they are most abundant and adhere tightly to the epithelium. This colonization with SFB resulted in augmented resistance to *Citrobacter rodentium*, an intestinal pathogen, and with increased expression of genes linked with inflammation and antimicrobial defenses [81]. TGF-β differentiate Th17 and Treg cells and are defined by the expression of lineage-specific transcription factors RORγt and Foxp3 [82–86]. Th17 cells are essential mediators of autoimmune diseases, as they have potent inflammatory effects; they have important roles in protection from bacterial and fungal infections, especially at mucosal surfaces, and secrete IL-17, IL-17F, and IL-22. The increased production of Th17 cell effector cytokines, for example, IL-17 and IL-22, and the consecutive increase in antimicrobial peptide production from epithelial cells augment the ability of the host to fight off intestinal infections. At the same time, however, this increase in proinflammatory cytokines may render the host more susceptible to chronic autoimmune inflammation [87].

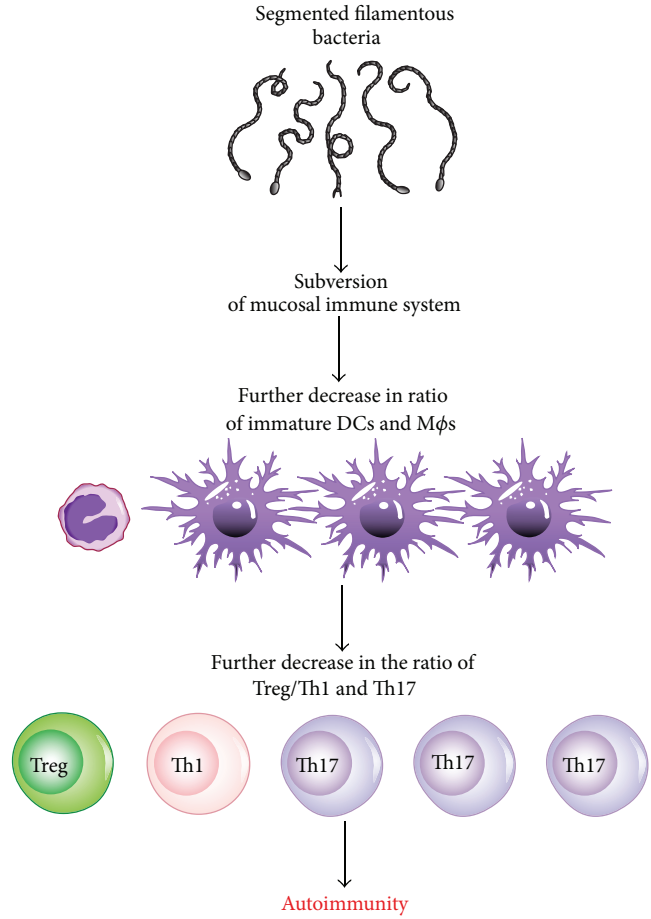

Figure 4: How segmented filamentous bacteria (SFB) can change the ratio between Th17 and Tregs, leading to autoimmunity.

6. The Gut and Rheumatic Disease

Rheumatoid arthritis (RA) is one of the most prevalent systemic autoimmune diseases targeting principally the joints. RA leads to joint deformity, disability, and increased mortality without treatment. It is a multifactorial and complex disease caused by genetic and environmental factors with increased production of self-reactive antibodies and proinflammatory T lymphocytes [88]. In RA there is a prolonged period of autoimmunity with circulating autoantibodies such as rheumatoid factor and anticitrullinated peptide antibodies. This preclinical state may last many years without any clinical signs or symptoms of inflammatory arthritis. However, there is an increase in antibody titers and epitope spreading with elevation in circulating proinflammatory cytokines before the onset of clinical disease. In these situations, environmental factors may be the triggering event for systemic joint inflammation. Microbes from the periodontal tissue, the airways, and the gut microbiota have been implicated [89, 90].

RA has pathogenic disease-specific autoimmunity to citrullinated proteins. Citrullination, a modification of arginine catalized by peptidylarginine deiminase enzymes, has the ability to change the structure, antigenicity, and function of proteins. Porphyromonas gingivalis, a major pathogenic bacterium related to gingivitis, is linked to RA in epidemiological studies and is the only bacterium that expresses endogenous citrullinated proteins [91].

The gut microbiota composition can be changed by antibiotics. Studies have shown that antibiotic use reduced Bacteroides and Bifidobacterium and led to the growth of Campylobacter, Streptococcus, Leuconostoc, or yeasts like *Candida Albicans* in the gut [92].

An alteration causing an imbalance in the gut microbiota can change T-cell responses and modulate systemic inflammation. Germ-free mice lack Th17 cells; when the gastrointestinal tract of these mice is colonized with segmented filamentous bacteria (SFB), Th17 cells are induced to accumulate in the lamina propria [81] (Figure 4). Mice raised in germ-free environments are persistently healthy. By introducing specific gut bacterial species, joint inflammation ensues. Treatment with antibiotics in these mice will prevent and negate a rheumatoid arthritis-like phenotype. When the gut of arthritis-prone K/BxN mice gut is colonized with SFB, the inflammatory disease is potentiated by Th17 cells [82]. An imbalance in gut microbiota with predominance of SFB may result in the reduction of functions of Treg cells and a predisposition to autoimmunity. This may affect systemic inflammatory processes and may partially be why

there is reduced Treg function in patients with RA. This demonstrates that T cells whose functions are under the control of the gut commensal microbiota can also be the effectors of pathogenesis in autoimmune disorders [83].

A recent study showed that 75% of patients with new onset RA (NORA) carried *Prevotella copri* in their intestinal microbiota. Furthermore, 37.5% of psoriatic arthritis patients also had *Prevotella copri* in their gut compared to 21.4% of healthy controls [93]. This again demonstrates the effects of the environment from the gut microbiota aspect on autoimmune disorders.

Patients with juvenile idiopathic arthritis have been shown to have increased intestinal permeability along with gastrointestinal symptoms, suggesting a role for intestinal changes in the pathogenesis of rheumatic diseases [94]. Arthritis is frequently found in patients with IBD, again suggesting the participation of the gut in immune-mediated rheumatic disorders [95]. IBD is an autoimmune disorder affecting the GI tract in two main forms: Crohn's disease and ulcerative colitis. The phyla of gut microbiota in patients with IBD greatly differ when compared with normal patients [96]. Studies have shown that antibiotics treatment benefits patients as well as animal models of IBD, indicating that bacteria play an important role in the pathogenesis [97]. A recent study has identified the specific microbiota in the dysbiosis of IBD patients. These patients have an overgrowth of proteobacteria and a reduction in Firmicutes and Bacteroides species [98].

Reactive arthritis and autoimmune reactions in joints may be triggered by infections with intestinal microbial pathogens, including Salmonella, Shigella and Yersinia [99]. Antibodies against antigens of certain species of gut bacteria, for example, Proteus, suggest that these bacteria and rheumatoid arthritis have a pathogenic relationship [100]. This parallels the findings in patients with ankylosing spondylitis having increased titers of anti-Klebsiella antibodies suggesting again a bacterial triggering factor [101].

7. The Gut and Neuroautoimmunity

The gut-brain axis acts as a bidirectional communication between the brain and the gut (Figure 5). The brain modulates gastrointestinal function and the gastrointestinal system is monitored by the brain via neural, immunological, and endocrine mechanisms. The development and function of the enteric nervous system are influenced by the intestinal microbiota [102]. The gastrointestinal system is directly controlled by the enteric nervous systems, the "second brain". This system consists of more neurons than the spinal cord, mainly in the myenteric and submucosal plexuses. Neuropeptides are able to increase the permeability of tight junctions to macromolecules and thereby modify the function of the mucosal barrier [103, 104].

In adults, chronic stress affects the composition of the gut microbiota with increase of *Bacteroides* spp. and *Clostridium* spp. Coupled with this are increasing levels of IL-6 indicating immune activation [105]. Chronic stress also makes the gut leaky, increasing circulating levels of LPS. Findings of altered

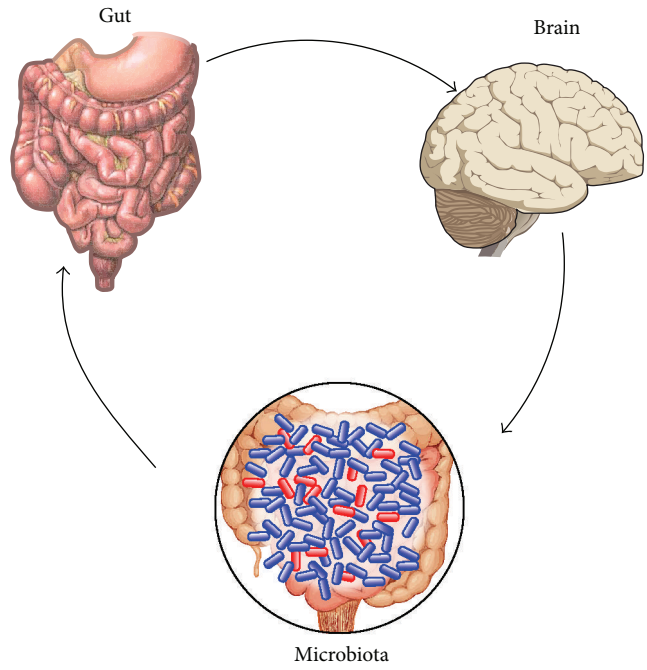

Figure 5: The interconnection of the gut, brain, and microbiota.

intestinal permeability (leaky gut) may play a pathogenic role in patients with depression and their first-degree relatives [106, 107].

Multiple sclerosis (MS) is one of the most frequent and severe demyelinating neurological diseases, mainly affecting young people, eventually leading to their becoming disabled. Increased intestinal permeability in these patients and in their relatives has been reported. MS has also been related to infections with bacteria and viruses [108]. Experimental autoimmune encephalomyelitis (EAE) is the animal model widely used for MS. A study in germ-free mice showed attenuated induction of EAE by myelin oligodendrocyte glycoprotein (MOG) peptide in complete Freund's adjuvant [109]. Another study with mice genetically predisposed to develop EAE showed that when they were housed in germ-free or pathogen-free conditions, they were protected from developing EAE. Once they reached adulthood and had normal gut colonization, the protection was lost [110].

There are increasing numbers of studies demonstrating the importance of the permeability of the gastrointestinal tract to large molecules and how this is linked to the development of various neurodegenerative disorders, including Parkinson's disease (PD). Lewy bodies, the pathological hallmark of Parkinson's disease, were found in intestinal biopsies of patients with PD [108–110].

8. The Other Side of the Same Coin

The gut microbiome can also help the host. There are commensal gut bacteria that can ameliorate disease. For example, in immunocompromised mice, *B. fragilis* can lessen the colitis induced by Helicobacter hepaticus via its production of PSA,

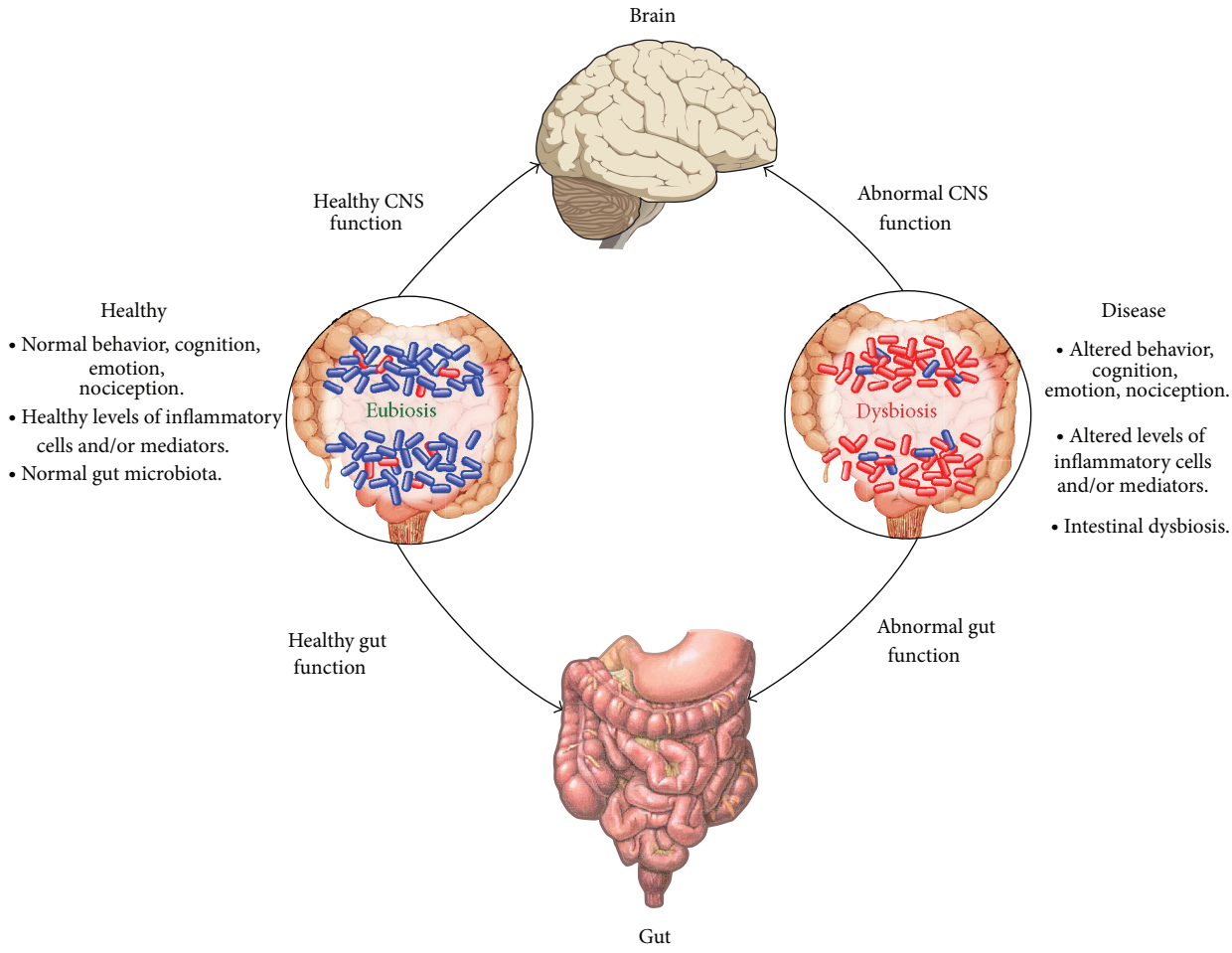

Figure 6: Impact of the gut microbiota on the gut-brain axis in health and disease. It is now generally accepted that a stable gut microbiota is essential for normal gut physiology and contributes to appropriate signaling along the gut-brain axis and, thereby, to the healthy status of the individual (a). On the other hand (b), intestinal dysbiosis can adversely influence gut physiology, leading to inappropriate gut-brain axis signaling and associated consequences for CNS functions and resulting in disease states. Conversely, stress at the level of the CNS can affect gut function and lead to perturbations of the microbiota. (Adapted from: Cryan J, Dinan T. Mind-altering microorganisms: the impact of the gut microbiota on brain and behavior. Nat Rev Neurosci., 13(10):701-12, 2012.).

which stimulates the anti-inflammatory IL-10 production from CD4+ T cells and the downregulating of proinflammatory IL-10 production in the colonic tissues. This, in turn, suppresses disease [111]. In another example, short-chain fatty acids (SCFAs) produced by the gut microbiota interact with G-protein-coupled receptors expressed on immune cells and reduce inflammation in the dextran sulfate sodium (DSS-)induced colitis model [112].

9. When Did It All Start?

Bacterial colonization during and shortly after birth plays a major role in the formation of gut microbiota. Factors affecting the communities in this microbiota include premature birth, Caesarean section versus vaginal birth, breast milk versus commercial formula, and many more. For example, premature infants were colonized principally by *C. difficile*. Infants born vaginally were colonized mostly by bacterial communities similar to their mother's vaginal microbiota, including *Lactobacillus*, *Prevotella*, or *Sneathia* spp, whereas Caesarean section born infants were colonized by bacteria found on the skin surface, including *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* species. Formula fed infants had colonization predominantly by *Staphylococci*, *E. coli*, *C. difficile*, *Bacteroides*, *Atopobium*, and *Lactobacilli* [113–117]. Infants delivered via Caesarean section have an increased risk of developing asthma, allergies, and autoimmune disease in later childhood [118, 119]. These are clear demonstrations of the importance of the gut microbiota starting at birth and affecting the patient years later.

10. Conclusion

Factors such as genetics, the environment, infections, and the gut microbiota all play a role in the mediation of autoimmune disorders. There have been tremendous recent advances in

our understanding of the interplay of these factors. It is clear that the gut microbiota has a profound and long-term effect starting at birth on the host immune system. It is also evident that it plays a significant role in autoimmune diseases both inside and outside the gut. There are still questions that remain to be answered: does the immune system shape the gut microbiota or vice-versa? This complex and dynamic symbiosis needs further elucidation and may help in determining the outcome of autoimmune diseases in patients. The clinician can assist the patient by being aware of the triggers of autoimmune disorders and monitoring immune and autoimmune markers in the peripheral blood, thereby being able to take preventive measures to hopefully avert the progression towards an autoimmune disease.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 239358, 10 pages
http://dx.doi.org/10.1155/2014/239358

Research Article

Elements of the B Cell Signalosome Are Differentially Affected by Mercury Intoxication

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Received 1 October 2013; Revised 7 January 2014; Accepted 20 January 2014; Published 4 May 2014

Academic Editor: K. Michael Pollard

It has been suggested that environmental exposures to mercury contribute to autoimmune disease. Disruption of BCR signaling is associated with failure of central tolerance and autoimmunity, and we have previously shown that low levels of Hg^{2+} interfere with BCR signaling. In this report we have employed multiparametric phosphoflow cytometry, as well as a novel generalization of the Overton algorithm from one- to two-dimensional unimodal distributions to simultaneously monitor the effect of low level Hg^{2+} intoxication on activation of ERK and several upstream elements of the BCR signaling pathway in WEHI-231 B cells. We have found that, after exposure to low levels of Hg^{2+}, only about a third of the cells are sensitive to the metal. For those cells which are sensitive, we confirm our earlier work that activation of ERK is attenuated but now report that Hg^{2+} has little upstream effect on the Btk tyrosine kinase. On the other hand, we find that signaling upstream through the Syk tyrosine kinase is actually augmented, as is upstream activation of the B cell signalosome scaffolding protein BLNK.

1. Introduction

Mercury is a potent immunotoxicant. Early experiments demonstrated that exposure to high or moderate levels of mercury induced immune cell death through either necrosis or apoptosis. Fortunately, exposure to high levels of mercury is no longer common, but large segments of the population continue to be exposed to low levels. In contrast to higher level exposures, low level exposure to mercury is not linked to immune cell death, but rather to immune disorders characterized by immunoproliferation and autoimmunity [1, 2].

Today in the US most exposure to mercury is the result of consumption of seafood contaminated with organic mercury. It has also been reported that high fructose corn syrup, a major constituent of most processed foods, often contains surprisingly high levels of inorganic mercury (Hg^{2+}), and so low-level Hg^{2+} exposure is now a source of concern [3]. However even with respect to dietary ingestion of organic mercury, the majority is fairly quickly metabolized to Hg^{2+}, so that over time mercury burdens in organs such as brain or spleen normally encompass both inorganic and organic species, even under conditions where exposure has been limited to organic mercury [4, 5]. In fact studies in normal individuals, as well as persons known to have been accidentally exposed to high levels of dietary organic mercury, have shown that eventually about 70% of the total mercury burden in spleen and brain is in the form of inorganic mercury [6, 7]. Most importantly, findings show that in mice exposed only to organic mercury, Hg^{2+} produced as a result of the metabolism of organic mercury, but not residual organic Hg, is the mercury species directly responsible for mediating an autoimmune inflammatory response. In other words, with respect to autoimmune disorders and inflammatory processes Hg^{2+} is the active metabolite [8, 9].

In principle, autoimmune disorders might result from a genetic defect in, or environmental interference with the control of central tolerance, the mechanism whereby the immune system neutralizes self-reactive immature lymphocyte clones. In immature B cells the B cell receptor (BCR) complex signaling pathway is of critical importance for this process [10, 11]. In fact it has been shown that there is a direct

connection between suppression of BCR signals and induced failure of central tolerance in the B cell compartment leading to increased mature autoimmune B cell clones [12]. The association of exposures to low levels (levels which are not immunosuppressive *in vivo* or trigger immune cell death *in vitro*) of Hg^{2+} with autoimmunity suggests that Hg^{2+} could potentially interfere with signal transduction pathways in immature B cells in a manner so as to compromise central tolerance.

WHEI-231 is a well-characterized mouse B cell line that exhibits many of the characteristics of immature B cells. In particular, in contrast to mature B cells for which stimulation of the BCR is associated with mitogenesis and increased proliferation, WEHI-231 in a process similar to immature B cells undergoes growth arrest and apoptosis in response to BCR stimulation [13, 14]. We have previously shown that exposure of WEHI-231 to low levels of Hg^{2+} interferes with the BCR signaling pathway in triggering growth arrest and apoptosis [15]. Furthermore in immature B cells, growth arrest and apoptosis consequent to BCR signaling are dependent on activation of ERK [16]. We have shown in WEHI-231 that, although ERK does not seem to be a direct target, low level exposure to Hg^{2+} attenuates activation of ERK1/2 during BCR signaling [17], thus suggesting that Hg^{2+} acts on an upstream element of the BCR signal transduction pathway to initially suppress ERK activation and subsequently growth arrest and apoptosis after BCR engagement of an agonist ligand [17].

Phosphoflow cytometry is a relatively new technology which has been shown to be far superior to traditional western blotting technology in investigating phosphorylation dependent signaling pathways in immune cells, and in particular B cells [18–21]. In the experiments outlined below we utilize multiparameter phosphoflow cytometry to simultaneously quantify the activity of several different elements of the BCR signal transduction pathway on a single cell basis. By capturing multiple interrelated phosphorylation patterns in Hg^{2+} intoxicated (exposed) and BCR stimulated WEHI-231 cells we have gained insight into the mechanism whereby Hg^{2+} at low exposure levels interacts with upstream elements of the BCR signal transduction pathway in B cells.

2. Materials and Methods

2.1. Reagents. $HgCl_2$ (≥99.5% pure) was obtained from Aldrich Chemicals (St. Louis, MO).

2.2. Cells. WEHI-231 cells were obtained from American Type Culture Collection (Manassas, VA). They are maintained in RPMI 1640 containing L-glutamine (HyClone, Logan, UT) and supplemented with 10% fetal bovine serum (FBS, HyClone), 50 μM 2-mercaptoethanol, and 1X antibiotic antimycotic solution (Mediatech, Herndon, VA) in a humidified 5% CO_2 atmosphere. Cells were passaged twice a week at a concentration of 1×10^5 cells/mL. Cell viability was monitored visually by Trypan blue exclusion.

2.3. Antibodies. Polyclonal goat affinity-purified antibody to mouse immunoglobulin mu chain was purchased from MP-Biomedicals-Cappel, Solon, OH. Fluorescently labeled antibodies used for cytometric analysis of phosphorylated ERK1/2, Syk, BLNK, and Btk were purchased from BD Biosciences (San Jose, CA).

2.4. Phosphospecific Flow Cytometry. WEHI-231 cells were counted, washed in serum-free RPMI medium, and resuspended at 3×10^6 cells/mL in the presence or absence of varying concentrations of $HgCl_2$ in polypropylene tubes. Subsequently, 1×10^6 cells were added to tubes containing goat anti-mouse immunoglobulin specific to the mu chain. Cells were incubated for the specified period of time at 37°C. At the end of stimulation (0.5, 1, 5, or 10 minutes), the cells were fixed by adding an equal volume of 4% paraformaldehyde, pH 7.4, and incubating for 10 minutes at 37°C. The cells were diluted in Dulbecco's phosphate-buffered saline, pH 7.4, pelleted by centrifugation, resuspended in 90% methanol, and then incubated on ice for at least 30 minutes to permeabilize the plasma membrane. Following permeabilization the cells were pelleted to remove the methanol and then washed twice in sample buffer (Dulbecco's phosphate-buffered saline, 1% FBS, and 0.1% sodium azide). The cells were resuspended in an antibody cocktail containing fluorescently labeled antibodies specific for the phosphorylated residues of ERK1/2, Syk, BLNK, or Btk according to the manufacturer's instructions. The cells were incubated for 1 to 2 hours on ice and then washed 3 times with sample buffer. The cells were kept in the dark at 4°C until being analyzed on a Beckman-Coulter Cyan ADP flow cytometer.

2.5. Data Analysis. For all experiments, 10,000 events were acquired per sample and the data were initially analyzed using Sumit (Beckman Coulter; Miami, FL) and/or FlowJo (Tree Star; Ashland, OR) software. Additional data analysis and graphing was done using Excel (Microsoft; Redmond, WA) and Sigma Plot (Systat Software; San Jose, CA) software. For any fluorescently labeled cell population the Mean Fluorescence Index (MFI) is defined as median fluorescence intensity of the population.

3. Results and Discussion

3.1. Phosphospecific Flow Cytometric Analysis of ERK Activation during BCR Signaling in WEHI-231 B Cells. In our previous investigation of the effect of mercury on BCR signal transduction we employed western blotting technology to show that ERK activation was impaired [17]. However we felt that the application of the newer phosphospecific flow cytometric technology might offer better delineation of the effects of Hg^{2+} on BCR signaling. Accordingly, we first employed phosphospecific flow cytometry to investigate the kinetics of BCR activation of ERK 1/2 in control WEHI-231 cells. ERK1/2 are serine/threonine kinases which in B cells are central to the BCR signal transduction pathway and upon BCR signaling become activated by dual phosphorylation of a canonical threonine/tyrosine motif [22]. In Figure 1, as described in the materials and methods section, WEHI-231 cells were or were not treated with anti-mu (25 μg/mL) to stimulate the BCR.

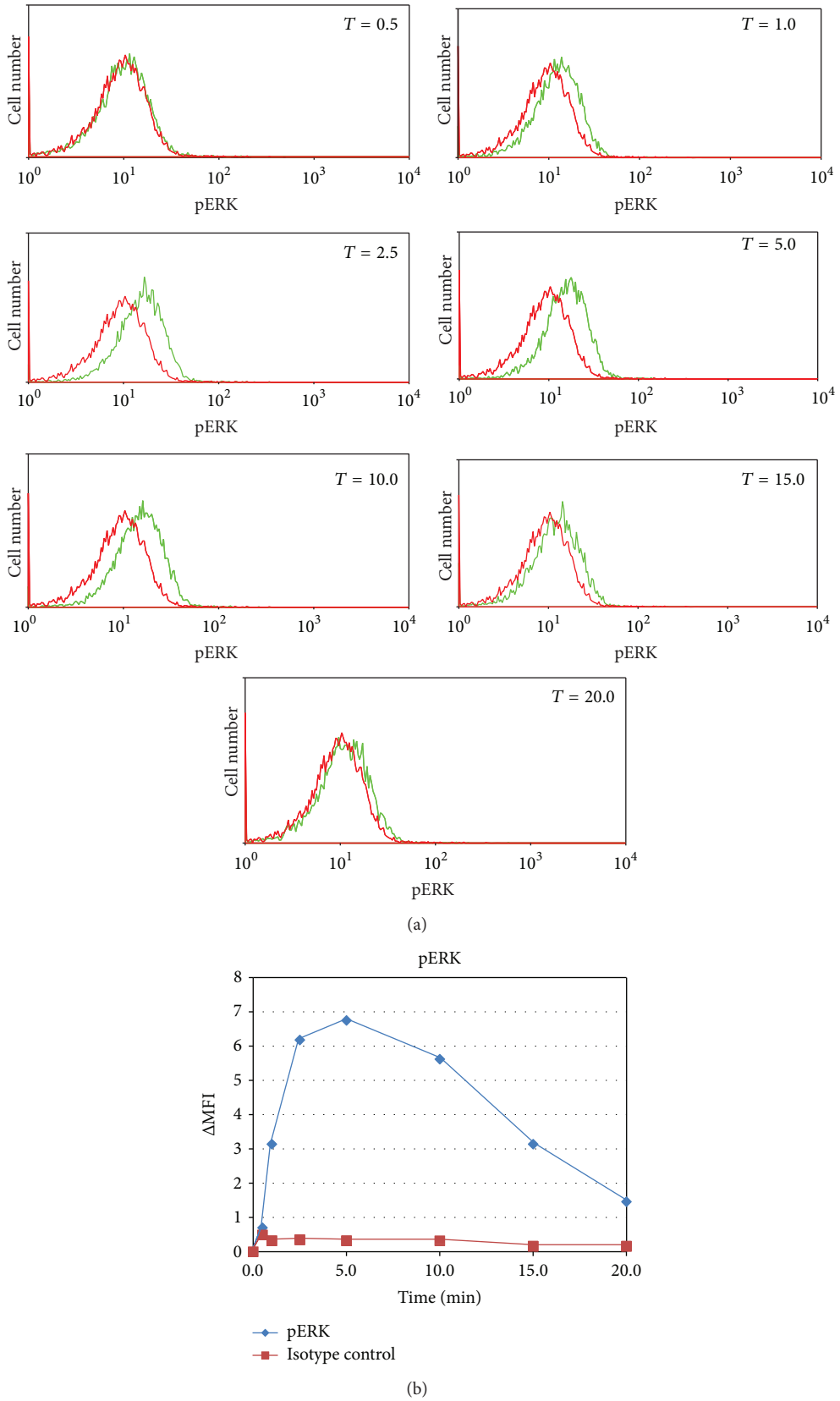

Figure 1: Time course of pERK in WEHI-231 B cells after stimulation of the BCR with anti-mu as determined with phosphoflow cytometry. (a) Histograms at various times for pERK (green, light hue) and isotype control (red, dark hue). (b) Change in MFI (as calculated from the histograms in (a) as a function of time after BCR stimulation.

At timed intervals the cells were probed with a fluorescently labeled antibody specific to dually phosphorylated ERK 1/2 (anti-pERK), or else an isotype control antibody. The level of ERK 1/2 activation was then determined by phosphospecific flow cytometry. In Figure 1(a) each of the plots represents a different time after BCR stimulation. In each plot the red line represents the control histogram for which no anti-mu was added, while the green line represents the histogram at that time point arising from BCR activated cells. Initially the two curves overlap and then begin to separate as ERK 1/2 becomes activated in stimulated cells. Maximum separation appears at the 5 minute mark, after which they move together as ERK 1/2 activation is attenuated.

The kinetics of ERK activation resulting from BCR stimulation are perhaps more easily appreciated in Figure 1(b). In Figure 1(b), for each of the distributions where the cells have been probed with anti-pERK (stimulated or unstimulated) shown in Figure 1(a), the Mean Fluorescence Index (MFI), defined as the median fluorescence intensity for that distribution, has been calculated. Then for each time point, the difference between the MFI for the untreated cells and the MFI for the BCR stimulated cells at that time has been plotted. The same procedure was then applied to cells which were probed with the isotype control antibody, and the difference in MFI between anti-mu treated cells and untreated cells plotted.

Generally speaking, because of the large number of cells examined, population differences in MFI determined by flow cytometry tend to be highly significant. For instance the standard error of the median for a normally distributed variable is given by $1.25\,\sigma/\sqrt{n}$, where σ is the standard deviation of the distribution, and n the number of independent measurements. For the distributions shown in Figure 1(a) (as well as in the other figures in this paper), the σ's are of the order of the MFIs, but since each distribution represents about 10,000 cells, the standard errors are of the order of 1% of the MFIs.

Another way of appreciating the significance of the MFI measurements in Figure 1(b) is to note that, in Figure 1, fluorescence intensity measurements are proportional to the log of the actual fluorescent signal. As a result relatively small changes in the MFI are often significant. By comparing the histograms shown in Figure 1(a) with the MFI differences plotted in Figure 1(b), it is evident that, for all time points between 2.5 and 15 minutes, the histograms of treated cells are significantly different from control cells. In other words, for this experiment, differences of MFI between control and BCR stimulated cells of at least 2 can be considered as meaningful.

Figure 1 demonstrates that in WEHI-231 the maximum pERK response to anti-mu stimulation of the BCR occurs in about 5 minutes, after which it trends towards baseline. However in Figure 1 we employed anti-mu at 25 μg/mL to stimulate the BCR. This initial 25 μg/mL figure was based upon our previous experience with this system [17]. However the anti-mu reagent we utilize is a polyclonal antibody, and the potential exists for batch to batch variations in the agonist response. To assure ourselves that we were utilizing an appropriate concentration of stimulating antibody, in Figure 2 we investigated the maximum pERK response at 5 minutes as a function of the stimulating antibody concentration. Accordingly, cells were treated with various concentrations of anti-mu for 5 minutes, and then pERK levels determined by flow cytometry utilizing the anti-pERK reagent used in Figure 1, or an isotype control antibody. pERK and isotype control staining profiles for each concentration of anti-mu were then compared to staining profiles of cells which had not been treated with anti-mu and the difference in MFI was determined and plotted.

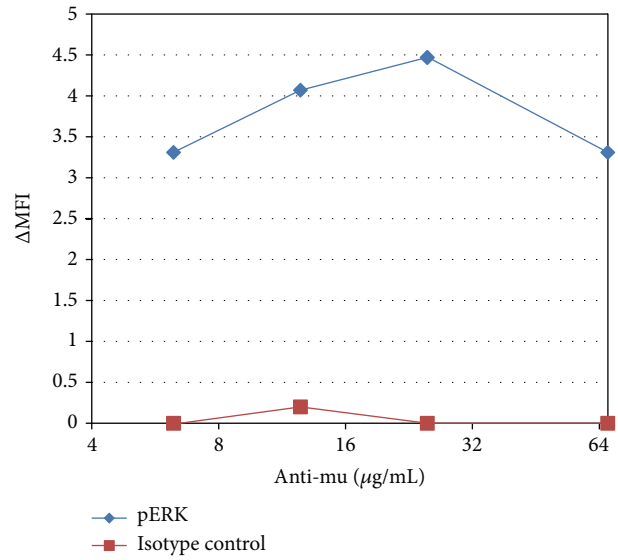

Figure 2: Change in pERK at 5 minutes after stimulation as a function of concentration of anti-mu used to stimulate the BCR.

3.2. Compared to Anti-Mu, Low Concentrations of Hg^{2+} Alone Only Marginally Perturb the Activity of ERK and Syk in the BCR Signaling Pathway. The tyrosine kinase Syk is an important element of the BCR signaling pathway upstream of ERK. During BCR signaling, activation of ERK is known to be dependent upon activation Syk, and, like ERK, Syk activity is increased by tyrosine phosphorylation [22, 23]. In later experiments we will be looking at the effect of Hg^{2+} on ERK and upstream elements of the BCR pathway during signaling, so for control purposes we measured the level of activated ERK and Syk (pERK and pSyk) which was induced by Hg^{2+}, alone, in the absence of specific BCR signaling, to those induced by anti-mu through binding to the BCR. Accordingly WEHI-231 cells were treated with (0 μg/mL, 2.5 μg/mL, and 5 μg/mL) Hg^{2+}, and, at various time points after addition of Hg^{2+}, cells were stained with different fluorescently labeled anti-pERK and anti-pSyk. We have previously shown that treating WEHI-231 cells with concentrations of 5 μg/mL $HgCl_2$ for 10 minutes results in cellular burdens of Hg^{2+} of the order of 1 ng/10^7 cells, and this cellular burden maximally impacts BCR signaling [17]. We have determined by propidium iodide uptake that these *in vitro* Hg^{2+} burdens are not toxic to WEHI-231 cells (NS), while similar *in vivo* Hg^{2+} burdens are not toxic to mouse spenocytes [24]. pERK and pSyk were then

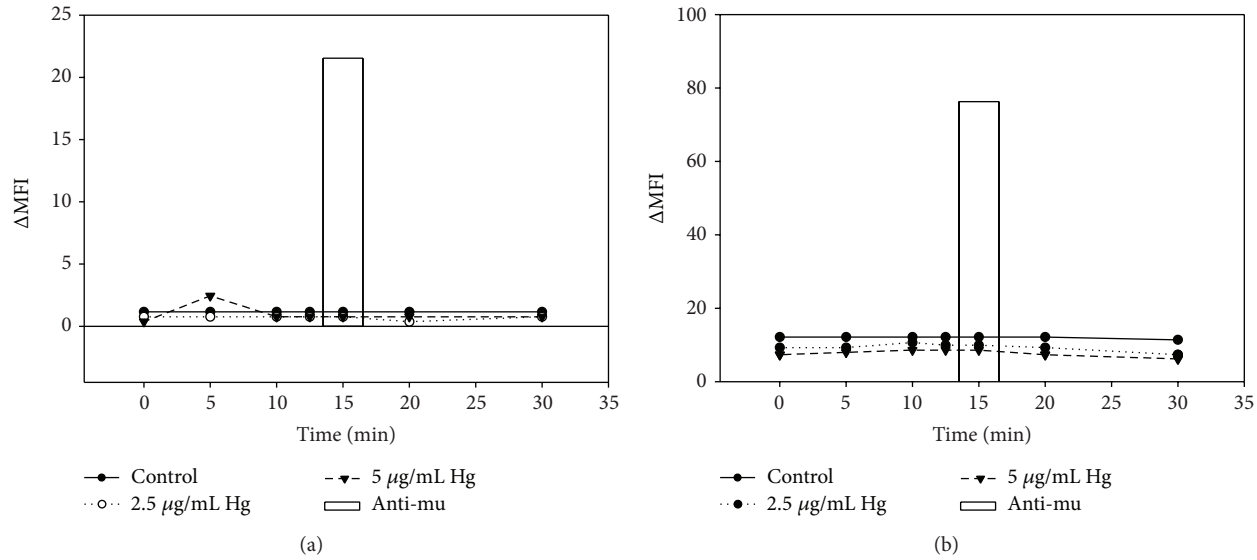

Figure 3: Effect of Hg^{+2} and anti-mu (12.5 μg/mL) as a function of time after addition of (a) pERK and (b) pSyk.

simultaneously measured by phosphospecific flow cytometry. At the 10 minute mark anti-mu was added to some of the cells which had not been treated with Hg^{2+}. Five minutes later at the 15 minute mark these cells were likewise assayed for pERK and pSyk by phosphospecific flow cytometry. Untreated cells were also labeled with isotype controls to pERK and pSyk antibodies and assayed by flow cytometry.

The results for pERK and pSyk are shown in Figures 3(a) and 3(b), respectively, and expressed as the change in MFI between cells which were labeled with the phosphospecific antibodies to those cells which were labeled with the appropriate isotype specific control. The results for the cells which were stimulated with anti-mu are displayed as a single bar plot at the 15 minute mark, to distinguish them from the results for cells which were treated with Hg^{2+}, which are displayed as line plots. Compared to anti-mu induced BCR signaling, Hg^{2+} alone has little effect on ERK or Syk.

3.3. Low Levels of Hg^{2+} Attenuate Phosphorylation of ERK, Accentuate Phosphorylation of Syk and BLNK, but Has Little Effect on Phosphorylation of BTK during BCR Mediated Signal Transduction in WEHI-231 Cells. We next investigated the effect of Hg^{2+} *on BCR* signaling upstream of ERK. BCR signaling is a complex process that is still not fully understood. However it is generally accepted that shortly after antigen engagement of the BCR complex that multimolecular cluster formation begins and assembly of the B cell receptor signalosome ensues [23, 25]. For the most part the structural integrity of the signalosome is anchored by the scaffolding protein BLNK, whose ability to bind other signalosome constituents is mediated by tyrosine phosphorylation of specific BLNK residues. These residues are substrates of the Btk, Lyn, and Syk kinases, and in an autocatalytic fashion the activity levels of these kinases, particularly Syk, are in turn regulated to some extent by whether or not they are bound to BLNK [23].

One of the advantages of phosphospecific flow cytometry is that it provides the ability to simultaneously monitor multiple elements of a phosphorylation dependent signaling pathway in individual cells. Accordingly in Figures 4(a)–4(d) WEHI-231 cells were incubated with or without Hg^{2+} for 5 minutes and then stimulated or not with anti-mu. At timed intervals cells were fixed and levels of activated ERK (pT202/pY204), Syk (pY436), Btk (pY551), and BLNK (pY84) simultaneously determined by multicolor phosphospecific flow cytometry. In each plot cells which have been preincubated with Hg^{2+} are compared to cells which have not. The results are expressed as the difference between the MFI of cells which have or have not been treated with anti-mu as a function of time after addition of anti-mu.

Figure 4(a) demonstrates that Hg^{2+} attenuates the normal ERK signal realized during BCR signal transduction, while Figure 4(b) shows that Hg^{2+} actually enhances the strength of the Syk signal. On the other hand BTK appears unaffected, as Hg^{2+} does not seem to have any appreciable effect on either the magnitude or kinetics of the BTK response. Finally phosphorylation of BLNK, like that of Syk, is enhanced by Hg^{2+}.

Although Figures 4(a) and 4(b) show that Hg^{2+} depresses and augments the activation of ERK and Syk, respectively, it is difficult to discern if there are differences in kinetics. To evaluate whether Hg^{2+} altered the kinetics of ERK or Syk activation during BCR signaling we replotted the data of Figures 4(a) and 4(b) in Figures 4(e) and 4(f). In Figure 4(e) we have plotted the change (between cells treated or not with anti-mu) in the MFI of ERK and Syk in (control) cells which have not been pretreated with Hg^{2+}. This figure demonstrates that under control conditions the kinetics of ERK activation is in phase with that of Syk activation. In Figure 4(f) we have plotted the same variables; only this time we have used the results from cells which were exposed to Hg^{2+}. Here it is clear

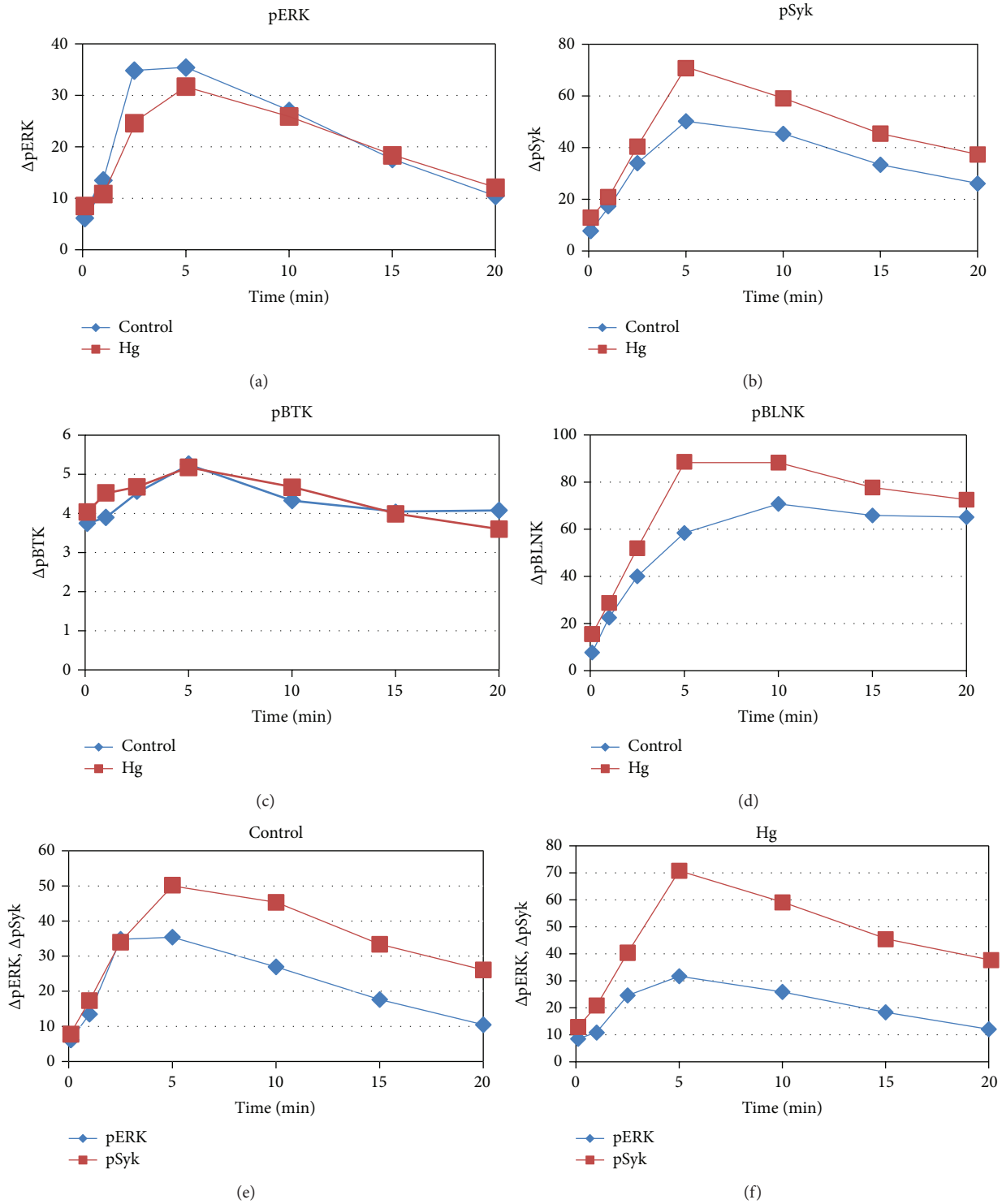

Figure 4: Effect of Hg^{+2} on stimulation of elements of the BCR signal transduction pathway after stimulation of the BCR with anti-mu (12.5 μg/mL). In Figure 4 the y-axis represents differences in MFI between (BCR stimulated cells treated or not with Hg^{2+}) and control cells which have neither been treated with Hg nor stimulated via the BCR. The x-axis represents time after stimulation of the BCR. (a) pERK versus time, (b) pSyk versus time, (c) pBtk versus time, (d) pBLNK versus time, (e) pERK versus pSyk versus time in the absence of Hg^{+2}, and (f) pERK versus pSyk in the presence of Hg^{+2}.

that although Hg^{2+} may decrease the magnitude (phosphorylation) of the ERK response while increasing the magnitude (phosphorylation) of the Syk response, exposure has little effect on the phase relationship between activation of the two kinases during BCR signal transduction.

3.4. Analysis of the Effect of Hg^{2+} on the Activation of ERK and Syk during BCR Signaling on the Single Cell Level. Although we had earlier pointed out that one of the advantages of phosphospecific flow cytometry is that it potentially permits analysis of signaling events on the single level, we did not take advantage of this in the analysis accomplished in Figure 4. In Figure 4 we reduced complex raw data sets obtained from the flow cytometery so as to characterize different cell populations solely with four numbers, the four MFIs associated with binding phosphospecific antibodies to ERK, Syk, BTK and BLNK. The effect of Hg^{2+} on BCR signal transduction was then essentially determined by comparing these numbers among differentially treated cell populations. However the initial data reduction to MFIs means that Figure 4 is in essence a population level analysis, and that similar results could in principle be obtained utilizing western blotting technology, albeit with much greater difficulty and likely less precision.

In Figure 5 we analyze the effect of Hg^{2+} on ERK and Syk activity during BCR signaling but forego initial reduction to MFIs in order to preserve single cell information. Accordingly Figure 5(a) is a 2-parameter histogram with respect to pSyk (y-axis) and pERK (x-axis) for the control population of WEHI-231 cells which have not been treated with Hg^{2+} or anti-mu. Here the number of cells expressing any particular pSyk, pERK value pair is proportional to the density of the plot in that region. Since Figure 4 indicated that the maximum response (pERK or pSyk) to anti-mu occurs at 5 minutes after stimulation, in Figure 5(b) we show the 2-parameter (pSyk, pERK) histogram for WEHI-231 cells which have been treated with anti-mu for 5 minutes. Finally Figure 5(c) is the histogram for cells which have been treated with anti-mu for 5 minutes but which have also been pretreated with Hg^{2+}. Each histogram is divided into quadrants, and the percent of the cell population falling into each quadrant is indicated.

In Figure 5(a) we have adjusted the quadrant settings so as to characterize 97.03% of the control population as $pSyk^{low}$, $pERK^{low}$ and only 0.18% $pSyk^{hi}$, $pERK^{hi}$. Utilizing the identical quadrant settings, upon anti-mu stimulation this changes after 5 minutes to 73.06% $pSyk^{hi}$, $pERK^{hi}$ and 5.18% $pSyk^{low}$, $pERK^{low}$. As we already determined in Figure 4, when cells are intoxicated with Hg^{2+}, the profile of pSyk and pERK is altered after anti-mu stimulation of the BCR from what is found for cells which are not intoxicated. This is reflected in Figure 5(c). After BCR stimulation, for Hg^{2+} intoxicated cells the percentages of cells in each of the quadrants do not drastically differ from those of cells which are not intoxicated, but nevertheless the bulk of the distribution seems to move to higher pSyk and lower pERK values.

Frequently in flow cytometry, cell populations characterized by a single fluorescent intensity are visualized as one-dimensional histograms. In order to analyze shifting population frequencies arising under different experimental conditions the Overton algorithm is often employed to subtract these one-dimensional histograms from one another [26]. In our case we would like to analyze the effect of Hg^{2+} exposure on the population shift that occurs simultaneously with respect to cellular levels of pERK and pSyk after BCR stimulation, and which has been visualized as two-dimensional histograms. Although we are working with two-dimensional histograms, just as for one-dimensional histograms, the effect of Hg^{2+} can be analyzed by subtracting the histogram for nonintoxicated cells (Figure 5(b)) from that for Hg^{2+} intoxicated cells (Figure 5(c)).

To accomplish this subtraction we have generalized the Overton algorithm from one to two dimensions. The result is shown in Figure 5(d) where discrete areas of the plot have been coded according to the legend on the right. Positive numbers indicate areas where there are excessive cell numbers in the Hg^{2+} exposed population with respect to the nonexposed population after BCR stimulation, while negative numbers represent the opposite. The absolute values of the numbers are proportional to the absolute differences, either positive or negative. In the one-dimensional Overton algorithm, the total number of positive cells is summed, and this number divided by the total population number is by definition the percent positive cells. Using an analogous procedure, we find that, in Figure 5(d), 37.6% of the cells in the mercury intoxicated population are designated "positive".

We have previously shown that intoxication of WEHI-231 B cells with low and nontoxic levels of Hg^{2+} (1 ng/10^7 cells) has little direct effect on ERK. However, at these levels Hg^{2+} interferes with BCR signal transduction so that activation of ERK is attenuated [17]. These earlier experiments relied upon western blot analysis. The phosphoflow cytometric findings in Figures 1, 3(a), and 4(a) are in complete support of these previous findings. Since there is a direct connection between suppression of BCR signals in immature B cells and increased mature autoimmune B cell clones [12], to the extent that WEHI-231 B cells can be taken as a model of immature B cells, Hg^{2+} attenuation of ERK activation implies that Hg^{2+} intoxication may permit some self-reactive B cell clones to escape central tolerance and contribute to autoimmunity or autoimmune disease upon maturation.

The fact that in both of these experiments and in our earlier work low levels of Hg^{2+} seem to have little direct effect on ERK implies that the target of Hg^{2+} is upstream of ERK, and that signaling through at least some elements of the BCR signal transduction pathway upstream of ERK should also be attenuated. Syk is one such possible element. It is a central tyrosine phosphokinase operating upstream of ERK in the BCR signal transduction pathway, and as such is necessary for the integrity of the BCR signal and the eventual elimination of self-reactive immature B cell clones [22, 23]. Our earlier analysis of Syk activation in Hg^{2+} intoxicated WEHI-231 B cells utilizing western blot technology was inconclusive as to whether Hg^{2+} intoxication attenuated Syk activation [17].

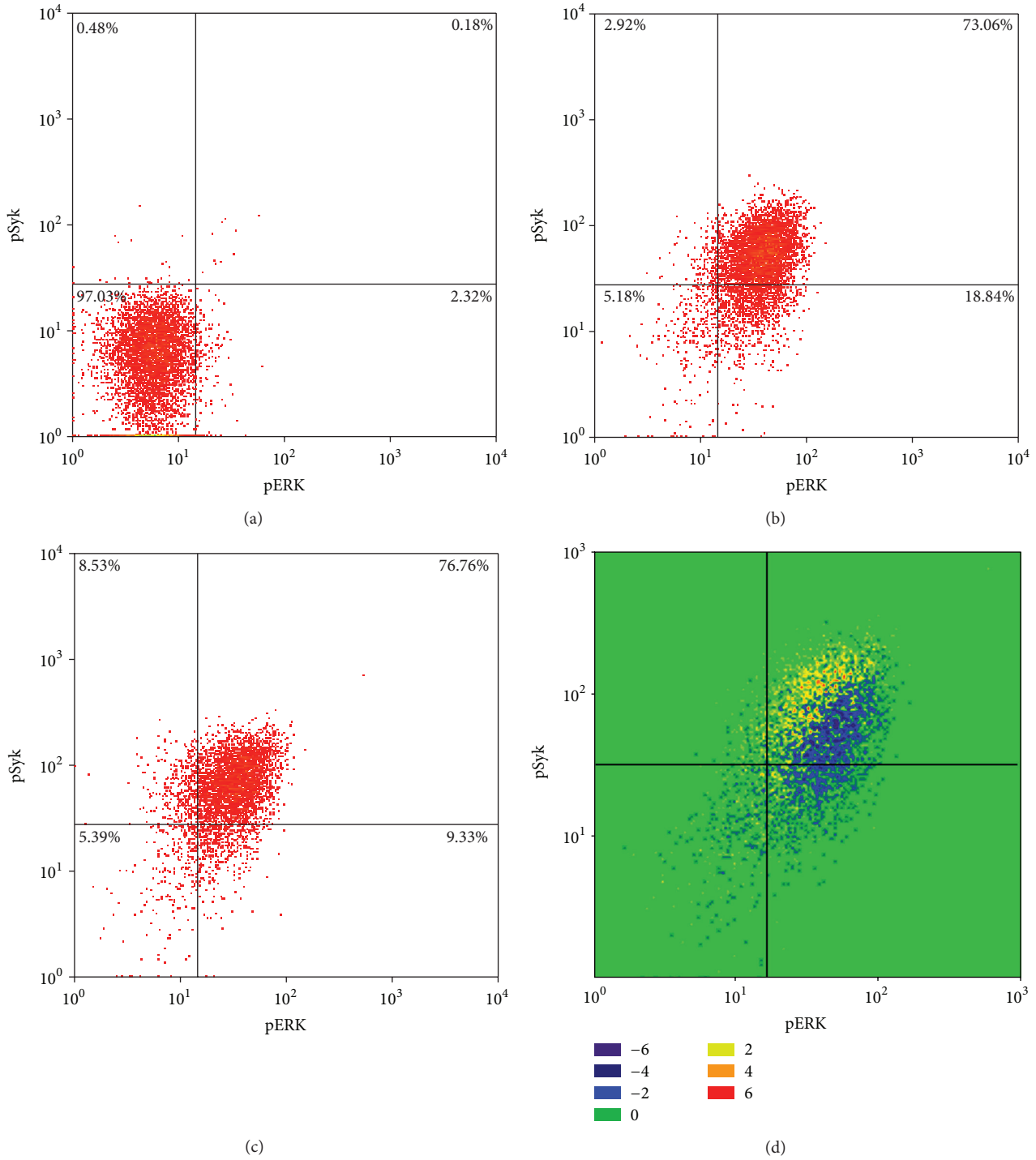

FIGURE 5: Two-dimensional histogram representation of pSyk versus pERK after stimulation of BCR with anti-mu (12.5 μg/mL). (a) Control cells at 0 minutes after stimulation of the BCR; (b) 5 minutes after stimulation of the BCR; (c) five minutes after BCR stimulation in Hg^{+2} intoxicated cells; (d) "Overton" subtraction of (c) from (b).

However utilization of phosphoflow cytometry (Figure 4(b)) now shows quite clearly that Hg^{2+} enhances the activation of Syk.

Aside from Syk, we have also looked at phosphorylation of BLNK and Btk, two other elements upstream of ERK in the BCR signal transduction pathway. BLNK is the scaffolding protein that is important for the structural integrity of the B cell signalosome, which forms consequent to antigen engagement of the BCR [23, 25]. During BCR signaling, phosphorylation of various BLNK tyrosine residues by the tyrosine kinases Syk, Btk, and Lyn is necessary for the proper formation and maturation of the signalosome, as

various signalosome constituents specifically recognize and bind to these phosphorylated motifs (primarily through SH2 domains) [25]. As Btk activation is itself associated with the phosphorylation of tyrosine 551 [27], both BLNK and Btk activities were assayed by phosphoflow cytometry to determine the effect of Hg^{2+} intoxication on their activity during BCR signaling (Figures 4(c) and 4(d)). We find that while Hg^{2+} has little effect on Btk activation during BCR signaling, similar to Syk, phosphorylation of BLNK is significantly enhanced. Considering that BLNK is a substrate of Syk, this was expected.

On the other hand, the finding that Syk activation is enhanced, while downstream activation of ERK is attenuated by Hg^{2+} was a bit surprising, especially considering our earlier work with T cells. BCR signaling has much in common with T Cell Receptor (TCR) signaling. In particular, in T cells TCR cross-linking leads to the transient activation of ERK, much like BCR cross-linking leads to the activation of ERK in B cells. We have found that in T cells intoxicated with low levels of Hg^{2+} ERK activation is attenuated during TCR signaling [28]. In T cells, TCR dependent ERK activation is dependent upon upstream activation of the phosphotyrosine kinase ZAP-70, a homologue of Syk. However in T cells we have found that attenuation of ERK by Hg^{2+} is associated with attenuation of ZAP-70, as well as LAT, the scaffolding protein homolog of BLNK [29].

While Figures 4(c) and 4(d) show that during BCR signaling, Hg^{2+} dependent attenuation of ERK activation is correlated with enhancement of Syk activation, these experiments only speak of what is happening on a population level. The question remains whether this correlation strictly holds on the cellular level. Utilizing the identical dataset used to generate Figure 4, Figures 5(a)–5(d) specifically take advantage of the ability of phosphoflow cytometry to simultaneously probe individual cells for Syk and ERK activity to answer this question. Figure 5(a) is the two-dimensional histogram plotting pSyk versus pERK activity levels in control cells, while Figure 5(b) plots pSyk versus pERK activity in cells which have been maximally stimulated with anti-IgM. Comparison of Figures 5(a) and 5(b) demonstrates that most cells (about 90%) respond to BCR stimulation by increasing Syk and ERK activity.

Figure 5(c) is the pSyk versus pERK histogram for cells which have been stimulated with anti-IgM as in Figure 5(b), but which have also been intoxicated with Hg^{2+}. Again, most cells still seem to be responding to anti-IgM as in Figure 5(b), but the distribution appears to be subtlety shifted to lower pERK and higher pSyk levels. This of course is consistent with Figure 4 demonstrating that on a population level Hg^{2+} intoxication leads on average to measurably lower pERK and higher pSyk levels. To assess the effect of Hg^{2+} intoxication on BCR signaling on the cellular level, we have utilized a generalized Overton algorithm to subtract the histogram in Figure 5(b) from that of Figure 5(c), resulting in Figure 5(d).

When one-dimensional histograms are subtracted it is common practice to display only the one-dimensional histogram of the resulting positive cells. However for the two-dimensional subtracted histogram in Figure 5(d) we display positive as well as negative areas. We interpret positive areas of the histogram (which are represented in brighter hues) as cellular positions which become more populated in Hg^{2+} intoxicated populations after BCR signal induction than in control populations. Likewise the negative areas of the histogram (represented by darker hues) represent cellular positions which become less populated as a result of Hg^{2+} intoxication. They essentially represent areas of the histogram from which cells (in the control) will move from as a result of Hg^{2+} exposure.

In Figure 5(d), the positive and negative areas are well segregated, with the positive areas having higher pSyk, but lower pERK, values. This finding supports the notion that on a cellular as well as population level, in Hg^{2+} intoxicated cells suppressed ERK activity is associated with enhanced Syk activity. Significantly, in Figure 5(d) we find that 37.6% of the cells are "positive." In this context we interpret positive to mean that only 37.6% of Hg^{2+} intoxicated cells respond to BCR cross-linking with depressed ERK and enhanced Syk activation when compared to control cells. The remaining 62.4% of Hg^{2+} intoxicated WEHI-231 cells respond to BCR cross-linking by enhancing pERK and pSyk, just as they would as if they had not been exposed to Hg^{2+}.

The overall finding that Hg^{2+} augments activation of Syk while it simultaneously attenuates downstream activation of ERK is at first glance a bit puzzling. However a recent report suggests that in B cells there are two functionally and topologically distinct ERK activation pathways [16]. Although both depend on Syk as an upstream element, they diverge shortly afterward. The first one is the familiar pathway previously described for B cells [22, 23], but it is postulated that this pathway is primarily operable in mature cells. Activation of this pathway is presumed to initiate cell proliferation. A second ERK activation pathway is described and postulated to be primary active in immature B cells. This pathway is connected to apoptosis and clonal deletion. The major difference between the two pathways is that this second pathway depends on a BCR mediated Icrac calcium current. If Hg^{2+} was to depress the calcium signal by interfering with an upstream regulator of Icrac channels, then it would be possible that ERK activation through this pathway could still be attenuated, even though upstream signaling through Syk was augmented. In any event it seems clear that Hg^{2+} initially interacts with a B cell target or targets upstream of Syk, possibly including Icrac channels.

4. Conclusions

After exposure of WEHI-231 B cells to low levels of Hg^{2+}, signal transduction initiated by the BCR is altered. Analysis on the single cell level shows that while activation of ERK is suppressed, upstream activation of Syk is enhanced and phosphorylation of BLNK is increased. On the other hand, activation of Btk is unaffected. As WEHI-231 is a model for immature B cells, suppression of ERK activation in these cells suggests that low level exposures to Hg^{2+} may also interfere with central tolerance in primary immature B cells. We suggest that interference with central tolerance may be one of

the mechanisms connecting low level mercury intoxication to autoimmune disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by a Grant from the National Institutes of Health: ES019228 (AR). The authors wish to express gratitude to Dr. Raymond Mattingly for his careful reading of the paper and thoughtful advice.

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 782045, 11 pages
http://dx.doi.org/10.1155/2014/782045

Review Article

Autoimmunity and Asbestos Exposure

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Received 3 January 2014; Accepted 10 April 2014; Published 29 April 2014

Academic Editor: K. Michael Pollard

Despite a body of evidence supporting an association between asbestos exposure and autoantibodies indicative of systemic autoimmunity, such as antinuclear antibodies (ANA), a strong epidemiological link has never been made to specific autoimmune diseases. This is in contrast with another silicate dust, crystalline silica, for which there is considerable evidence linking exposure to diseases such as systemic lupus erythematosus, systemic sclerosis, and rheumatoid arthritis. Instead, the asbestos literature is heavily focused on cancer, including mesothelioma and pulmonary carcinoma. Possible contributing factors to the absence of a stronger epidemiological association between asbestos and autoimmune disease include (a) a lack of statistical power due to relatively small or diffuse exposure cohorts, (b) exposure misclassification, (c) latency of clinical disease, (d) mild or subclinical entities that remain undetected or masked by other pathologies, or (e) effects that are specific to certain fiber types, so that analyses on mixed exposures do not reach statistical significance. This review summarizes epidemiological, animal model, and *in vitro* data related to asbestos exposures and autoimmunity. These combined data help build toward a better understanding of the fiber-associated factors contributing to immune dysfunction that may raise the risk of autoimmunity and the possible contribution to asbestos-related pulmonary disease.

1. Introduction

Autoimmune disease is the clinical manifestation of abnormalities in immune regulation that lead to tissue damage by self-reactive lymphocytes and autoantibodies, resulting in debilitating symptoms and death when vital organs are affected. The cause(s) of most autoimmune diseases remain uncertain, although environmental factors are strongly indicated through studies in animal models [1]. Systemic autoimmune diseases (SAID) including systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and rheumatoid arthritis (RA) appear to have complex etiologies with gene-environment interactions [2]. Silicate dusts, including crystalline silica and asbestos, increase production of autoantibodies, possibly through the production of excess cellular debris in the context of a highly inflammatory environment [2–4]. However, the exact mechanisms (apoptotic pathways, cytokine patterns, and redox regulation) by which exposure to silicate dusts drives autoimmune responses are not clearly elucidated, and it is not known whether this is a universal response to inhaled mineral dusts (Figure 1).

Exposure to crystalline silica leads to increased antinuclear autoantibodies (ANA) in both mice and humans and increases the risk of SLE, RA, and SSc [2, 5, 6]. While this association with silica exposure is widely accepted, asbestos exposure has not yet been strongly linked with any particular autoimmune or connective tissue disorder. Nevertheless, there are reports of immune abnormalities and humoral indices consistent with autoimmune mechanisms, including a variety of autoantibodies such as ANA and rheumatoid factor (RF) (detailed below). Several factors could be contributing to the inability to associate asbestos with SAID epidemiologically, including (a) a lack of statistical power due to relatively small or diffuse exposure cohorts, (b) exposure assessment issues, (c) the latency of the clinical disease, and (d) mild clinical or subclinical entities that remain undetected or masked by other pathologies. In addition, a key factor may center around the definition of asbestos in these studies.

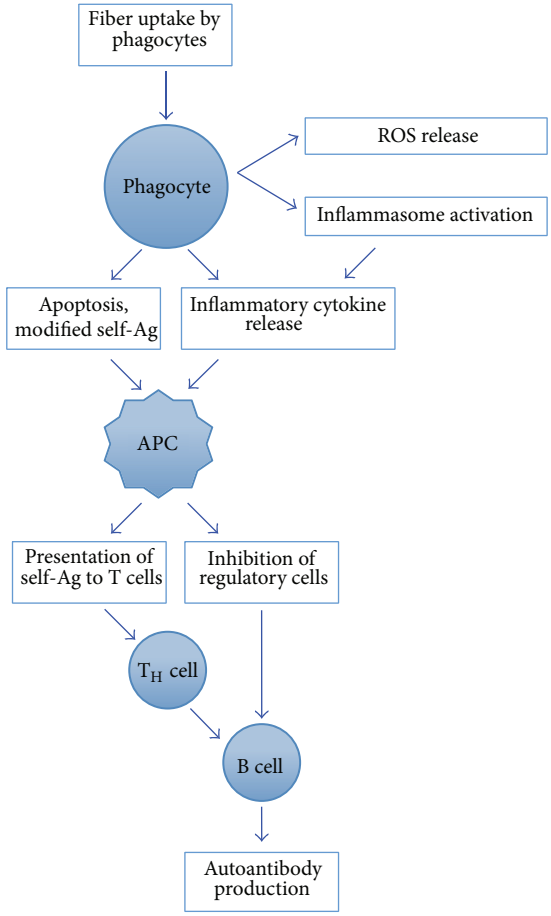

Figure 1: Schematic of possible players in the immune dysfunction by mineral fibers. These are putative mechanisms only. More details on mode of action are covered in excellent reviews mentioned in the text [2, 3, 16, 17].

The term "asbestos" is generally regarded as broadly descriptive of mineral fibers used commercially due to their durability and heat resistance. Specifically, they are defined as being long and thin (having an aspect ratio greater than 3 : 1), and falling into categories of either "serpentine" (chrysotile) or "amphibole" (tremolite, amosite, crocidolite, actinolite, and anthophyllite) [7]. As a group, asbestos has been classified as a carcinogen and is known to cause a pulmonary fibrotic disease called "asbestosis." Despite this generalization, all of these fiber types have distinct physicochemical properties (shape, durability in physiological fluids, surface chemistry, and aerodynamic properties), making the term "asbestos" mineralogically imprecise [7] (see Table 1). In 2010, the U.S. Environmental Protection Agency (EPA) and the National Institute of Environmental Health Science (NIEHS) jointly convened a workshop to invite experts from all areas of asbestos research and toxicology in order to address these issues of nomenclature and dosage and to better understand the modes of action (MOA) behind asbestos-induced health effects [8, 9]. Part of the impetus behind this effort was the awareness of severe health problems that have occurred as a result of exposure to mineral fibers in contaminated vermiculite mined just outside of Libby, Montana. Much of the fibrous material, including winchite and richterite, did not fall into the definition above, despite containing long, thin "asbestiform" mineral fibers. Since then, another mineral fiber in the zeolite family called erionite has been shown to be highly carcinogenic and causing pulmonary diseases similar to those seen with asbestos [10–12]. In addition, over the last few decades the manufacture and use of nanomaterials called "nanotubes" and "nanowires" have dramatically increased, leading to health concerns due to similarities to asbestos [13, 14]. The imprecision in asbestiform fiber classification means that it is impossible to generalize about associated health outcomes, since one type of fiber might have very different health effects based on its ability to be inhaled into deep regions of the lung, surface properties that affect the interaction with cells, and the amount to which it is cleared by the innate immune system [15]. Until recently, much of the literature on the health effects of mineral fibers was focused on occupational exposures to asbestos. Industrial hygiene and work records data available for occupationally exposed cohorts enable quantitative exposure assessment methods, but such methods are typically focused on one fiber type such as chrysotile. Such analyses do not allow for the possibility of the mixed fiber exposures or account for the potentially disparate health outcomes associated with different fiber exposures. Occupational exposures also are comprised primarily of men, but autoimmune diseases are often more common among women. It is very possible, therefore, that the lack of epidemiological evidence in support of an association between asbestos exposure and autoimmunity is because the studies exploring this issue have been focused on different (or mixed) mineral fiber types in occupational, rather than general, populations.

This review describes the evidence for induction of autoantibodies following asbestos exposure, the enigmatic epidemiological data regarding an association with SAID, and then explores hypotheses that might help explain the discord between the two types of data. Finally, we present emerging data that support the presence of tissue specific autoantibodies that may play a critical role in the severity or progression of asbestos-associated pulmonary disease. Identification of weaknesses and limitations within available epidemiological data are important to help strengthen design of future studies since exposures to mineral fibers will continue to present public health challenges long into the future.

2. Asbestos Exposure and Autoantibodies

A small number of epidemiological studies explore an association between asbestos exposure and autoantibody responses (see Table 2). Cross-sectional associations between humoral responses, including rheumatoid factor (RF) and ANA, among asbestos workers were initially reported in 1965 [18]. Subsequent reports described increased ANA frequency with asbestos exposure, as well as increased serum IgG/IgA and immune complexes [19–25]. A few studies indicate no increase in ANA [25–27]. Most recently, subjects exposed

Table 1: Description of mineral fibers discussed.

Fiber family	Fiber names	Chemistry	Location/use
Serpentine	Chrysotile	$Mg_3(Si_2O_5)(OH)_4$ (idealized), rolled sheets of Si oxide tetrahedra	Many commercial uses [7], Sumas Mtn [30]
Amphibole	Actinolite Amosite Anthophyllite Crocidolite Tremolite	Various Mg, Fe, Ca, and Na ions on double chains of silicon oxide tetrahedra	Igneous and metamorphic rock, many commercial uses [7, 31, 32]
Asbestiform	Winchite Richterite	Similar to amphibole, not specifically classified as asbestos	Similar to amphiboles, contaminant [8, 31]
Nanomaterials	Nanotubes Nanowires	Many metal formulations, formed into very long, thin chains or tubes	Synthetic, many commercial uses [13, 14]
Zeolite	Erionite	$(Na_2,K_2,Ca)_2Al_4Si_{14}O_{36}\cdot 15H_2O$ (idealized), chains of silicate "cages" or rings	Igneous rock: Turkey [11, 12]; S. Dakota [11, 12]

Table 2: Selected studies evaluating antinuclear antibodies (ANA) and rheumatoid factor (RF) among asbestos exposed subjects.

Study, year [reference]	Exposure context, fiber type	Exposed group			Comparison group			Associated w/radiologic changes
		n	ANA+	RF+	*n*	ANA+	RF+	
Pernis et al. 1965 [18]	Insulation workers, chrysotile	315	—	25%	103	—	14%	
Turner Warwick and Parkes 1970 [23]	Medical screening, mixed	80	28%	27%				Yes
Turner Warwick 1973 [33]	Medical screening, mixed	196	20%	11.7%	—	—	—	Yes
Turner Warwick 1973 [33]	Factory workers, unknown	252	7.5%	5.3%	—	—	—	Yes
Turner Warwick 1973 [33]	Naval personnel, mixed	334	8.4%	3.6%	—	—	—	Yes
Lange 1980 [29]	Textile workers, unknown	58	21%	—	19	0%	—	Yes
Toivanen et al. 1976 [34]	Asb. miners, anthophyllite	66	1.5%	10.7%	—	—	—	
Kagan et al. 1977 [35]	Subjects with asbestosis	26	7.7%	35%	45	0%	11%	
Haslam et al. 1978 [36]	Subjects with asbestosis	28	35.7%	17.9%	—	—	—	Yes
Huuskonen et al. 1978 [25]	Varied: asbestos sprayers, insulators, cement, quarry	169	11.8%	22.5%	504	11%	—	No
Lange 1980 [29]	Asbestos textile workers	242	21%	10%	181	9%	—	Yes
de Shazo et al. 1983 [26]	Asbestos cement workers	31	0%	0%	51	0%	—	No
Doll et al. 1983 [37]	Asbestos cement workers	144	15%	3%	–	–	—	No
Lange 1980 [29]	Asbestos workers	39	50%	—	9	0%	—	
Zerva et al. 1989 [24]	Whitewash, tremolite (amphibole)	109	14%	—	34	34%	—	Yes (pleural)
Tamura et al. 1993, Tamura et al. 1996 [22, 38]	Asbestos plant workers	220	15%	3.2%	—	—	—	Yes (interstitial)
Nigam et al. 1993 [19]	Asbestos factory milling	71	12%	1.4%	28	7%	0%	
Pfau et al. 2005 [20]	Contaminated vermiculite Amphiboles	70	70%	33%	50	40%	36%	Yes

Table 3: Animal model studies of asbestos and autoimmunity.

Reference	Strain (all inbred)	Disease model	Sex used	Treatment (fiber, route, duration)	Notes
Ferro et al., 2013 [39]	C57BL/6 mice	None	Female	LA, Chry, i.t., 7 mo.	LA (not Chry) increased ANA and IL-17
Pfau et al., 2008 [40]	C57BL/6 mice	None	Female	LA, i.t., 7 mo.	LA increased ANA, anti-Ro52, anti-dsDNA, IC
Salazar et al., 2012 [41]	Lewis rat	None	Female	LA, amosite, i.t., 13 weeks	Both increase ANA, anti-Jo-1. No IC, no anti-dsDNA
Salazar et al., 2012 [42]	Lewis rat	Antigen-induced arthritis (CIA, PG-PS)	Female	LA, amosite, i.t., 13 weeks	Both fibers increase ANA; no exacerbated disease
Pfau et al., 2011 [43]	C57BL/6 mice	None	Female	LA, tremolite, i.t., 7 mo.	Both induced antifibroblast antibodies

LA: Libby amphibole; ANA: antinuclear antibodies; Chry: chrysotile; i.t.: intratracheal; CIA: collage-induced arthritis; PG-PS: peptidoglycan/polysaccharide induced arthritis; IC: immune complexes in kidneys. Amosite and tremolite are both amphiboles.

to the Libby, MT, amphibole were shown to have elevated frequency and titers of ANA compared to a reference population [20]. Among the autoantibodies detected were those that target common SLE autoantigens, including dsDNA, SSA/Ro52, and ribonuclear proteins (RNP) [20, 28]. An increased frequency of positive RF tests among asbestos workers compared to the general population has been reported in several studies [21, 23, 29], while others reported no association [20, 24, 27]. It is highly likely that differences in serum dilutions and technical approaches can explain some of these differences. An early, sensitive detection marker for RA, antibodies to cyclic citrullinated proteins (anti-CCP), was not elevated in a subset of the Libby amphibole-exposed population [28].

Exposure to amphibole asbestos increases the frequency of positive ANA tests in nonautoimmune prone mice and rats [39–41] (Table 3). Mice exposed to amphibole asbestos (tremolite) exhibited immune complex deposition in the kidneys and mild glomerular changes suggestive of lupus nephritis [40]. The amphibole initially obtained by the U.S. Geological Survey (USGS) from the Libby mine site has been described as "6-Mix" because it was collected from six different sites, combined and characterized [31]. It is a combination of amphiboles including winchite, richterite, tremolite, and amosite and is very likely similar to the material to which the miners and townspeople were exposed over decades of mining the asbestos-contaminated vermiculite [31]. This material (LA (Libby amphibole)) has also been shown to induce ANA in intratracheally exposed mice [39] and rats [41, 42]. In the rat studies, a more pure sample of amphibole asbestos (amosite) was also shown to induce ANA in the rats [41, 42].

The combined human and animal data suggest that there are autoimmune responses associated with asbestos exposure that include autoantibodies characteristic of SAID, particularly SLE. Although autoantibodies are often present prior to onset of clinical disease [44], it might be expected that epidemiological data would report SAID in asbestos-exposed populations.

3. Systemic Autoimmune Disease (SAID) and Asbestos

Like the serological studies, previous epidemiological assessments of SAID in asbestos-exposed cohorts were fairly small studies and tended to suffer from problems with exposure assessment [45]. Rheumatoid arthritis has been the SAID most frequently associated with asbestos exposure [46–48]. Other SAIDs are extremely rare with prevalence estimates ranging from 4 to 24 per 100,000 populations, resulting in challenges to statistical power for studies conducted among relatively small asbestos-exposed populations. Nevertheless, one study described an increased risk for SSc deaths among persons having occupations with likely exposure to asbestos [49]. A recent case-control study of self-reported SLE or SSc patients nested within a medically screened general population cohort in Libby, MT, showed associations for both diseases with amphibole exposure [47].

An association with ANCA-associated vasculitis has been described in two studies of asbestos exposures [50, 51] but was not found in at least one study despite an association with silica exposure [52]. Because the interstitial pneumonia that is common in this form of vasculitis can be mistaken for asbestosis, this link may simply be overlooked. Several studies also report an association between asbestos exposure and periaortitis and retroperitoneal fibrosis, both of which are considered autoimmune diseases [53–57]. This pathology is of interest due to the fiber burden of tissues in this area of the body following asbestos exposure [58].

Two groups have examined symptoms of systemic autoimmune disease in animal models after asbestos exposure. In addition to inducing ANA in C57BL/6 mice, tremolite was shown to increase immune complex deposition in

the kidneys of exposed mice [40]. In that study, the autoantigen targets for the ANA included dsDNA, Ro52, and RNP, which are common in human SLE. However, neither proteinuria nor overt kidney disease was significantly increased over the experimental period. In rats, despite production of ANA after exposure to Libby amphibole or amosite, there was no evidence of exacerbated disease in a model of induced RA [42]. These fibers increased proteinuria in the rats but did not increase immune complex deposition or kidney pathology [41]. Therefore, to our knowledge there have been no studies that clearly demonstrate induction or exacerbation of SAID by mineral fibers in animal models.

Taken together, these studies make a compelling, but not definitive, case for an association between "asbestos" and immune dysfunction relevant to autoimmunity. Many of the human studies suffer from technical issues such as small study sizes, predominantly male occupational cohorts and limited exposure data. For example, one study indicated no association of positive ANA tests with asbestos exposure, but that study only consisted of 25 asbestos workers, and there was no clear definition of the type of asbestos [27]. A small study of 66 anthophyllite miners showed no induction of ANA, but the method of measurement is unclear [34]. As indicated in Table 2, most studies indicate the occupation but not the fiber types. Incidences where persons are exposed to pure chrysotile or amphibole are rare, so most of these studies represent mixed exposures of unknown proportions. However, a recent review reported on the perceived strength of the literature support for the association of asbestos exposure with autoimmunity, and the strongest data was shown to be in studies of tremolite, an amphibole asbestos, or mixtures with heavy amphibole content [59]. This therefore raises the issue of the different mineralogy of these fibers and whether they have similar effects in immune dysfunction.

4. Hypotheses Regarding the Discordant and Inconsistent Results

There are several possible explanations for the lack of strong epidemiological data supporting a link between asbestos and autoimmune disease. First, asbestos exposure cohorts tend to be small and composed predominantly of males. With the possible exception of rheumatoid arthritis, SAIDs are rare with estimated prevalence in the U.S. general population of 24 per 100,000 for SLE, 5 per 100,000 for polymyositis/dermatomyositis, and 5 per 100,000 for systemic sclerosis [45]. Prospective epidemiological studies of rare disease require large cohorts followed for extended periods of time. Case-control studies can overcome some of these challenges, but asbestos is a relatively rare exposure and difficult to adequately assess retrospectively in the general population. Thus, epidemiological studies of asbestos exposure and risk of SAID often have limited statistical power even when evaluating associations with large effects sizes. SAIDs, including rheumatoid arthritis, are also more prevalent among women who account for 67% to 92% of SAID prevalence [45]. By

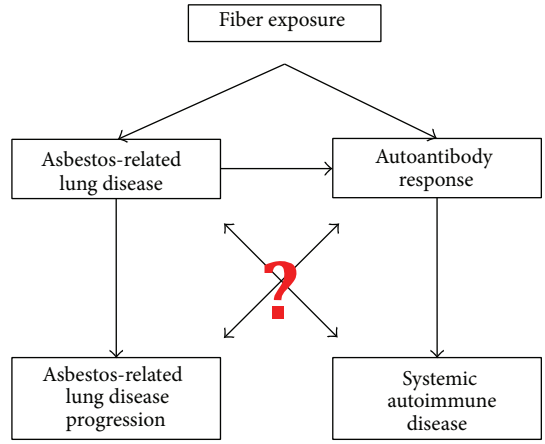

Figure 2: Proposed relationships between asbestos exposure, autoimmunity, and fibrotic lung disease progression. Data (as mentioned in the text) support the connections indicated, but questions remain regarding (a) the types of fibers that are responsible and (b) the etiological and mechanistic bases for the outcomes.

contrast, occupational asbestos-exposed cohorts are predominantly male. Several studies have evaluated respiratory disease outcomes among women exposed to take-home asbestos from their male occupationally exposed spouses [60], but epidemiological studies of autoimmune disease outcomes among exposed women have rarely been conducted [61].

Second, autoantibodies may not contribute significantly to pathology and may be the result of chronic damage and inflammation associated with asbestos-related pleural disease. The long, but uncertain and variable, latency of autoimmune changes further limits the epidemiological approaches that can be employed to elucidate these relationships (Figure 2). Longitudinal studies are required to disentangle this potential issue of reverse causality. To date only one study specifically addressed the temporal nature of the asbestos/autoimmune/lung pathology complex by following a cohort of workers in an asbestos plant [22, 38]. The baseline study demonstrated the presence of an increased frequency of ANA in this cohort, along with radiological changes in the workers' lungs [22]. The follow-up study demonstrated that subjects with ANA were more likely to develop radiologic abnormalities than subjects who were ANA negative [38]. These results, along with the knowledge that, in general, autoantibodies occur quite early in SLE patients, before clinical onset [62], argue against the hypothesis that autoantibodies associated with asbestos exposure occur after lung disease is already apparent clinically. A general population cohort that has been environmentally and occupationally exposed to amphibole asbestos is currently being followed to further examine the temporal relationship between autoantibodies and lung disease [63].

Third, limited attention to fiber type in epidemiological studies may result in fiber-specific exposure misclassification. Bernstein et al. have shown that chrysotile is less biopersistent than amphibole [64], likely leading to a shorter time in contact with immune system. It might take extended periods

in the presence of fibers to create the local environment of accumulating cell debris combined with a combination of cytokines that stimulate self-reactive lymphocytes [2, 3]. While the definition of asbestos includes both families, amphiboles and chrysotile, the fibers are clearly distinct morphologically and have unique physicochemical properties [65]. Common health outcomes of asbestos inhalation include lung carcinoma, interstitial fibrosis (asbestosis), pleural scarring, and mesothelioma, but there is no clear distinction regarding the toxicology of individual fiber types [15]. There is, however, quite a bit of evidence that amphibole asbestos seems to be more pathogenic, especially in terms of scarring of the lung parenchyma and pleura and possibly cancers as well [64, 66]. Because two recent studies from the Libby, MT cohort have indicated an association between the presence of autoantibodies and more severe disease, this makes it even more important to determine the immunotoxicological properties of specific forms of asbestos [20, 67]. There is a great deal of disagreement in the literature regarding the relative impact of different fiber types on cancer, pulmonary fibrosis, pleural disease, and immune parameters. A study in rats showed that chrysotile (Sumas Mountain) induced worse lung fibrosis compared to Libby amphibole and tremolite [68]. Dosages were made comparable by elutriation for rat-respirable fibers and by comparing exposure by mass, length, and aspect ratio. Other studies have reported significantly worse pulmonary and pleural fibrosis among amphibole-exposed subjects compared to chrysotile [64]. Therefore, there is clearly not a simple relationship between fiber type and specific disease end points.

In addition, there is evidence that chrysotile may induce long-term immunosuppressive effects among lymphocytes subsets of mesothelioma patients, leading to susceptibility to cancer but not autoimmune responses [35, 69, 70]. Comparisons with silica support the hypothesis that chrysotile does not induce the chronic immune activation/inflammation seen with silica that seems to drive the elevated risk for autoimmune diseases among silica exposed subjects [70]. This hypothesis is also supported by the work by a Japanese group [30, 71] that has shown immunosuppression in chrysotile exposed cells *in vitro* and *ex vivo*. Particular cells affected included cytotoxic T cells and NK cells, which were both suppressed by chrysotile, but not crocidolite, an amphibole [30]. The section below further reviews the literature comparing immunological parameters affected by amphibole versus chrysotile asbestos.

5. Amphibole versus Chrysotile: Autoimmunity

A recent *in vitro* comparison of the effects of Libby amphibole (6-Mix) and chrysotile on THP-1 monocytic cells and epithelial cells showed differential effects on inflammation/inflammasome activation [72]. Although both fibers activated the NLRP-3 inflammasome, amphibole appeared to do so via reactive oxygen species, while the response with chrysotile may have been mediated through lysosomal rupture. Therefore, these fibers induce very early innate immune responses for which these differences could greatly impact downstream consequences.

C57BL/6 mice were used to compare exposure to amphibole with chrysotile asbestos in terms of autoimmune responses [39]. While Libby amphibole induced ANA in a significantly higher proportion of the mice compared to controls (saline), chrysotile did not [39]. In addition, serum cytokines profiles in the mice exposed to amphibole were quantitatively and qualitatively different than in the chrysotile-exposed mice, including a dramatically elevated mean concentration of serum IL-17. The serum cytokines for chrysotile exhibited a T_H1 profile, suggestive of mild chronic inflammation, with no elevation of T_H2 cytokines or of IL-17. However, the results in the amphibole mice clearly suggest a T_H17 response. The T_H17 response is characterized by high levels of IL-17, triggered or maintained by other cytokines such as IL-6, IL-23, and TGF-beta [73]. T_H17 responses have been implicated in a variety of diseases, including RA, SSc, and SLE [74–76]. In the above experiments, dosages were on a mass basis [39]. Therefore, due to differences in length and width of the different fiber types, mice were exposed to different numbers of fibers and total fiber surface area, dependent on fiber type. Since the surface area per mass of chrysotile is higher than for the amphiboles used, one might expect the effects of chrysotile to be greater, based on studies showing that surface area may be a critical factor in the pathogenicity of fibers [15, 77]. However, the results suggest the opposite: in these mice, chrysotile exposure is not associated with autoimmune responses. The only mechanistic hypothesis that emerged from this study seemed to support the idea of an immunosuppressive effect of chrysotile; in that an increased frequency of B suppressor cells was found in both the spleen and lungs of the chrysotile-exposed mice, but not amphibole [39]. Because the evidence suggests a very different kind of immune dysfunction induced by different fiber types, it is critical to examine the possible mechanisms by which autoantibodies might impact disease processes in asbestos-exposed patients.

6. Targets of Autoantibodies and Mechanisms of Disease

It has been suggested that identification of the specific targets of the autoantibodies might help in the development of hypotheses regarding mechanism of action, as well as diagnosis and progression of SAID [28]. Few studies have attempted to identify specific targets for asbestos-induced ANA, but one commonality has been the presence of anti-dsDNA in both mice and humans [20, 40, 78], but not rats [41]. Antibodies to neutrophils (ANCA) have been associated with silica and asbestos exposure [51, 79], but the asbestos exposure data came from an occupational exposure questionnaire, so the exposures likely included mixed chrysotile and amphibole. Pfau et al. did not find an association with ANCA in their amphibole-exposed cohort [20]. Recently, extractable nuclear antigen (ENA) specificities were reported for amphibole and chrysotile-exposed mice [39], but the number of ENA positive animals was too low

to show any statistically significant differences. Interestingly, however, the Libby amphibole exposed mice showed a high frequency of anti-Jo-1 antibodies, similar to the rat study that showed significantly elevated positive tests for anti-Jo-1 with amphibole exposure [41]. Jo-1 autoantibodies have been shown to be associated with pulmonary disease [80], but the mechanism is not known.

Excellent reviews have explored the immunological effects of asbestos and attempted to link the various pathologies via a unified immune dysregulation [16, 17]. One of the recurring ideas regarding silica and asbestos immunotoxicology is that there are two events that converge to perpetuate autoimmune responses. The first is silicate-induced apoptosis, particularly of phagocytic cells, leading to accumulation of cellular debris. The second event is immune activation via "adjuvant" or inflammasome-activating effects, which drive antigen presentation in an environment that is no longer tolerized to self-material (Figure 1). Recent studies describe activation of inflammasomes by asbestos, driving proinflammatory effects such as IL-1β secretion [81, 82]. The inflammasome cascade activation, which can trigger a wide range of effects, may help explain the extremely diverse effects of asbestos in surface markers and cytokines that have been reported over the years [70, 83–86]. Despite the appeal of this 2-hit theory to link asbestos pathologies, the literature so far supports association, but not necessarily causation [87, 88]. However, there is the one study recently suggesting differential inflammasome activation by chrysotile and amphibole [72], which supports the idea that a key early trigger involves the inflammasome. This study demonstrated that although caspase cascade, oxidative stress, and the NLRP3 inflammasome were activated by both fibers, there were important differences in the specific pathways that were activated.

Interestingly, the murine SLE-like disease induced in mice by Libby amphibole was characterized by the production of autoantibodies to dsDNA and Ro52, similar to what was seen in the Libby asbestos human exposures [20, 28]. Such studies may be critical to discovery of mechanism of action. For example, it has been postulated that autoantigens become antigenic due to proteolytic degradation or apoptotic processes [2, 3]. During cell stress or death, Ro52 undergoes intracellular translocation and accumulates in apoptotic blebs during programmed cell death induced by a variety of oxidant challenges including asbestos [4, 89]. One study demonstrated that autoantibodies from asbestos-exposed mice bind to apoptotic blebs in which Ro52 had accumulated [88]. Ro52 has been identified as an E3 ubiquitin ligase [90], so it is possible that exposure to fibers causes upregulation of Ro52 expression, protein misfolding, and/or altered ubiquitination by Ro52 (including self-ubiquitination of Ro52 itself) and ineffective proteasomal degradation. Alteration or poor removal of target proteins could support such proteins becoming antigenic. One hypothesis, therefore, regarding the differences between immune dysfunction with amphibole and chrysotile relates to increased biopersistence of amphibole compared to chrysotile, so that long-term exposure to the fibers leads to accumulation of antigenic cell debris in an inflammatory environment, supporting the development of highly activated APCs that could then trigger autoreactive T and B cells. Alternatively, since both amphibole and chrysotile asbestos can cause oxidative stress and cell death in macrophages and mesothelial cells [91, 92], the mechanism of cell activation and apoptosis may be different [72], leading to different pathways of protein degradation.

Much more work is clearly needed to understand the mechanistic etiologies of the differential immune dysfunction by chrysotile and amphibole. The importance of this ongoing discovery is illustrated in an examination of the relationship between autoantibodies and pulmonary disease, which strongly suggests exacerbation of disease.

7. Relationship between Autoimmunity and Pulmonary Disease

Several of the studies reporting ANA following asbestos exposure also indicated that having a positive ANA test was associated with either more severe or more rapid progression of lung disease (see Table 2, Figure 2) [20, 33, 38, 93]. The significance of this requires careful scrutiny, since it is possible that this association exists simply because high levels of exposure to asbestos may lead to both lung disease and autoantibodies, but that the latter two are not causally related. At least one study has shown no association between the presence of autoantibodies and radiological changes [37]. As mentioned above, it could also be that the autoantibodies follow the lung disease due to tissue damage, although the longitudinal studies by Tamura et al. argue against this since the autoantibodies were present prior to lung disease in many cases [22, 38]. Others have concluded that the lack of autoantibodies in other chronic pulmonary diseases also argue against the idea of the autoantibodies being only secondary to pulmonary disease [33, 93]. There are some clues among the various studies that might help elucidate whether there is an autoimmune component driving severity or progression of asbestos-related pulmonary disease. In the Tamura studies, where an association existed between increased ANA frequency with pulmonary lesions among asbestos-exposed workers, the association was only significant for interstitial, not pleural, lesions [22]. Although not clearly indicated, these were occupational exposures that were likely primarily chrysotile or a mixture of fibers. Another study, however, suggested that ANA in a tremolite (amphibole) exposed cohort were associated with pleural abnormalities [24]. Among former and current Libby, Montana residents, radiographic abnormalities were seen in 18% of the total population; however, among those with suspected SAID, nearly twice as many (35%) had radiographic abnormalities [94]. A follow-up study of this cohort revealed that LA-exposed individuals testing positive for ANAs were nearly 3.55 times more likely to have pleural or interstitial abnormalities than were those testing negative ($P = 0.004$) [67]. In the Libby cohort studies to date, the analyses were done simply for radiographic abnormalities, whether pleural or interstitial, primarily due to the fact that the vast majority of Libby subjects exhibit pleural disease, making analysis of interstitial disease alone

very difficult [94]. Thus, these studies suggest the possibility that studies of cohorts (or animal models) exposed to pure chrysotile or amphibole asbestos might reveal very different autoantibody profiles that contribute to different forms of disease.

A possible role of autoantibodies to fibroblasts, endothelial, and epithelial cells in vascular and fibrotic disorders is receiving increasing attention as the evidence of autoantibody pathogenicity expands. Autoantibodies to endothelial cells have been implicated in vasculitis [95], SSc [96], and SLE [97]. Antifibroblast antibodies (AFA) are also considered a possible factor in pathogenesis of SSc [98–100]. However, data on the role of autoantibodies in fibrotic disease is emerging slowly, due to difficulties in assigning etiology in these complex disease processes (Figure 2). Autoantibodies are thought to contribute to fibrosis by activating target cells to produce profibrotic or proinflammatory cytokines [98], to secrete extracellular matrix proteins such as collagen I [43, 101], or by activating profibrotic cell signaling pathways [102]. Antifibroblast antibodies have been demonstrated in amphibole-exposed mice, and these AFA activate a phenotype change to myofibroblasts in mouse primary lung fibroblasts [43]. Based on the phosphorylation of PDGF-R alpha following treatment of these cells with serum antibodies from these mice, it was postulated that this receptor could be one of the targets for the autoantibodies [43]. In fact, AFA have been shown to bind to PDGF-R in SSc subjects, inducing profibrotic signaling [102]. Recently, mesothelial cell autoantibodies (MCAA) were found in sera of Libby amphibole-exposed subjects, and there was a positive and significant correlation between MCAA presence and pleural, but not interstitial, disease [67]. MCAA bind to the surface of pleural mesothelial cells (Met5A) and induce the production of collagen matrix in the absence of mesothelial-mesenchymal transition [101]. Thus, AFA and MCAA are found in the serum of amphibole-exposed mice and humans, respectively, and potentially contribute directly to the fibrotic disease process.

8. Conclusions

The limited number of epidemiological studies exploring a causal association between asbestos exposure and autoimmune disease makes it difficult to draw conclusions. First, as with most studies of asbestos, the observations of immune dysfunction described above are focused primarily on male, occupationally exposed populations. This could be a limitation when evaluating clinical outcomes such as autoimmune diseases that are more prevalent among women. Second, many studies are retrospective, introducing limitations in terms of exposure assessment and in clarifying the temporal relationship between exposure, autoimmune response, and pulmonary manifestations of disease. It is possible that asbestos exposure is associated with autoimmune disease processes that are not yet clinically recognized. Asbestos exposure in general, or exposure to specific fibers, may be associated with distinct autoimmune pathologies and serological responses that fall outside standard diagnostic criteria. This presents a unique challenge for epidemiological studies that often rely on medical records, physician assessment, death records, or other documentation to assess clinical endpoints. Nevertheless, the data summarized here provide compelling evidence of an association between asbestos exposure and autoimmunity, including a possible contribution of autoantibodies to the fibrotic disease process. It will be critical for future studies to carefully examine immune dysfunction following specific types of asbestos since there are important clues already suggesting unique pathologic mechanisms with chrysotile compared to amphibole. Such studies will need to include asbestos-like fibers such as erionite and nanofibers, which could significantly expand the potential public health impacts of environmental autoimmunity if such fibers induce similar immune dysfunction. Importantly, if there is an autoimmune component to asbestos-related lung diseases, specifically targeting the adaptive immune system may provide better therapeutic approaches for fibrotic processes, leading to far better health outcomes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors gratefully acknowledge the intellectual contributions of the Libby Epidemiology Research Program (LERP): Icahn School of Medicine at Sinai including Raja Flores, M.D., Jaime Szeinuk, M.D., and Stephen Levin, M.D. and the Center for Asbestos Related Diseases: Brad Black, M.D. This work was funded in part by the LERP Grant from CDC/ATSDR TS000099-01.

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 325461, 12 pages
http://dx.doi.org/10.1155/2014/325461

Research Article

Mercury, Autoimmunity, and Environmental Factors on Cheyenne River Sioux Tribal Lands

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Received 7 November 2013; Accepted 17 February 2014; Published 24 April 2014

Academic Editor: Aristo Vojdani

Mercury (Hg), shown to induce autoimmune disease in rodents, is a ubiquitous toxicant throughout Cheyenne River Sioux Tribe (CRST) lands. CRST members may be exposed to Hg through fish consumption (FC), an important component of native culture that may supplement household subsistence. Our goals were to ascertain whether total blood Hg levels (THg) reflect Hg exposure through FC and smoking, and determine whether THg is associated with the presence of anti-nuclear antibody (ANA) and specific autoantibodies (sAuAb). We recruited 75 participants who regularly consume fish from CRST waters. Hg exposure through FC and smoking were assessed via questionnaires. Whole blood samples were collected from participants, and THg was measured using ICP-MS. ANA and sAuAb in serum were modeled using demographic and exposure information as predictors. Female gender, age, and FC were significant predictors of THg and sAuAb; self-reported smoking was not. 31% of participants tested positive for ANA $\geq 2+$. Although ANA was not significantly associated with Hg, the interactions of gender with Hg and proximity to arsenic deposits were statistically significant ($P < 0.05$). FC resulted in a detectable body burden of Hg, but THg alone did not correlate with the presence of ANA or sAuAb in this population.

1. Introduction

For more than a century, mining from greater than 900 mines in the Black Hills, including gold mines in which Hg was used for amalgamation purposes, has released contaminants into watersheds draining onto CRST lands [1]. Additionally, approximately one ton of airborne Hg is emitted per year from coal power plants in Montana, Wyoming, North Dakota, and South Dakota [2] and carried downwind to CRST lands where precipitation and dust wash this mercury out of the air into water and soil. Thus, Hg is virtually ubiquitous throughout the CRST reservation. Studies over the last decade conducted by the tribe, United States Environmental Protection Agency (USEPA), and University of Colorado [3] have documented high mercury concentrations in mid-flow water samples and sediment [4], invertebrates [5], and fish [5–7]. As a result of the widespread presence of Hg in the environment, fish consumption warnings have been posted along the Cheyenne River since 1974, yet no comprehensive health studies have ever been conducted in the CRST population to assess the health effects of consuming fish from tribal waters. In spite of posted warnings, CRST members still consume locally caught fish for complex reasons. Fishing and fish consumption are not only important in Lakota culture,

but high rates of poverty (~50%) [8, 9] and unemployment (88%) [10] on the CRST reservation increase the community's likelihood of using fish to supplement household subsistence. Therefore, the safety of eating mercury-contaminated fish caught on tribal lands was a prime concern for CRST members. To address the CRST's environmental health concerns, a research partnership, *Environmental Justice on Cheyenne River*, was established in 2003 among the CRST Department of Environment and Natural Resources (DENR), the Black Hills Center for American Indian Health, and the University of New Mexico Community Environmental Health Program (UNM CEHP). Through community forums and discussions with tribal leaders, the partnership identified a major concern that a perceived increase in autoimmune disease (AD) prevalence in the CRST population might be related to Hg exposures through fish consumption, as well as a widespread frustration that actual health studies had not occurred in spite of Hg warnings posted for nearly 40 years. Although deidentified numbers of autoimmune cases were obtained from Indian Health Service (IHS) data sources, interpretation of the prevalence is difficult in identification of an appropriate denominator and determination of an appropriate comparison figure for Native American populations. Data on antinuclear antibody (ANA) prevalence in Native populations has not been evaluated. Prevalence of ANA in other US populations was recently derived from National Health and Nutrition Survey data (NHANES) [11, 12], but values for Native American populations could not be extracted due to no representation in that sample. Reference values for specific AD in tribal populations relative to the US total population are also not readily accessible.

Since tribal populations are comparatively more homogenous than other studied US populations, it may be tempting to ascribe any elevations in AD in the CRST merely to genetics. However, while genetic susceptibility has long been acknowledged as an important causative factor in the development of AD and evidence [13, 14] exists that genetic composition may predispose CRST members to AD, it is estimated that genetic factors only account for one-third of disease risk and that gene-environmental interactions play a vital role in the onset of autoimmunity [15]. The growing role of environmental factors, including aluminum metal compounds and thimerisol in vaccines, as adjuvants to the pathogenesis of autoimmunity has been studied extensively [16]. In addition, studies [17, 18] indicate that Hg toxicity and autoimmunity may be synergistically enhanced by various infectious and noninfectious triggers. It is reasonable that chronic stimulation of the immune system by environmental Hg may act through similar mechanisms. To address the community's concerns and begin to address existing gaps in knowledge about the effects of chronic low-level environmental exposures to metals, we sought to systematically examine the relationships among fish consumption, THg, and basic immune system markers in the CRST population in this study.

Existing knowledge about the effects of metals on the immune system comes mainly from the use of rodent models. In these models, relatively high doses of inorganic mercury administered to genetically susceptible mouse strains lead to the development of lupus-like autoimmune syndrome, which includes increased circulating antibodies to nuclear targets (antinuclear autoantibodies, ANA) [16, 17]. Further, exposure to inorganic *or* organic mercury exacerbates and accelerates the development of lupus-like disease in susceptible mouse strains [18–21]. Rodent models of mercury-induced autoimmunity [22–24], as well as their consistency with sex differences in autoimmune disease incidence observed in humans, suggest it is biologically plausible that Hg and other metals contribute to autoimmune pathogenesis in humans. Yet, with the exception of a few epidemiologic studies investigating the role of mercury amalgam fillings in multiple sclerosis [25, 26] and studies of ANA and cytokines in mercury-exposed Amazonian Brazil populations [27–30], too few [31, 32] have investigated the potential role of chronic environmental metal exposures as risk factors in the development of AD in humans. While relationships between metal exposure and immune dysfunction have been demonstrated in animals, limited data exist in humans. Since Hg has long been linked to development of AD-like symptoms in animal models [17], we hypothesized that increased mercury exposure, primarily through fish consumption, would be associated with higher levels of circulating autoantibodies in the CRST population. In order to test this hypothesis and respond to community concerns, we modeled ANA and specific autoantibody concentrations in blood collected from CRST community members using THg, fish consumption, smoking, age, gender, and proximity to high-concentration arsenic sediment deposits as predictors.

2. Materials and Methods

2.1. Human Subjects. The protocol and study design were approved by the Executive Committee of the Cheyenne River Sioux Tribe Tribal Council (Tribal Resolution number: E-302-08-CR and extended under E-343-2009-CR) and by the University of New Mexico Health Sciences Center Human Research Protection Office (HRPO number: 08-486). As deidentified serum samples were sent to the Scripps Research Institute Department of Molecular and Experimental Medicine, the Scripps Research Institute's Institutional Review Board provided approval for an analysis of serum ANA and specific autoantibodies.

Participants were recruited by using community-based communication tools and procedures previously developed by this team and applied in the *Environmental Justice on Cheyenne River* study. Outreach, enrollment, and sampling were conducted in conjunction with local collaborators, notably Missouri Breaks Industries Research, Inc. (MBIRI), who were crucial contributors in several previous federally funded research projects among Cheyenne River Tribal communities, and collaborating staff from the CRST DENR. The recruitment was targeted toward fishermen and their family members, who were known to local collaborators as regular consumers of fish caught from the Cheyenne River and its tributaries.

Written informed consent was obtained from a total of 75 adults living on the CRST Lands during the peak of fishing season. The study population includes members from

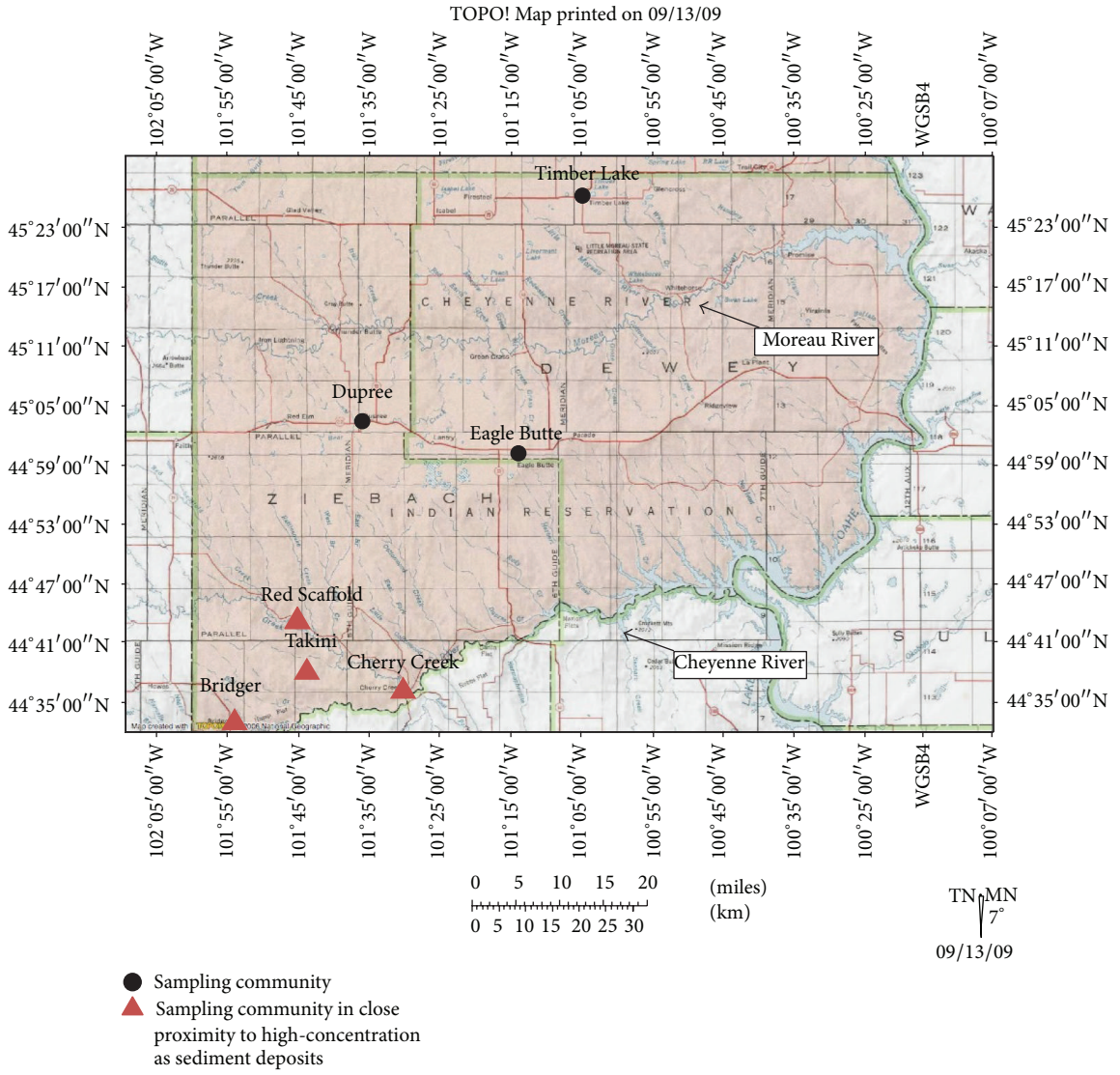


Figure 1: Map of Cheyenne River Sioux Tribal lands and sampling communities.

multiple communities including Eagle Butte, Cherry Creek, Dupree, Timber Lake, Red Scaffold, Bridger, Takini, and Howes (Figure 1). At each location, enrollment was conducted and biological samples were collected in community centers. These communities, some of which are in close proximity to rivers, lakes and ponds on CRST lands, encompass both commercial centers and rural areas, as well as members whose primary source of food is store-bought versus acquired from the local environment (subsistence lifestyle), and therefore reflect a wide range of potential exposures to Hg through fish consumption. Smoking status was a concern as an alternate contributor to THg based on previously reported increases in smoking on the CRST reservation [33] and the demonstrated contributions to THg from cigarette smoking [34, 35]. MBIRI team interviewers collected demographic (e.g., age, gender), health condition, fishing, and smoking habit information through personal interviews conducted in English using a Centers for Disease Control- (CDC-) developed fish consumption survey and our own short smoking exposure questionnaire. When participants needed information or clarification spoken in their native language, the community-certified nurse interviewers provided the answers.

2.2. Surveys Used in the Study

2.2.1. Fish Consumption. A CDC questionnaire, as well as local collaborators' knowledge of CRST community members' fishing habits, was used to assign a categorical rating of 1, 2, or 3 to each participant's fish consumption, with 1 designating minimal to no fish consumption, and 3 corresponding to high fish consumption. For reference to the local environment and consumption patterns, the safe amount of fish intake per month was previously recommended by our *Environmental Justice on Cheyenne River* study using DENR Hg measurements from local fish and USEPA guidelines [37].

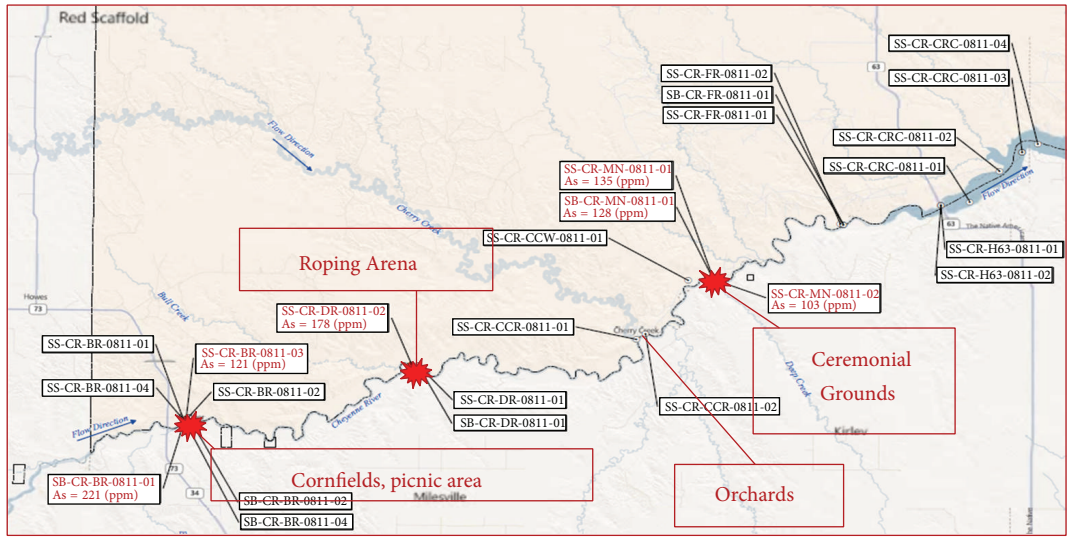


Figure 2: Map of arsenic sampling conducted by USEPA and CRST DENR. Concentrations of arsenic in sediment exceeding 100 ppm are marked with a burst pattern. Exposure-relevant sites are labeled with activities frequently conducted in those areas.

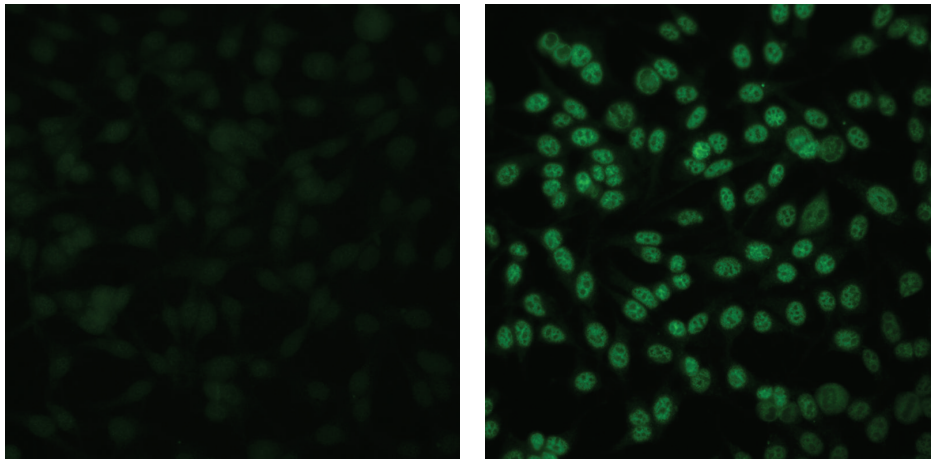
One monthly recommended serving was defined as one northern pike, two bass or perch, three walleye, or four catfish. A rating of (1) denotes consumption of <1 serving of fish per month; (2) denotes 1-2 servings/month; and (3) denotes >2 servings/month.

2.2.2. Smoking. To account for smoking as both a potential source of Hg and contributor to immune system effects, participant smoking data were collected via questionnaire. The questionnaire was based on coauthor PNH's previous work [33] on smoking among tribal members and was given to all participants in order to obtain self-reported information regarding smoking exposures. The questionnaire encompassed both direct and second-hand exposure to cigarette smoke. There were seven questions total; smoking score was coded as low (1) when fewer than two questions were answered affirmatively; medium (2) when 3-4 questions were answered affirmatively; and high (3) when greater than five questions were answered affirmatively. A participant was considered an "active smoker" if he/she answered "yes" to the included question, "Do you smoke currently?"

2.2.3. Arsenic Proximity. During the analytic phase of this study, elevated sedimentary arsenic deposits were discovered in land-use areas in close proximity to several of the sampling-site communities in this study (Figure 2). Ongoing collaborations among DENR, Dr. Lewis, and USEPA Region 8 are surveying residents and characterizing exposure pathways, frequencies, and duration. However, as these deposits were identified subsequent to consent for this study, no arsenic biomonitoring data were obtained from the population in the original design nor were exposure activities involving these sedimentary deposits identified. Due to studies in humans and animals indicating that arsenic suppresses autoimmunity [38, 39], while mercury may either suppress or increase autoimmune response [28, 32], a surrogate of participant arsenic exposure was incorporated into models to address potentially competing exposures. A binary surrogate for arsenic exposure was derived; the designations of "near" or "far" proximity to known quantified environmental arsenic deposits by USEPA were given according to self-reported participant residence data. The designation of "near" was given to participants who live in the communities of Cherry Creek, Takini, Bridger, and Red Scaffold (Figure 1). Surveys of residents have identified potential exposure pathways which include common land-use practices such as fishing; herb, fruit, and firewood gathering; inhalation of wood combustion products during sweat lodge and ceremonial practices; and roping/other horseback riding activities along the Cheyenne River near the identified alluvial arsenic deposits (Figure 2) (personal communication C. Ducheneaux and J. Lewis). Participants residing in the Eagle Butte, Dupree, and Timber Lake (Figure 1) communities more distal to the arsenic deposits were given a designation of "far" for arsenic proximity in this pilot assessment. This binary variable was incorporated to determine if further studies on the relationship of these exposures to AD were warranted.

2.3. Biological Sample Collection

2.3.1. Blood and Serum Samples. Venous blood samples were collected by venipuncture at community centers or during home visits by a trained and certified phlebotomist or registered nurse. One red top (9 mL) for serum collection and one purple top (7 mL) Vacutainer tube were collected for biomonitoring from each participant. After clotting, serum samples were spun at 2,500 rpm for 10 minutes and separated into cryovials and placed into a −80°C freezer. At a later time point, sera were shipped to the UNM HSC laboratory and subsequently to the Scripps Research Institute.



(a) (b)

Figure 3: Examples of ANA determination by immunofluorescence. Human sera were incubated with HEp-2 cells followed by fluorescent anti-human IgG. The sample on the left (a) is ANA negative, while the sample on the right (b) was considered 2+ ANA positive, showing fine speckled nuclear staining sparing the nucleolus.

2.4. Experimental Use of Collected Biological Samples

2.4.1. Biomonitoring. The EDTA-containing whole blood samples were transported to the CDC ONDIEH/NCEH Environmental Health Laboratory where inductively coupled plasma mass spectrometry (ICP-MS) was used to determine THg concentrations. The limit of detection was 0.32 μg/L.

2.4.2. Detection of Autoantibodies

(1) Antinuclear Antibodies (ANA). The presence of ANA was determined by indirect immunofluorescence (IIF) microscopy using HEp-2 cells as substrate (MBL-BION, Des Plaines, IL) and Alexa Fluor 488 Goat Anti-Human IgG (H + L) (Life Technologies, NY, USA) as detecting reagent. Sera were diluted 1 : 100 in serum diluent, and detecting reagent 1 : 200 with anti-Ig diluent as previously described [40]. Slides were viewed by a single observer (KMP) blinded to participant identity on a BH2-RFCA fluorescence microscope (Olympus, Lake Success, NY). Intensity of fluorescence was graded on a scale of 0–4+. A reading of ≥2+ was considered significant and further used in our statistical modeling. This cut-off value reflects a stricter value based on the literature [36, 41]. Example immunofluorescence images for ANA determination can be found in Figure 3 for negative (0) and ANA ≥ 2+ readings.

(2) Specific Autoantibodies (sAuAb). Commercially available kits (INOVA Diagnostics, San Diego, CA) were used as described by the manufacturer to detect and quantify serum autoantibodies to the following antigens: chromatin, Sm, RNP, SSA, SSA-52, SSB, Scl-70, RNA Pol III, CENP-A/B, Ribo-P, Jo-1, M2 EP (MIT3), and primary biliary cirrhosis (PBC) screen, a panel of antigens (M2 EP, gp210 and sp100 IgG/IgA). Assay-specific positive controls were used to convert optical density values to units in order to determine whether the results of assays for Sm, RNP, SSA, SSA-52, SSB, Scl-70, RNA Pol III, Ribo-P, and Jo-1 were negative/equivocal (<20 units), weakly positive (20–39 units), moderately positive (40–80 units), or strongly positive (>80 units). The tests for M2 EP and the PBC screen were interpreted as being equivocal from 20.1 to 24.9 units and positive for >25 units. Centromere-A/B (CENP-A/B) has negative/equivocal results for <20 units, weak positive for 20–30 units, and strong positive for >30 units. Chromatin has a negative/equivocal reading <20 units, moderate positive between 20 and 60 units, and a strong positive >60 units.

Additional assays to chromatin, denatured DNA (single-stranded, dDNA), native DNA (nDNA), and histones were quantified by enzyme-linked immunosorbent assays (ELISA) as previously described in [42, 43]. Briefly, Immulon 2HB microtiter plates (Dynex Laboratories, Inc., Alexandria, VA) were coated with antigen at 2.5 μg/mL concentrations. For the antichromatin assays, in-house-prepared H1-stripped chromatin was used as the solid-phase antigen. S1-nuclease (Invitrogen-) treated DNA (Calbiochem) was used in the antinative DNA assay, and DNA was heated for 10 min and then quickly cooled for preparation of the dDNA antigen. Prior to coating plates with nDNA or dDNA, plates were pre-coated at 2 μg/mL with the synthetic 20–50 kDA polypeptide poly(lys-phe) (Sigma-Aldrich), comprising a co-polymer of lysine and phenylalanine at a 1:1 ratio as previously described [43]. Total histone was from Worthington. Serum samples were diluted 1 : 200 and incubated on the plate for 2 hours at room temperature with gentle shaking. Each sample was run in duplicate. The bound antibodies were detected with peroxidase-conjugated anti-human IgG (Southern Biotech, AL) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (MP Bioproducts) as the secondary substrate. Optical densities (OD) beyond the range of direct measurement at 1 h in the ELISA were extrapolated from OD at earlier time-points as described [44]. Positive and negative control sera were always included in each assay, and values determined in different assays were normalized by

multiplying by the ratio of the reactivity of the positive control sera tested in each assay.

2.5. Statistical Analysis: Modeling. Total blood mercury results lower than the limit of detection (LOD = 0.32 μg/L) were analyzed with the value of LOD/$\sqrt{2}$, because fewer than 50% of participants had a biomonitoring value < LOD. Total blood mercury results are presented as median values with the interquartile range, since the median is a better indicator of the true population value for the distribution of the collected data. The mean and 95% confidence interval for THg are also presented for ease of comparison with published NHANES population data. When comparing groups (e.g., male versus female) in relation to THg and ANA status, Fisher's exact test was used.

To characterize the complex exposures on CRST lands and their relationships to immune system responses and autoantibody production, several statistical models incorporating biomonitoring data, fish consumption score, smoking exposure score, distance to arsenic contamination, and immune system markers were developed. The approaches included multiple linear, logistic, and Poisson regression models to evaluate relative contributions of environmental exposures to circulating autoantibodies. They also included accepted risk factors such as age and gender. Multiple linear regression was used to model THg in relation to environmental exposure and risk factors, while logistic regression was used to model ANA $\geq$ 2+ in relation to predictors. Poisson regression was used to model the numbers of specific autoantibodies (both determined via INOVA and additional assays) with environmental exposure and risk factors. Poisson models accommodate count information with nonnormal distribution, thereby enhancing the analytical capacity to understand the exposure factors' underlying contribution to risk. Full models were fitted using all demographic, biomonitoring, and exposure data as predictors. Reduced models were selected using the Akaike information criterion (AIC), which is a measure of the relative quality of a statistical model. The openly available statistical software R [45] and the stepAIC function from the package MASS [46] were used to complete this AIC model selection, where

$$\mathrm{AIC} = 2k - 2\ln(L). \quad (1)$$

And $k = 2$ and L is the likelihood of each model. The AIC selection criterion minimizes the distance between the predicted values of the model and the true values while also favoring models with fewer parameters.

2.5.1. Specific Approach to Analyze Specific Autoantibody Results from INOVA Assays. Since positivity for individual-specific autoantibodies was expected to be lower in frequency, we pooled all participants who tested positive for any specific autoantibodies to examine an overall prevalence of specific autoantibodies and the contributing exposure factors by summing the number of specific autoantibodies for which each participant tested positive and then conducting Poisson regression on the count variable generated. Biomonitoring data and exposure data were incorporated as well. This count variable makes biological sense because it follows established clinical AD diagnosis criteria; individuals diagnosed with AD present with varying combinations of specific autoantibodies.

2.5.2. Specific Approach to Analyze Specific Autoantibodies Results from Additional Autoantibody Assays. Poisson regression was used to model several combinations of autoantibodies that would otherwise be rarely detected. The combinations modeled were selected in order to examine different possible scenarios of positive autoantibody response. Individuals with detectable autoantibody response were classified into groups according to the following scenarios based on the literature [42–44]:

(a) presence of any autoantibody response (nDNA, dDNA, histone, chromatin);

(b) detectable levels of potentially environmentally related autoantibodies (dDNA and histone);

(c) disease-associated autoantibodies (nDNA and chromatin).

Results of these models were summarized in several tables presented in the next section.

Because anti-chromatin autoantibodies were detected using both INOVA and in-house assays, we evaluated the reproducibility of this antigen. We applied a nonparametric correlation (Spearman r-value) and used a z-score.

2.5.3. Reporting of Significant Results. While our primary results will follow a more standard reporting cutoff of $P < 0.05$, we will report those with probabilities up to 0.1 to guard against Type 2 error and to ensure comprehensive consideration of predictors in designing follow-up investigations. This decision is warranted given (1) the importance of the results to the communities, (2) the lack of prior studies in this area and in this population, and (3) the lack of biomonitoring data at this time on other potential environmental exposures including arsenic resulting in imprecise measures for that variable.

3. Results

3.1. Mercury Exposure and Population Characteristics. Population characteristics, including gender, smoking score, fish consumption score, community size, and proximity to identified high-concentration sedimentary arsenic deposits, are summarized in Table 1. Total blood mercury concentrations (THg) ranged from below the limit of detection (LOD, 0.32 μg Hg/L) to 4.14 μg Hg/L, with a median lower than the LOD (Figure 4). For most population characteristic categories, the median THg was below the LOD, with the exception of males (0.37 μg Hg/L) and participants with "medium" or "high" fish consumption scores (0.35 and 0.54 μg Hg/L, resp.).

Total blood mercury in the CRST depended on gender, age, and fish consumption but not smoking. The reduced multiple linear regression modeling results for THg as a response with demographic and exposure information as

Table 1: Biomonitoring and ANA ≥2+ results linked with study participant characteristics.

Population characteristic	*N*	Participants with THg >LOD*	Hg biomarker median (interquartile range)	Hg biomarker mean (95% CI)	ANA reading ≥2+ (*n*, %)
All participants	75	36 (36%)	<LOD (<LOD–0.87)	0.75 (0.55–0.95)	23 (31%)
Gender					
Male	38	23 (61%)	0.37 (<LOD–1.81)	1.01 (0.67–1.37)	2 (5%)
Female	37	13 (35%)	<LOD (<LOD–0.56)	0.48 (0.32–0.63)	9 (24%)
Smoking score					
(1) Low	32	17 (53%)	0.37 (<LOD–0.74)	0.67 (0.41–0.92)	8 (25%)
(2) Medium	18	8 (44%)	<LOD (<LOD–1.24)	0.82 (0.36–1.28)	3 (17%)
(3) High	25	11 (44%)	<LOD (<LOD–1.21)	0.81 (0.39–1.23)	7 (28%)
Active smoker					
Yes	42	19 (45%)	<LOD (<LOD–0.96)	0.77 (0.49–1.05)	4 (10%)
No	31	16 (52%)	0.37 (<LOD–0.87)	0.76 (0.40–1.06)	7 (23%)
Fish score					
(1) Low	41	17 (59%)	<LOD (<LOD–0.60)	0.59 (0.36–0.82)	7 (17%)
(2) Medium	18	10 (56%)	0.35 (<LOD–1.53)	0.90 (0.38–1.43)	2 (11%)
(3) High	16	9 (56%)	0.54 (<LOD–1.89)	0.99 (0.51–1.48)	2 (13%)
Arsenic proximity					
Yes	23	7 (30%)	<LOD (<LOD–0.52)	0.45 (0.25–0.64)	7 (30%)
No	52	29 (56%)	0.37 (<LOD–1.27)	0.89 (0.62–1.16)	4 (8%)

*LOD = 0.32 μg/L.

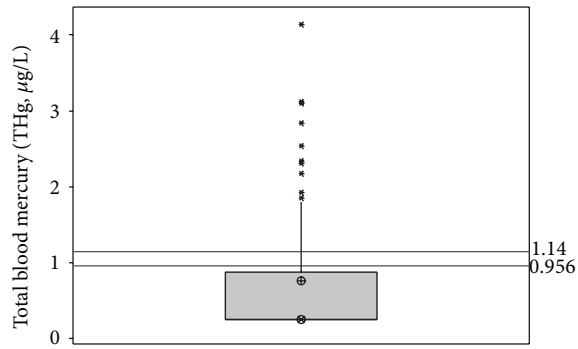


Figure 4: Scatterplot of total blood mercury (THg) for sample population with median denoted by an encircled "X" and mean denoted by an encircled cross. The reference lines at 0.956 μg/L and 1.14 μg/L indicate the 95% CI for THg in the US population from NHANES [36].

Table 2: Reduced model (multiple linear regression) for total blood mercury.

	Estimate	*P* value	Std. error
Intercept	0.412	0.46	0.56
Gender*	−0.545	*0.0084*	0.2
Age	0.0181	*0.022*	0.0077
Smoking score	−0.0127	0.91	0.12
Fish score	0.246	0.053	0.13
Arsenic proximity	−0.526	*0.020*	0.22

P values less than or equal to 0.05 are italicized.
*Male gender was used as the reference, so the estimate describes the effect of being a female.

predictors are summarized in Table 2. Male gender and older age were significant predictors of THg ($P = 0.0084$ and 0.022, resp.); fish consumption approached significance as a predictor for THg ($P = 0.053$).

3.2. Prevalence of ANA in the CRST Population. Anti-nuclear antibodies (ANA) were analyzed in serum samples from all participants. Data are presented in Table 1 and representative images of negative and ANA ≥ 2+ readings are shown in Figure 3. Approximately thirty-one percent of participants had an ANA reading of ≥2+. For readings ≥2+, ANA prevalence was significantly higher in women than in men (24% versus 5%; $P = 0.025$). ANA prevalence was also larger in community members living in proximity to high-concentration sedimentary arsenic deposits (30% versus 8%; $P = 0.028$).

Gender and fish consumption were significant predictors of ANA ≥2+, and gender modifies the effects of environmental exposures with respect to ANA. The logistic regression model information is shown in Table 3. Age and fish consumption are borderline predictors ($P < 0.10$) of ANA ≥ 2+ ($P = 0.081$ and $P = 0.092$, resp.), with age and fish consumption positively associated with the probability of ANA ≥ 2+ level of circulating ANA. Gender, THg, and proximity to arsenic, by themselves, do not strongly correlate with the probability of ANA ≥ 2+; however, the *interactions* of gender with THg and arsenic proximity are significant, and

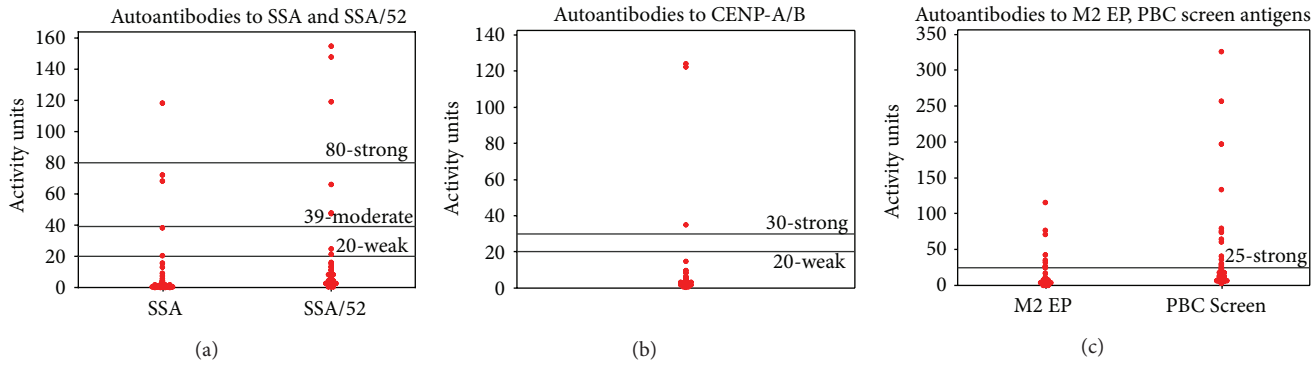


Figure 5: Dot plots of detectable autoantibodies in participant serum measured in activity units with labels for clinical cutoffs. Note: In clinical practice, there are no "moderate" positive readings for CENP-A/B; there are no "weak" or "moderate" positive readings for M2 EP and the PBC Screen.

Table 3: Point estimates and 95% confidence intervals (CI) for coefficients and odds ratios (OR) for fitting logistic regression models for ANA ≥2+.

	OR	95% CI	P value
Intercept	0.011	N/A	0.93
Age	1.1	0.96–1.17	0.081
Gender	2.4	0.046–645.5	0.37
THG	0.4	0.045–1.75	0.89
Fish score	2.9	0.56–20.70	0.092
Gender: THG	13.8	0.97–487.8	*0.026*
Gender: fish score	0.1	0.0013–1.12	0.97
Gender: arsenic proximity	27.1	0.68–2101	*0.040*
Arsenic proximity	0.3	0.015–4.26	0.82

P values less than or equal to 0.05 are italicized.

their odds ratios are greater than one (OR = 13.83, $P = 0.026$ and OR = 27.71, $P = 0.04$, resp.).

3.3. Specific Autoantibodies in the CRST Population. Of the specific autoantibodies for which participant sera were tested, SSA, SSA/52, CENP-A/B, M2 EP, and the autoantibodies detected by the primary biliary cirrhosis (PBC) panel were noteworthy. These results are summarized in Figure 5 and Table 4. Fifteen percent of participants tested positive for autoantibodies to M2 EP, while 24% were positive for autoantibodies to the PBC panel.

The number of specific autoantibodies detectable by INOVA kit increased with female gender and fish consumption score. Information for the reduced Poisson model for the number of detectable specific autoantibodies using INOVA assays can be found in Table 5(a). The number of specific autoantibodies detectable from INOVA assays was associated significantly with female gender ($P = 0.0064$). The model indicated that the mean number of specific autoantibodies detectable in serum is increased by a factor of 6.5 in female versus male community members. Age and fish consumption had significant ($P = 0.012$ and $P = 0.0073$, resp.) but smaller effects on the number of specific autoantibodies in the collected serum samples. In particular, the mean number of specific autoantibodies detectable by INOVA assay was 2.6 times greater in participants with a high (3) versus a low (1) fish consumption score. The number of participants positive for autoantibodies to native DNA, histone, and chromatin using in-house assays was small in our study. No significant associations were found between any demographic or exposure predictors, including smoking, and various combinations of in-house autoantibodies except in the case of dDNA and histone. The values for the reduced model can be seen in Table 5(b). Fish score was a significant predictor ($P = 0.035$) of the number of subjects with elevated anti-dDNA and anti-histone autoantibodies detectable using in-house assays. An increase of one fish score category predicts a 2.5-factor increase in the number of dDNA and histone autoantibodies. Smoking was a borderline predictor ($P = 0.065$) with a 0.4-factor decrease in the number of dDNA and histone autoantibodies for an increase of one smoking category.

Antichromatin positivity and reproducibility between INOVA and additional assays were confirmed in all positive serum samples (5/75); there was 100% agreement in detection using INOVA and in-house assays.

4. Discussion

We assumed that, due to consumption of locally caught fish, community members would have elevated levels of total blood mercury (THg). We hypothesized that THg would correspond to an increased level of autoantibodies, as has been shown in animal models [17]. Contrary to expectations, although Hg deposition in fish tissue had been documented in CRST sources by DENR, the detected THg levels in participants were low with a median THg < LOD, despite sampling during the middle of fishing season when fish consumption was considered to be maximal. The median THg for all participants (<LOD) was lower than the published results of the NHANES survey [47]. NHANES reported a mean THg of 0.944 μg/L for those 12 years and older [47]. Native American populations were not stratified in that study; the data were compiled under "other" ethnicity. The low levels of blood mercury among the CRST members

Table 4: Results of selected specific autoantibody results from the CRST population sample.

$n = 75$ Autoantibody	Negative	Moderate positive	Strong positive	Total positive
SSA	72 (96%)	2 (3%)	1 (1.3%)	3 (4%)
SSA-52	70 (93%)	2 (3%)	3 (4%)	5 (7%)
CENP-A/B	72 (96%)	0 (0%)	3 (4%)	3 (4%)
M2 EP	64 (85%)			11 (15%)
PBC panel	57 (76%)			18 (24%)

Table 5: (a) Model (Poisson regression) for the number of detected specific autoantibodies using INOVA assays. (b) Model (Poisson regression) for number of detected specific denatured DNA and histone autoantibodies from in-house assays.

(a)

	Factor of change	95% CI	P value
Intercept	0.0	0.00–0.18	0.0012
Gender*	6.5	1.69–24.78	*0.0064*
Age	1.0	1.01–1.04	*0.012*
Fish score	1.6	1.13–2.16	*0.0073*
Smoking score	1.9	0.60–5.77	0.29
Gender: smoking score	0.5	0.25–1.01	0.053

P values less than or equal to 0.05 are italicized. * Male gender was used as the reference, thus, the factor of change reflects the effect of being female.

(b)

	Factor of change	95% CI	P value
Intercept	0.2	0.004–9.831	0.406
Age	1	0.919–1.055	0.658
Smoking	0.4	0.127–1.064	0.065
Fish score	2.5	1.067–5.980	*0.035*
Arsenic proximity	0	0.000–3.752	0.117
Age: arsenic proximity	1.1	0.984–1.244	0.092

P values less than or equal to 0.05 are italicized.

found in our study confirmed the THg levels reported in a 2008 collaborative study between our team and CDC [48, 49]. Potential reasons for the observed low THg include variation due to race/ethnicity and possible physiological and metabolic changes among CRST community members, or possible alterations in deposition and clearance with repeated exposure in this population.

While gender, age, and fish consumption showed an impact on THg levels in the CRST population, smoking did not (Table 2). This finding is puzzling since 56% of the participants reported current smoking, yet only 45% of that group had THg above the detection level. The trends in our data parallel those seen in the NHANES survey [50]. Males have greater mean THg versus females, and THg increases with age. Males may consume larger quantities of locally caught fish or engage in activities that increase dust and particulate exposures to mercury (e.g., agricultural work, horse-tending). The age-dependent increase in THg found in this study, as well as in Wolkin et al. [48] and the NHANES survey [50], is likely due to the accumulation of metals in the body over time.

Thirty-one percent of participants had an ANA reading of ≥2+. ANA production could be associated with chronic toxicant exposure, which introduces self-antigens to antigen presenting cells, resulting in the breakdown of T-cell tolerance. While no single predictor was significantly associated ($P < 0.05$) with ANA ≥ 2+, fish score was a borderline predictor ($P = 0.092$). A larger proportion of ANA-positive participants were female, which concurs with the literature and clinical findings about autoimmune diseases [24], and may support a possible role for female hormones in AD and immune dysregulation. Although THg and proximity to high-concentration arsenic deposits, by themselves, did not correlate with the probability of ANA ≥ 2+, the *interactions* of female gender with THg and female gender with arsenic proximity are significant ($P = 0.026$ and $P = 0.040$, resp.) and the odds ratios were large (OR = 13.8 and 27.1, resp.). Gender differences may reflect alterations in the molecular mechanisms by which gender-specific detoxification occurs within the human body.

Another interesting finding is that current smokers were less likely to have ANA ≥ 2+ results (Table 1). Additionally, the specific autoantibody model estimates for smoking were negative with ORs less than one (Tables 5(a) and 5(b)), suggesting a protective effect of smoking. The fact that fewer autoantibodies were detected in this subgroup of smokers sheds light on probable molecular mechanisms by which smoking induces immunosuppressive effects.

There were significant associations between predictor exposure variables and the presence of autoantibodies to dDNA and histone (Table 5(b)). This is potentially similar to previously observed instances of xenobiotic-induced antibody responses such as drug-induced lupus [42]. Autoantibody production to dDNA and histone may also be linked to epigenetic changes triggered by environmental stimuli.

The CRST population exhibited strong positivity for M2 EP autoantibodies and autoantibodies detectable with the PBC screen, both of which are associated with liver diseases. It is possible that the medical problem of high rates of idiopathic liver cirrhosis in Sioux communities (personal communication J. Henderson) may have environmental etiology. Similar findings were reported among Alaskan Natives [51]. As with ANA ≥ 2+ models, fish score was a significant predictor of *specific* autoantibodies using both detection methods ($P < 0.01$ for INOVA kit detection and $P < 0.05$ for in-house ELISA).

As to the specific mechanisms responsible for mercury and metal/metalloid-induced autoimmune responses in the CRST population, several mechanisms should be considered. In susceptible individuals, environmental metals may behave as adjuvants that prolong or enhance antigen-specific immune response through various mechanisms such as molecular mimicry [52], polyclonal activation of B cells [53], bystander activation [54], and epitope spreading [55]. Additionally, chronic exposures to environmental metals, including Hg and arsenic, are well known to induce oxidative stress. As has been characterized with thimerisol [56], this oxidative stress could lead to sensitization of inositol 1,4,5-triphospate (IP3) receptors, resulting in enhanced intracellular calcium release and subsequently the dysregulation of immune cells and autoimmunity. Another possibility includes the role of chronic gut exposures to ingested dietary nanoparticles of soil and minerals, which induce inflammasome production and the breakdown of immune tolerance via enhanced gastrointestinal antigen presentation. Since fish consumption was an important predictor of antibody production in this study, dietary exposure may be one potential pathway through which molecular markers of autoimmunity are generated, especially among native community members who are more likely to inhale and ingest large quantities of dust and metals due to their rural location, cultural practices, and subsistence and agricultural activities.

We hypothesize that fish consumption reflects multiple exposures, including coexposures to mercury, arsenic, and other environmental toxicants, such as pesticides, pharmaceuticals, and infectious agents. In animal and cell studies, Hg toxicity and autoimmunity are synergistically enhanced by coexposure to additional xenobiotics. These ideas will be explored in future studies, and additional activities that increase inadvertent exposure to toxicants will also be examined. Future studies will include a larger sample size, participant AD medical record history, and biomonitoring for arsenic and cotinine (indicator of smoking exposures) in order to address this study's limitations. We also acknowledge that technical issues with indirect immunofluorescence assays (IIFA) for the detection of ANA limit the comparability of these data to other population information and previous publications. However, IIFA is the gold-standard technique for ANA detection [57], and we attempted to minimize variability by using only one evaluator of staining (KMP, coauthor).

In this study, compelling evidence that the CRST population exhibited elevated levels of both ANA and specific autoantibodies was found. The observed results highlighted environmental toxicants that may contribute to autoantibody production in this population and also underscored the need to characterize the CRST communities' lifestyles and behaviors to better understand how complex exposures contribute to autoimmune health effects. There is a large knowledge gap concerning environmental influences on the development of AD, and it is imperative that they be addressed within the context of environmental health disparities issues, particularly in tribal communities. Information will empower CRST community members and leaders by aiding them in making informed decisions about health, health services, the environment, and the preservation of their culture, in which fishing plays a vital role.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jennifer Ong and Esther Erdei contributed equally to this work.

Acknowledgments

Colleen Martin and the Centers for Disease Control are acknowledged for their help with data collection and biomonitoring. Dr. Jeffrey A. Henderson from the Black Hills Center for American Indian Health is acknowledged for his continuing support, close relationship with CRST community members, and clinical knowledge. Esther Erdei received Community Engaged Research Pilot Grant from University of New Mexico (UNM) (2008) Center for Participatory Research under the Institute for Public Heath, Department of Family and Community Medicine in partnership with the Community Engagement and Research Unit of the Clinical and Translational Science Center (CTSC). Johnnye Lewis received theUniversity of New Mexico Clinical and Translational Science Center Pilot Grant *NIEHS R25 ES013292* Environmental Justice on Cheyenne River (2010).

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 260613, 10 pages
http://dx.doi.org/10.1155/2014/260613

Research Article

A Tandem Repeat in Decay Accelerating Factor 1 Is Associated with Severity of Murine Mercury-Induced Autoimmunity

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Received 4 January 2014; Accepted 21 February 2014; Published 10 April 2014

Academic Editor: Aristo Vojdani

Decay accelerating factor (DAF), a complement-regulatory protein, protects cells from bystander complement-mediated lysis and negatively regulates T cells. Reduced expression of DAF occurs in several systemic autoimmune diseases including systemic lupus erythematosus, and DAF deficiency exacerbates disease in several autoimmune models, including murine mercury-induced autoimmunity (mHgIA). *Daf1*, located within *Hmr1*, a chromosome 1 locus associated in DBA/2 mice with resistance to mHgIA, could be a candidate. Here we show that reduced *Daf1* transcription in lupus-prone mice was not associated with a reduction in the *Daf1* transcription factor SP1. Studies of NZB mice congenic for the mHgIA-resistant DBA/2 *Hmr1* locus suggested that *Daf1* expression was controlled by the host genome and not the *Hmr1* locus. A unique pentanucleotide repeat variant in the second intron of *Daf1* in DBA/2 mice was identified and shown in F2 intercrosses to be associated with less severe disease; however, analysis of *Hmr1* congenics indicated that this most likely reflected the presence of autoimmunity-predisposing genetic variants within the *Hmr1* locus or that *Daf1* expression is mediated by the tandem repeat in epistasis with other genetic variants present in autoimmune-prone mice. These studies argue that the effect of DAF on autoimmunity is complex and may require multiple genetic elements.

1. Introduction

Decay accelerating factor (DAF [the gene and protein designations for decay accelerating factor in this paper are *DAF* for the human gene and DAF for the human protein; the mouse genes are *Daf1* and *Daf2* and corresponding proteins DAF1 and DAF2] or CD55) is a surface-expressed member of the complement-regulatory protein family that protects cells from attack by autologous complement proteins [1]. DAF inhibits the neoformation and accelerates the dissociation of preformed C3/C5 convertase complexes generated by the classical and alternative pathways, thus blocking both complement split product activity and the formation of the membrane attack complex [2]. DAF is present on inflammatory cells and at sites of tissue inflammation where it most likely inhibits bystander complement-mediated cell lysis (13). In addition, recent studies suggest DAF regulates T cell activity [3–6].

In humans, DAF is a single gene on chromosome 1q32 encoding a glycosylphosphatidylinositol- (GPI-) anchored cell surface glycoprotein [7]. In contrast, mice have two tandem Daf genes positioned head-to-tail on chromosome 1 [8] with the GPI-linked *Daf1* (Daf-GPI) located 5′ to the transmembrane containing *Daf2* (Daf-TM). Expression of DAF varies depending on tissue [9] and cell type [10] with DAF widely expressed on the surface of all major circulating blood cells and epithelial and endothelial cells [11, 12]. Studies with *Daf1* knockouts showed that absence of the GPI form results in the loss of DAF expression in most tissues, except testis and spleen where *Daf2* is expressed (27, 28). In the

spleen, DAF2 is expressed primarily in CD11c$^+$ dendritic cells (28). In human cells, DAF expression is modulated by cytokines such as IL-1, IL-6, TNF-α, TGF-β1, and IFN-γ [13–15], prostaglandin PGE2 [16], and tissue specific factors [17]. Although there is evidence that *DAF* mRNA stability can be affected by tissue specific factors [17] and inflammation [18], most studies suggest that expression is primarily modulated at transcription [15–17, 19, 20]. The human *DAF* promoter has been identified, the transcription start site mapped, and regions of potential transcriptional regulation proposed [10, 21]. Analysis of the key transcriptional regulatory elements controlling basal expression of mouse *Daf1* showed that transcriptional activity requires the functional cooperation of two Sp1-binding sites and is enhanced by the presence of a CREB site [22].

Evidence supports a protective role for DAF in autoimmunity [6, 23]. *Daf1* deficient mice exhibit increased CD4$^+$ T cell proliferation and greater secretion of IFN-γ, IL-2, and IL-4 but reduced IL-10 [4]. Furthermore, DAF1 is reduced on T and B cells in autoimmune prone NZB mice [24], and its deletion in lupus prone MRL-*Fas^{lpr}* mice accelerates disease [25]. During induction of murine mercury-induced autoimmunity (mHgIA) DAF1 is specifically reduced on CD4$^+$ T cells resulting in an accumulation of activated (CD44^{high}Daf^{low}) CD4$^+$ T cells [24]. *Daf1* deficiency also exacerbates mHgIA via increased levels of IFN-γ, IL-2, IL-4, and IL-10 but not IL-17 [26]. DAF mediated complement regulation does not appear to contribute to mHgIA as neither the accumulation of CD44^{high}Daf^{low} CD4$^+$ T cells nor the downregulation of DAF1 expression on CD4$^+$ T cells was influenced by a lack of C3 [27]. Additionally, *Daf1* deficiency exacerbates organ specific disease in models of experimental autoimmune encephalomyelitis (EAE) [4], glomerulonephritis in antibody-induced nephritis [28, 29], and experimental myasthenia gravis [30]. Thus DAF impacts the expression of disease in both idiopathic and induced models of autoimmunity.

In a previous study, we showed that resistance to mHgIA resides at a single major quantitative trait locus on chromosome 1, designated *Hmr1*, which was shown to be linked to glomerular immune complex deposits but not autoantibody production [31]. *Hmr1* encompasses a region containing several lupus susceptibility loci as well as *Daf1* and *Daf2*. As DAF regulates complement activation it is possible that differences in DAF expression may impact the deposition of immune complex deposits and contribute significantly to the *Hmr1* phenotype. In this study, we show that *Daf1* expression is reduced in multiple murine strains susceptible to spontaneous autoimmunity and identified a pentanucleotide tandem repeat in the second intron of *Daf1,* which in the mHgIA resistant DBA/2 consisted of eleven repeats while most other strains had 10, except for MRL-*Fas^{lpr}* and SJL/J, which lacked the repeat. Comparison of the presence or absence of the tandem repeat, in a (DBA/2xSJL/J)F2 intercross, with several disease parameters showed that presence of the DBA/2 repeat was associated with less severe disease. Analysis of NZB mice congenic for the *Hmr1* locus of DBA/2, however, showed that *Daf1* expression is controlled by trans elements not within *Hmr1* and the reduction in *Daf1* expression was not associated with changes in levels of its major transcription factor SP1. These studies document lower levels of *Daf1* in lupus-prone mice and show that this is not directly caused by cis elements within the *Daf1* gene or by differences in constitutive Sp1 expression.

2. Materials and Methods

2.1. Mice. DBA/2, NZB, MRL/Fas^{lpr}/J, BXSB, and C57BL/6 mice were obtained from the Scripps Research Institute Breeding Colony (La Jolla, CA). NZW/LacJ, A.SW/SnJ, BALB/cJ, SJL/J, and 129S6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). (SJL/JxDBA/2)F2 intercross mice have been previously described [31]. NZB.DBA/2-*Hmr1(Daf1$^{DBA/2}$)* and DBA/2.NZB-*Hmr1(Daf1^{NZB})* interval congenic mice that contained the relevant *Daf1* locus were generated by marker-assisted breeding using *D1Mit21* (67 Mb) and *D1Mit17* (190 Mb) to define the outer limits of the chromosome 1 interval. Breeding and maintenance were performed under specific pathogen-free conditions at the Scripps Research Institute Animal Facility (La Jolla, CA). All procedures were approved by the Scripps Research Institute's Institutional Animal Care and Use Committee.

2.2. RNA Isolation and Real-Time PCR. Total RNA extraction from splenocytes was performed using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was denatured at 65°C for 5 minutes, placed on ice, and reverse transcribed in a total volume of 20 ml using random hexamers, dNTPs, RNase inhibitor (RNase-OUT; Invitrogen), and 200 units of SuperScript III reverse transcriptase (Invitrogen). Real time PCR primers, probes, and methods were as previously described [24]. *Daf1* was expressed relative to cyclophilin A or 18sRNA [24]. Levels of Sp1, Sp3, CREB, and CREM mRNA were determined in spleen cells of naïve female autoimmune prone NZB and healthy DBA/2 mice by real time PCR using iQ SYBR green Supermix (Bio-rad, Hercules, CA). The following primers were used for amplification: Sp1 forward, 5′-CAAACACCCCAGGTGATCATGGAAC-3′, and Sp1 reverse, 5′-CAGTGAGGGAAGAGCCTCAGGAG-3′; Sp3 forward, 5′-GGCAGCTCAGTGGTGACTCTAC-3′, and Sp3 reverse, 5′-GGTGGTGGGAGAGGTACCAATC-3′; CREB forward, 5′-GTGGGCAGTACATTGCCATTACCC-3′, and CREB reverse, 5′-GTTGTTCAAGCTGCCTCAGGCG-3′; CREM forward, 5′-CACAGGTGACATGCCAACTTACCAG-3′, and CREM reverse, 5′-CGGGAGTGTCGCAGGAAGAAG-3′. All PCR reactions were performed using an iCycler iQ (Bio-Rad). The reactions were run in duplicate and relative expression mRNA levels were determined by the ΔΔCT method and normalized against cyclophilin A. Data are expressed as fold change compared to mRNA levels measured in DBA/2 samples.

2.3. Genomic DNA. Genomic DNA was isolated from 5 mm sections of mouse tail incubated in 500 μL lysis buffer (0.1 M Tris, pH 8.0; 5 mM EDTA; 0.2 M NaCl and 0.4% w/v SDS) containing 200 μg/mL proteinase K (Sigma, St Louis, MO)

overnight at 55°C. Samples were spun down and supernatants containing genomic DNA were purified using the ZR Genomic DNA™-Tissue MiniPrep kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol.

2.4. Tandem Repeat PCR. The tandem repeat sequence, which can be represented by either (CTTTT)n or (TTTTC)n, was identified within the second intron of Daf1 using the following primers: 5′-GCTTAAGGCATTACTGTCTGC-3′ (forward) and 5′-GCCATCCTAATGTAAAGTAACTCC-3′ (reverse). PCR amplifications were performed with the KOD Hot Start Polymerase (EMD Millipore, Billerica, MA) using the following conditions: an initial 2 min denaturation step at 94°C and then 35 cycles of denaturation (94°C), annealing (57°C), and extension (68°C) followed by a final 10 min 68°C incubation step. The PCR products were separated by agarose gel electrophoresis, extracted using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and submitted for sequencing.

2.5. DNA Sequencing. Purified PCR products were submitted to the Scripps Research Institute DNA Core Facility and analyzed with an ABI PRISM 3100 sequencer using appropriate primers. Sequencing data were analyzed with the BioEdit Sequence Alignment Editor software.

2.6. Induction and Assessment of mHgIA in (SJL/JxDBA/2)F2 Intercross Mice. Induction and features of mHgIA including immune deposits in kidney and spleen and serum autoantibodies and MHC class II genotypes in (SJL/JxDBA/2)F2 intercross mice were described previously [31]. Use of mercuric chloride was approved by the Scripps Research Institute Department of Environmental Health and Safety.

2.7. Statistics. Unless otherwise noted, all data is expressed as mean and standard error. Statistical analysis was done using GraphPad Software, San Diego, CA. Mann-Whitney U test was used for comparisons between individual mouse strains. Analysis of variance (ANOVA) with Bonferroni's Multiple Comparison test was used for comparisons between features of mHgIA in (SJL/JxDBA/2)F2 intercross mice. $P < 0.05$ was considered significant.

3. Results

3.1. Daf1 mRNA Expression is Reduced in Autoimmune Prone Mice. In a previous study, we found that autoimmune prone NZB mice have reduced endogenous DAF1 expression [24]. To determine if this is common to other lupus-prone strains we analyzed *Daf1* mRNA expression in spleen cells from naïve female autoimmune prone MRL-*Fas*^{lpr} (MRL/lpr), NZB, NZW, and BXSB mice and healthy DBA/2 and BALB/c mice (Figure 1). All the autoimmune prone strains had lower *Daf1* mRNA levels than the DBA/2 confirming that reduced DAF1 expression is coupled to a predisposition for autoimmunity. *Daf1* expression in BALB/c mice was no different from DBA/2 but was higher than the other strains tested ($P < 0.01$). Interestingly, SJL/J mice also had reduced *Daf1* mRNA expression. This may reflect the propensity of SJL/J mice to develop autoimmunity with age [32] but may also be a manifestation of dysferlin deficiency in these mice [33]. Thus, *Daf1* is reduced in strains with a predisposition to autoimmunity.

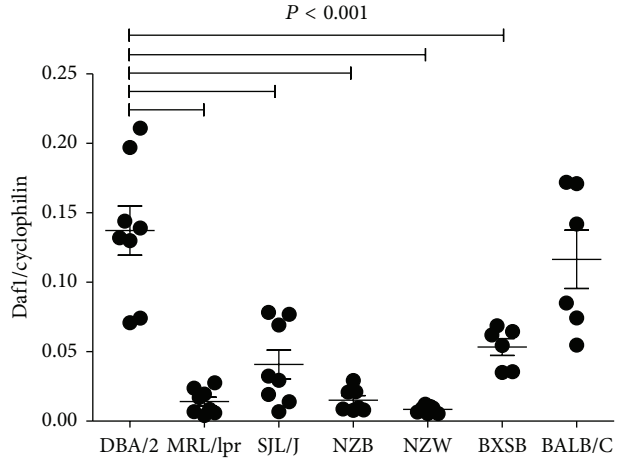
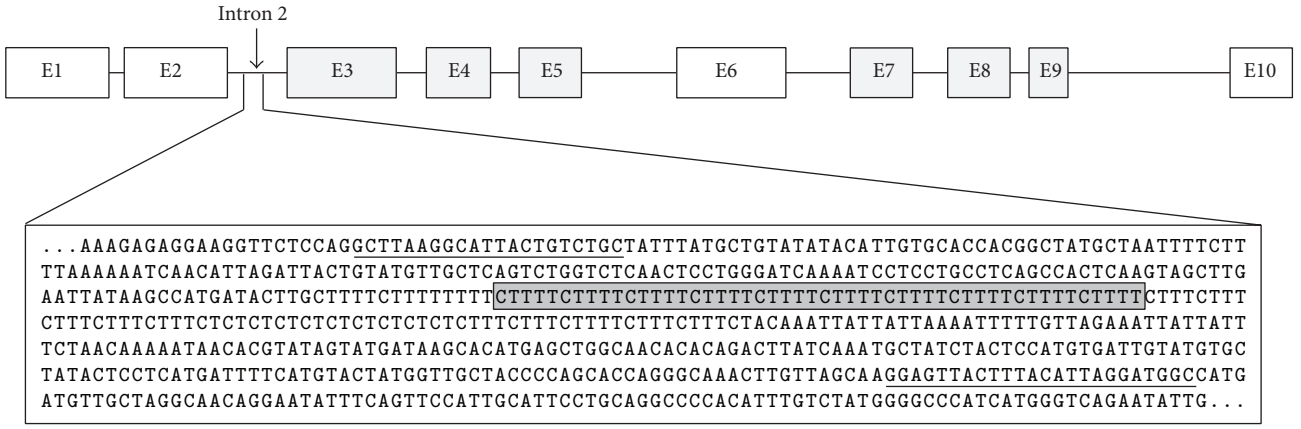


Figure 1: *Daf1* mRNA is reduced in autoimmune prone mice. Real time PCR was used to determine *Daf1* mRNA in spleen cells of naïve female autoimmune prone MRL-*Fas*^{lpr} (MRL/lpr), NZB, NZW, and BXSB mice and healthy DBA/2 and BALB/c mice. *Daf1* was expressed relative to cyclophilin A. N = 4-5 mice/strain.

3.2. Intron 2 of Daf1 Contains a Pentanucleotide Tandem Repeat. Differences in expression of DAF1 might reflect genetic polymorphisms among the different strains. Sequencing of *Daf1* transcripts and 2.5 kb of genomic DNA 5′ of the *Daf1* ATG start site in NZB and DBA/2 mice revealed no differences compared to the C57BL/6 genome. However, further examination identified a tandem repeat in the second intron of *Daf1* (Figure 2). Sequencing of this region in a number of mouse strains revealed three different genotypes. DBA/2 mice had the longest repeat sequence with CTTTT (or TTTTC) being repeated 11 times. NZB, NZW, BXSB, B10.S, C57BL/6, A.SW/Sn, BALB/c, and 129S6 mice had 10 repeats while MRL-*Fas*^{lpr} and SJL/J lacked the tandem repeat. Tandem repeat length did not show a strict correlation with *Daf1* mRNA expression, although the longest repeat was found in the strain with the highest expression (DBA/2) while the two strains lacking the tandem repeat, MRL-*Fas*^{lpr} and SJL/J, did have significantly lower expression than the DBA/2 (Figure 1). The inability of mercury exposure to decrease DAF1 expression in mHgIA resistant DBA/2 mice [24] suggested the possibility that the DBA/2 tandem repeat may influence the expression of *Daf1* and, in turn, the severity of autoimmunity.

3.3. Presence of the DBA/2 Daf1 Tandem Repeat Is Associated with Reduced Immune Deposits. To determine if the DBA/2 tandem repeat variant is associated with facets of mHgIA we examined archived DNA samples from 133 mice from a (SJL/JxDBA/2)F2 intercross which had been used to identify



CTTTT (or TTTTC) repeat length

11-DBA/2

10-NZB, NZW, BXSB, B10.S, C57BL/6, A.SW/Sn, BALB/c, and 129S6

0-MRL-*Fas*^{lpr} and SJL/J

Figure 2: *Daf1* gene organization showing location of the $CTTTT_n$ tandem repeat. Diagrammatic representation of exons (E) in *Daf1* (top) and the expanded region of sequence in intron 2 (bottom) showing the CTTTT tandem repeat (shaded box) and PCR primers sequences (underlined). DNA sequence is from the C57BL/6 genomic sequence which has ten CTTTT repeats. Note that the repeat can also be represented by TTTTC by simple removal of the C residue at the 5′ end and inclusion of the C at the 3′ end. DBA/2 mice had the longest repeat sequence with CTTTT (or TTTTC) being repeated 11 times. NZB, NZW, BXSB, B10.S, C57BL/6, A.SW/Sn, BALB/c, and 129S6 mice had 10 repeats while MRL-*Fas*^{lpr} and SJL/J lacked the repeat.

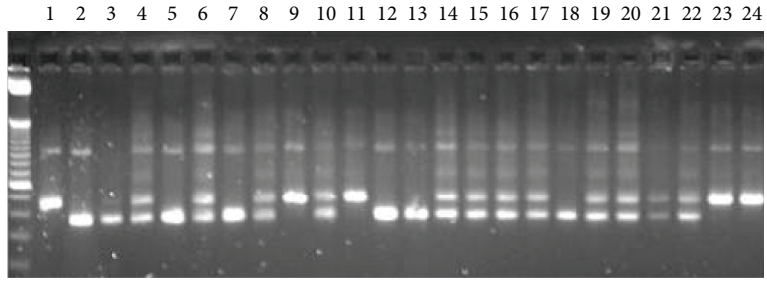


Figure 3: PCR determination of presence or absence of CTTTT tandem repeat. Archived DNA from mercury treated (SJL/JxDBA/2)F2 intercross mice was subjected to PCR using appropriate primers (see Figure 2) and products separated by agarose gel electrophoresis. Lane 1, base pair marker; lane 2, homozygous D tandem repeat; lanes 3 and 4, homozygous S tandem repeat; lane 5, heterozygous D/S tandem repeat; lanes 6–25, (SJL/JxDBA/2)F2.

the *Hmr1* locus [31]. PCR analysis determined that there were 32 mice with the DBA/2 (D/D) tandem repeat: 28 had the SJL/J (S/S) genotype as they lacked the tandem repeat and 73 were heterozygous (D/S) animals (Figure 3). Tandem repeat status was then compared to previously obtained data [31] of immune deposits in kidney and spleen and serum autoantibodies to determine the relationship between presence or absence of the tandem repeat and severity of mHgIA.

Comparison of autoantibody responses revealed that D/D, S/S, and D/S tandem repeat groups showed no differences in antinucleolar autoantibody (ANoA) response but the antichromatin autoantibody (ACA) response was greater in S/S than D/D animals ($P < 0.05$) (Figure 4). Comparison of immune deposits found that D/D animals had reduced glomerular IgG deposits ($P < 0.05$) compared to S/S animals but no differences were found for C3 deposits (Figure 4) or glomerular IgM. Immune deposits in the spleen were also affected by the presence of the D/D tandem repeat with D/D animals having reduced IgG and C3 deposits in splenic vessels compared to S/S animals ($P < 0.05$) (Figure 4). Splenic vessel deposits of C3 were also reduced in D/S animals compared to S/S animals ($P < 0.05$). Of the 133 animals, 13 had IgG deposits in both kidney glomeruli and splenic vessels and of these 6 (46%) were S/S, 5 (38%) D/S, and 2 (15%) D/D. When expressed as a percentage of each genotype this revealed that 21% of the S/S mice had deposits in both organs, while only 7% of D/S and 6% of D/D had such deposits. None of 8 DBA/2 mice had deposits in either organ while 5/8 SJL/J had deposits with 4 of these having deposits in kidney and

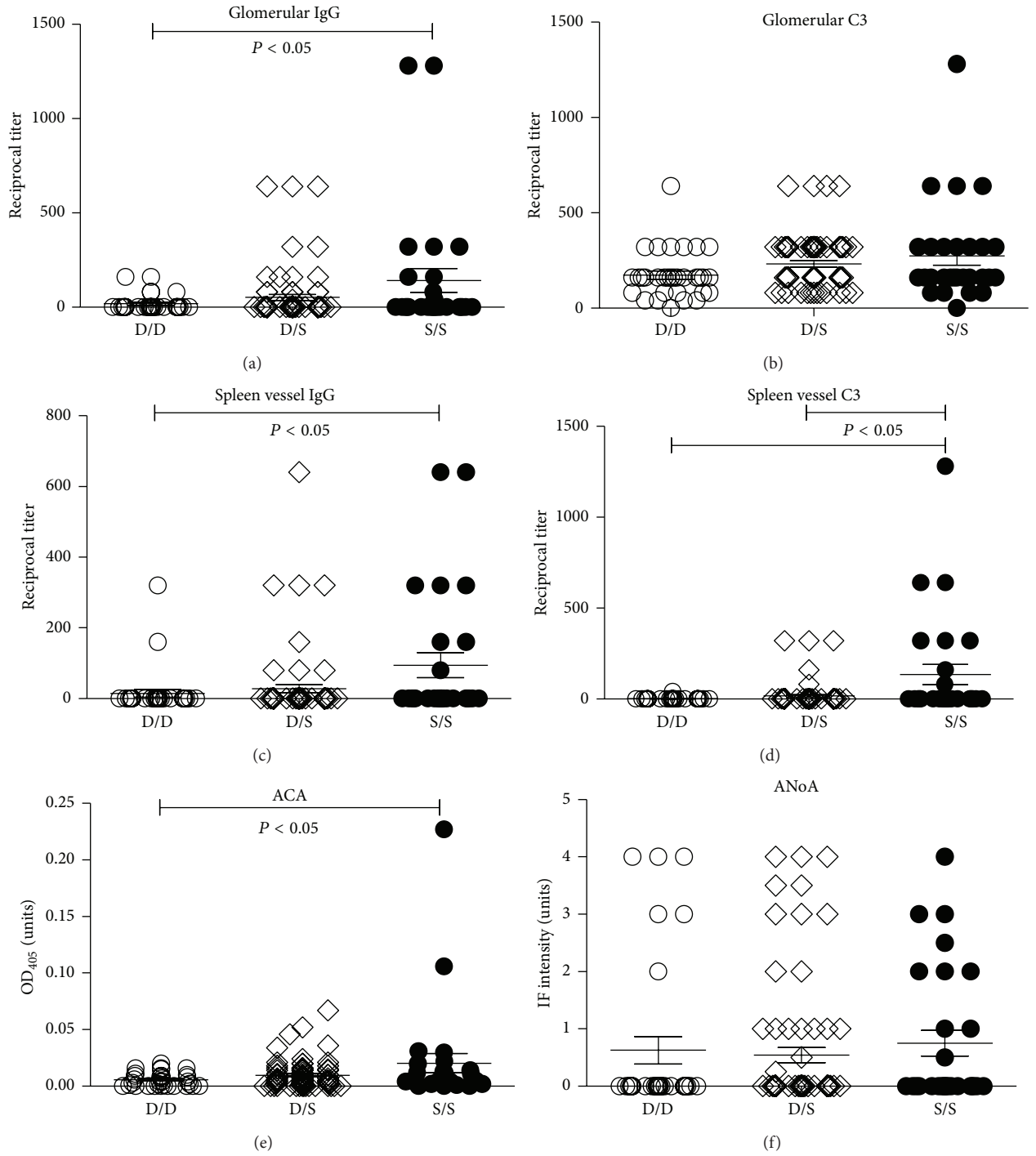


Figure 4: Presence of the DBA/2 tandem repeat is associated with reduced immune deposits. Tandem repeat genotypes (D/D, S/S D/S) were compared with features of mHgIA including glomerular deposits of IgG and C3, splenic vessel deposits of IgG and C3, antichromatin autoantibodies (ACA), and antinucleolar autoantibodies (ANoA) in 133 (SJL/JxDBA/2)F2 intercross mice.

spleen. These observations suggest that heterozygous and particularly homozygous presence of the D tandem repeat of *Daf1* is associated with less severe disease.

3.4. MHC Class II Genotype Is Not Associated with Reduced Immune Deposits. Autoimmunity, including mHgIA [31], is associated with class II genes of the MHC [34]. Therefore, differences in severity of mHgIA in the (SJL/JxDBA/2)F2 intercross could simply reflect distribution of DBA/2 and SJL/J MHC class II genes. To examine this possibility comparison was made between the MHC class II genotypes and the presence of immune deposits in kidney and spleen and serum

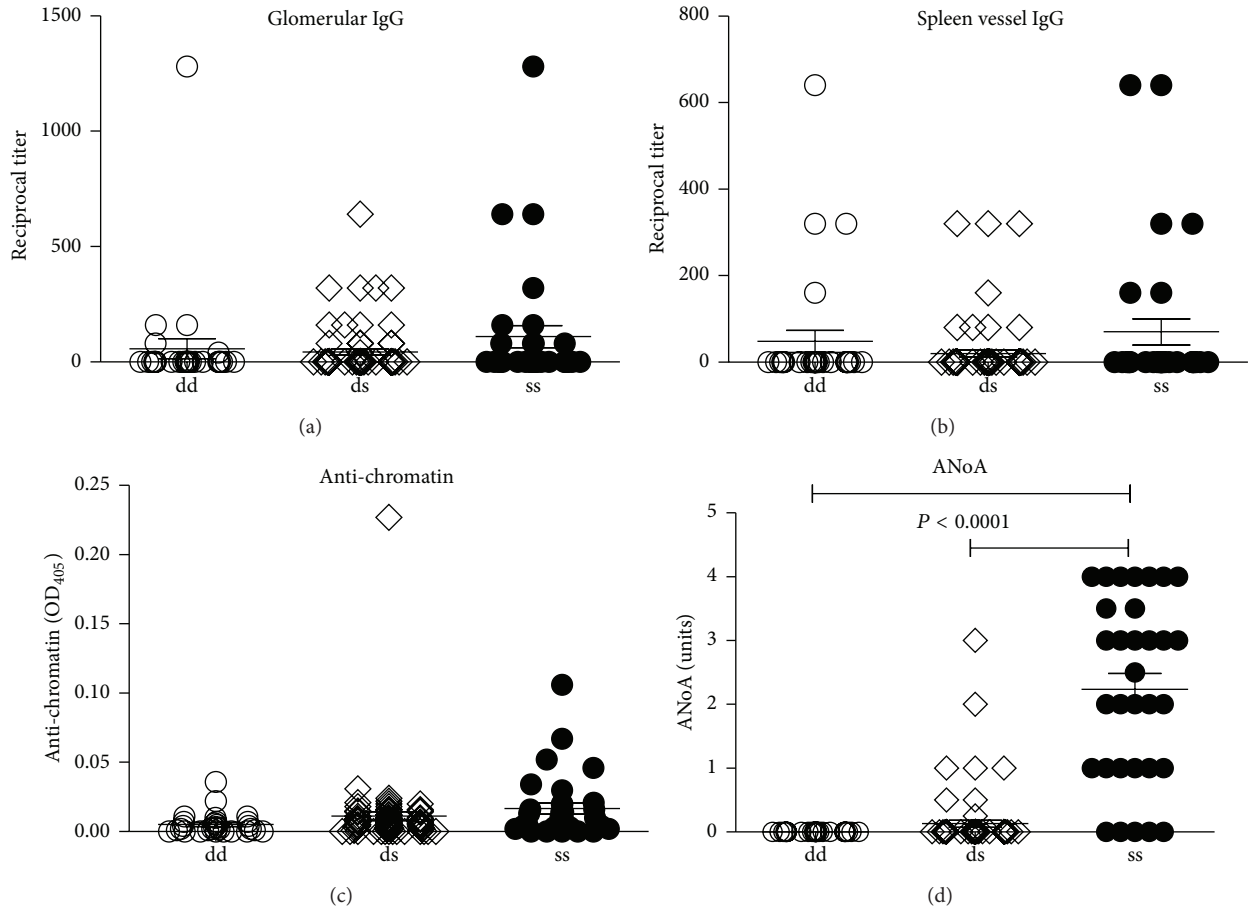


Figure 5: MHC class II is not associated with reduced immune deposits. MHC class II genotypes of (SJL/JxDBA/2)F2 intercross mice (H-2^d, dd; H-2^s, ss H-2^{ds}, ds) were compared with features of mHgIA including glomerular deposits of IgG, splenic vessel deposits of IgG, antichromatin autoantibodies (ACA), and antinucleolar autoantibodies (ANoA). $N = 133$.

autoantibodies. MHC class II was not associated deposits of IgG (Figure 5) or C3. Antichromatin autoantibodies were also not associated with MHC class II but, as described previously [31, 35], ANoA was highly associated with mice that were homozygous for H-2^s ($P < 0.0001$) (Figure 5). Therefore immune deposits do not reflect of the distribution of DBA/2 or SJL/J MHC.

3.5. Expression of Daf1 Is Not Regulated within the Hmr1 Locus. Strain specific differences in the expression of *Daf1* [24] and the relationship of *Hmr1* to disease severity [31] suggested that transfer of the DBA/2 *Hmr1* locus into autoimmune susceptible mice may help determine if *Daf1* expression was controlled within the *Hmr1* locus. NZB mice made congenic for the DBA/2 *Hmr1* locus (NZB.DBA/2-*Hmr*1(*Daf*$1^{DBA/2}$) or ND) still had reduced *Daf1* expression, while DBA/2 mice congenic for the NZB locus (DBA/2.NZB-*Hmr*1(*Daf*1^{NZB}) or DN) retained the elevated expression of *Daf1* (Figure 6). Thus, *Daf1* expression is affected by genetic elements outside of the *Hmr1* locus.

3.6. Daf1 Transcription Factor Expression Is Not Reduced in Autoimmune Prone Mice. We previously determined that constitutive expression of *Daf1* is under the control of the transcription factor SP1 [22] which suggested that the reduced expression of *Daf1* in autoimmune prone mice may be due to reduced expression of *Sp1*. Real time PCR analysis of *Sp1* in splenocytes revealed increased expression in NZB compared to DBA/2 mice although Sp3, another member of the Sp1 family, showed no difference in expression (Figure 7). The *Daf1* promoter also contains a CREB binding site [22], however expression of this transcription factor was not different between the two mouse strains although the closely related CREM was increased in NZB mice (Figure 7). Thus reduced *Daf1* in NZB mice could not be attributed to the lack of putative transcription factors, including SP1.

4. Discussion

In this study, we extended our observation that lupus-prone NZB mice have reduced DAF1 [24] to other major lupus strains including MRL-*Fas*^{lpr}, NZW, and BXSB mice. Thus,

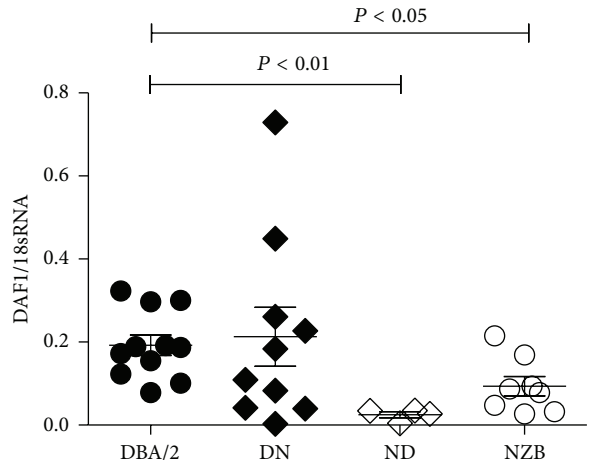


Figure 6: *Hmr1* locus does not control *Daf1* expression. NZB and DBA/2 mice were made congenic for the *Hmr1* locus and *Daf1* expression determined from spleen cells. NZB.DBA/2-*Hmr*1($Daf1^{DBA/2}$) = ND; DBA/2.NZB-*Hmr*1 ($Daf1^{NZB}$) = DN; $N = 4$–11.

reduction of DAF1 is closely associated with susceptibility to autoimmunity. Examination of the *Daf1* sequence revealed a pentanucleotide tandem repeat of either $(CTTTT)_n$ or $(TTTTC)_n$ in intron 2, with DBA/2 mice having the most repeats while most other strains had one fewer repeat except for MRL-Fas^{lpr} and SJL/J mice, which completely lacked the repeat. These observations suggested that resistance of DBA/2 to mercury-induced DAF1 downregulation and subsequent mHgIA might be related to the presence of the longer tandem repeat. Comparison of the presence and absence of the tandem repeat with features of mHgIA supported this possibility by revealing that absence of the repeat was linked with more severe disease particularly IgG deposits. However, mice congenic for the *Hmr1* locus [31], which contains *Daf1*, demonstrated that presence of the DBA/2 tandem repeat in NZB mice and vice versa did not influence *Daf1* expression. Furthermore, *Daf1* expression was not related to an increase in the transcription factor SP1 which has been shown to regulate constitutive expression of *Daf1* [22].

Several lines of evidence have previously suggested that *Daf1* is the most likely gene within the *Hmr1* to explain the association with glomerular immune complex deposits. First, gene expression profiling of NZB and DBA/2 mice exposed to mercury identified 12 differentially expressed genes within the *Hmr1* including *Daf1* [36]. As expected, *Daf1* had greater expression in mHgIA-resistant DBA/2 mice relative to the autoimmune-prone NZB. Moreover, *Daf1* was the only gene with a functional activity, inhibition of complement activation, which offers an explanation for the phenotype displayed by the *Hmr1* locus. Thus the biological role of DAF1 as a negative regulator of complement activation points to its association with deposition of immune complexes. Second, the DBA/2 mouse does not develop mHgIA [31, 37] and Hg exposure does not affect its expression of DAF1 [24]. Third, the SJL/J, which lacks the tandem repeat, has significantly reduced *Daf1* [33], is highly susceptible to mHgIA, and develops significant immune deposits [37]. Our finding that mice congenic for the *Hmr1* do not display any difference in *Daf1* expression compared to their original strain suggests that the association of the absence of *Daf1* tandem repeat with immune deposits might simply reflect the presence of lupus-predisposing genetic variants within the *Hmr1* locus [31]. However, it is also possible that the *Daf1*-expression mediated by the tandem repeat is in epistasis with other genetic variants present in lupus mice.

Our previous studies showed that constitutive expression of *Daf1* is regulated by the transcription factor SP1 [22], but we were unable to demonstrate a correlation of constitutive *Sp1* expression with DAF1 levels. Thus, the reduced expression of DAF1 in autoimmune-prone mice likely involves multiple factors. One intriguing possibility is that the TTTTC pentanucleotide sequence may contribute to *Daf1* expression as it has been identified as an IFN-γ and IRF1 response element of the mouse RANTES promoter [38], both IFN-γ and IRF1 are required for mHgIA [39], and deficiency of *Daf1* is associated with increased IFN-γ [4]. It can be speculated that the lack the pentanucleotide repeat in lupus-prone MRL-Fas^{lpr} and the mHgIA-sensitive SJL/J might reduce IRF1s influence on *Daf1* transcription. This is supported by the observation that mice deficient in IFN-γ or IRF1 have reduced DAF1 on activated $CD4^+$ T cells following mercury exposure even though they do not develop mHgIA [39]. Other mice that are sensitive to mHgIA, such as the B10.S, do have the tandem repeat, and naïve mice have approximately equivalent DAF1 on $CD4^+$ T cells as DBA/2 mice [24]. However, induction of mHgIA in the B10.S results in a reduction of DAF1 to levels found in naive NZB while DBA/2 are unaffected [24]. This suggests the possibility that the presence of an additional cis-acting element in the DBA/2 is even more efficient at maintaining *Daf1* expression.

Although the role of the tandem repeat sequence, (CTTTT)n or (TTTTC)n, in *Daf1* expression and mHgIA remains to be resolved, similar sequences in other genes have been shown to influence biological responses. CTTTT or TTTTC repeats have been found in human HLA [40], the CD4 locus [41], and the murine RANTES promoter [38]. The repeat at the CD4 locus has been associated with type I diabetes [42] and vitiligo [43]. A similar sequence, (CCTTT)n, has been found in the promoter of human inducible nitric oxide synthase (NOS2A) where 14 repeats are associated with absence of diabetic retinopathy [44]. IL-1β induction of NOS2A with 9, 12, or 15 repeats was inhibited by the presence of 25 mM glucose while the 14 repeats maintained transcription. Whether the size of the tandem repeat influences *Daf1* promoter activity in idiopathic and/or mercury-induced autoimmunity remains to be determined.

5. Conclusions

These studies show that expression of decay accelerating factor 1 is reduced in mice susceptible to systemic autoimmunity. Control of *Daf1* expression appears to be multifactorial and is not primarily mediated by a pentanucleotide tandem repeat

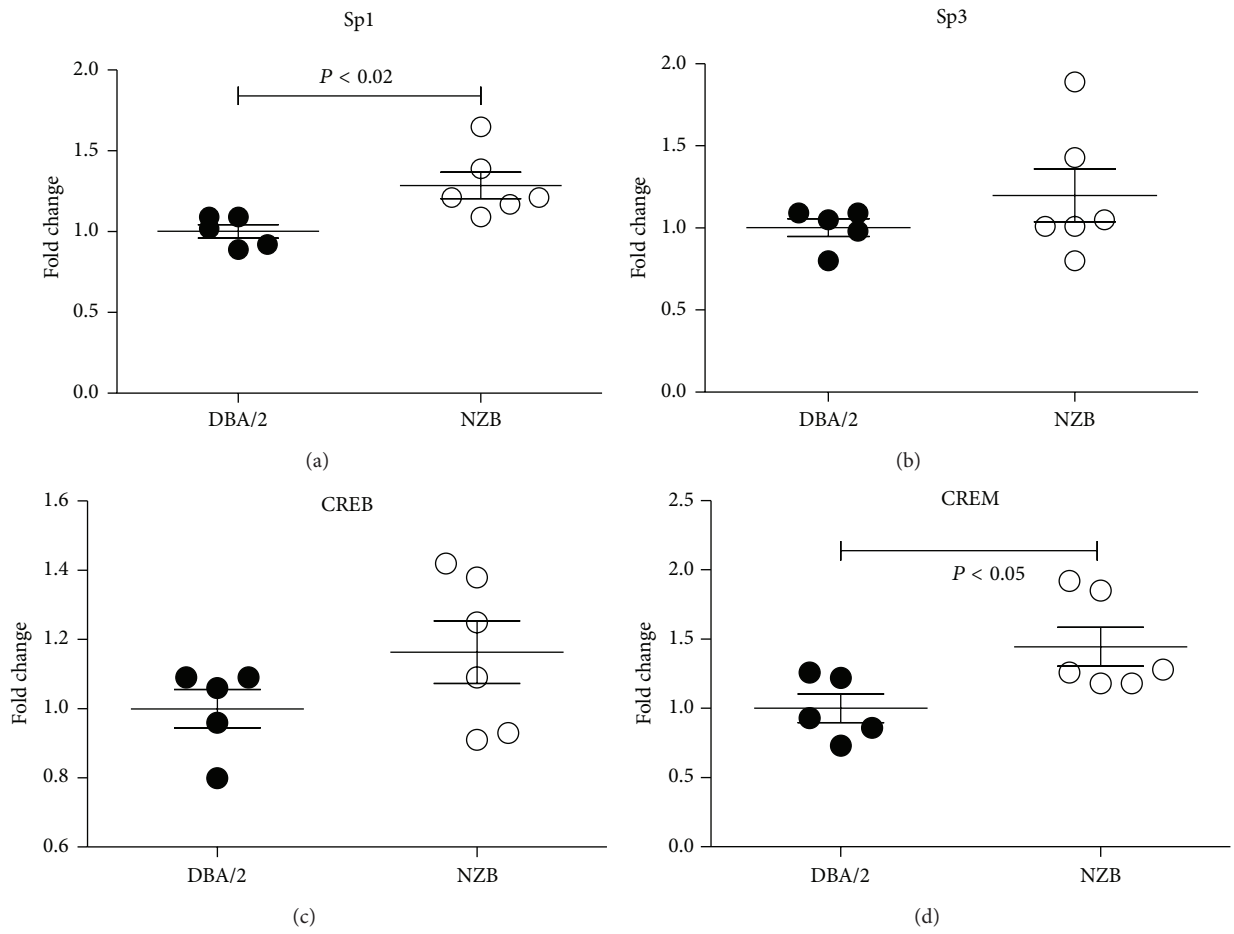


Figure 7: *Daf1* transcription factor expression is not reduced in autoimmune prone mice Real time PCR was used to determine *Sp1, Sp3, CREB,* and *CREM* mRNA in spleen cells of naïve female autoimmune prone NZB and healthy DBA/2 mice. mRNA levels were determined by the ΔΔCT method and normalized against cyclophilin A. Data are expressed as fold change compared to mRNA levels measured in DBA/2 samples. $N = 5$ DBA/2 and 6 NZB.

in intron 2 nor constitutive differences in SP1 expression. The absence of the tandem repeat was associated with increased severity of immune deposits in mercury-induced autoimmunity, which may be due to linkage of the repeat with other predisposing variants or epistasis with other genes that promote *Daf1* expression.

Conflict of Interests

The authors report no conflict of interests.

Acknowledgment

This work was supported by NIH Grants ES014847, ES020388, ES021464 (KMP), ES08666, and AR053731 (DHK).

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 743616, 12 pages
http://dx.doi.org/10.1155/2014/743616

Review Article

The Potential Roles of Bisphenol A (BPA) Pathogenesis in Autoimmunity

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Received 19 November 2013; Revised 20 January 2014; Accepted 12 February 2014; Published 7 April 2014

Academic Editor: Aristo Vojdani

Bisphenol A (BPA) is a monomer found in commonly used consumer plastic goods. Although much attention in recent years has been placed on BPA's impact as an endocrine disruptor, it also appears to activate many immune pathways involved in both autoimmune disease development and autoimmune reactivity provocation. The current scientific literature is void of research papers linking BPA directly to human or animal onset of autoimmunity. This paper explores the impact of BPA on immune reactivity and the potential roles these mechanisms may have on the development or provocation of autoimmune diseases. Potential mechanisms by which BPA may be a contributing risk factor to autoimmune disease development and progression include its impact on hyperprolactinemia, estrogenic immune signaling, cytochrome P450 enzyme disruption, immune signal transduction pathway alteration, cytokine polarization, aryl hydrocarbon activation of Th-17 receptors, molecular mimicry, macrophage activation, lipopolysaccharide activation, and immunoglobulin pathophysiology. In this paper a review of these known autoimmune triggering mechanisms will be correlated with BPA exposure, thereby suggesting that BPA has a role in the pathogenesis of autoimmunity.

1. Introduction

Bisphenol A [2, 2 bis(4-hydroxyphenyl) propane; BPA] is a monomer used in the manufacture of polycarbonate plastics. BPA is used in diverse forms of plastic products in the food and electronic industries and in various types of commonly used consumer goods, such as plastic containers, utensils, toys, water bottles, and fax paper. BPA has been shown to leach out of products, and high levels of the monomer have been identified in human and animal samples [1]. The extensive use of BPA-containing products has resulted in high human exposure worldwide [2], with studies reporting that more than 90 percent of the US population has detectable levels in urine samples [3]. It appears that increased temperature leaches BPA into food and water products as does acidic pH of liquids [4]. Additionally, dermal contact with sales receipts and printer paper containing BPA compounds can lead to BPA exposure [5].

BPA has been studied extensively as an endocrine disruptor, and numerous papers have shown how BPA may impact perinatal, childhood, and adult health [6]. BPA has the ability to bind to estrogen receptors and promote both agonist and antagonist activity [7]. It also has the ability to bind to aryl hydrocarbon receptors and exert diverse adverse endocrine effects on human physiology [8]. Its impact on hormone signaling and endocrine dysfunction continues to be an area of research.

BPA also has been shown to have potential adverse neurological effects, especially with respect to fetal brain development and promotion of neurodegenerative diseases [9]. Mice models showing perinatal exposure to BPA inhibits synaptogenesis and affects synaptic structural modification after birth [10]. The impact of BPA on brain health and neurodevelopment also continues to be an area of research.

This paper explores the worldwide exposure to BPA and its potential role in the growing epidemic of autoimmune disease. Although no human or animal studies have been published linking BPA to the onset of autoimmune disease, the potential seems very high due to the physiological influences of BPA and current immunological models regarding loss of self-tolerance and autoimmunity. In addition to known immune mechanisms promoted by BPA that overlap with

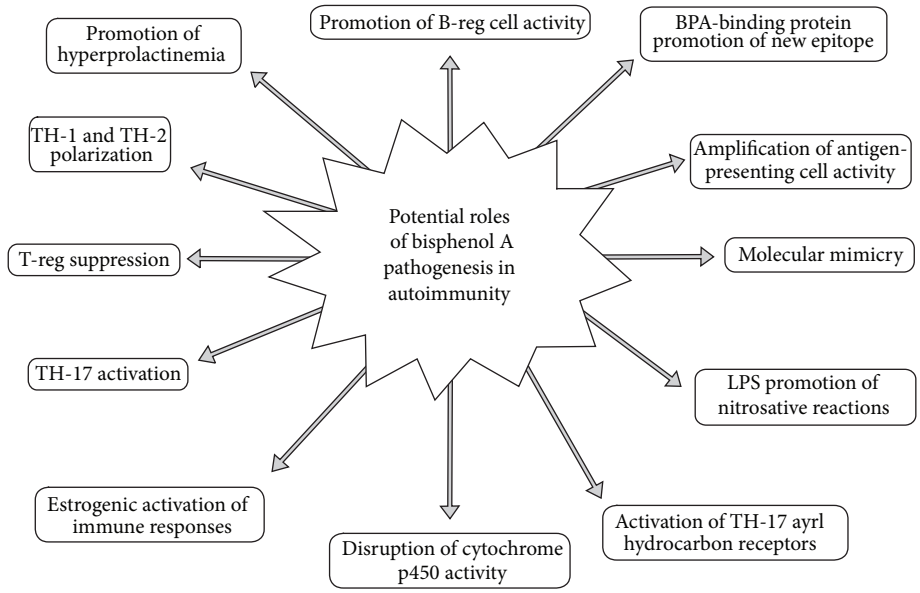

Figure 1: This diagram illustrates the potential mechanisms of bisphenol A's promotion of autoimmunity. BPA: bisphenol A; B-reg cell: regulatory B cell; LPS: lipopolysaccharide; TH: T-helper; T-reg: regulatory T cell.

autoimmune generation, some early evidence also indicates that BPA may contribute to mechanisms that promote autoimmune expression and progression (Figure 1).

2. BPA, Hepatic Biotransformation, and Autoimmunity

The hepatic biotransformation of BPA depends on phase I oxidation/reduction involving glutathione and phase II glucuronidation, glutathione, and sulfate conjugation [11]. Healthy humans exposed to BPA appear to have an accumulated body burden of BPA and monitoring studies that measure urinary BPA showed it stored in lipid reservoirs [12]. Despite proper hepatic biotransformation of BPA, the accumulation of BPA in body reservoirs may set the stage for immune reactivity and the onset of autoimmunity. Also, impaired hepatic clearance of circulating immune complexes in response to environmental compounds may induce autoimmunity. In a study of mice exposed to inorganic mercury, those mice that demonstrated reduced hepatic clearance of immune complexes also showed increased levels and altered quality of circulating immune complexes in mercury-induced autoimmunity [13]. Patients with abnormal hepatic biochemistries also have been shown to have a higher frequency of autoimmune disease [14].

A growing body of evidence shows increased toxic loads deplete hepatic tolerance, which leads to over activation of the innate and adaptive immune response and the development of autoimmune disease [15]. Higher BPA concentrations were associated with increased abnormal liver function tests [16]. Animal studies demonstrate that BPA has the ability to generate reactive oxygen species (ROS) and reduce antioxidant reserves and enzymes that are critical for hepatic phase I and II biotransformation, including glutathione, superoxide dismutase, glutathione peroxidase, glutathione S-transferase, glutathione reductase, and catalase activity [17].

BPA disruption of cytochrome P450 enzymes may be a potential mechanism for autoimmune pathophysiology. The cytochrome P450 (CYP) monooxygenases play a crucial role in the liver and various other tissues and are involved with oxidation of organic substances and the bioactivation of drugs and xenoestrogens [18]. CYP activity is necessary for the conversion of xenoestrogens into inactive metabolites that are both noninflammatory and biologically inactive. However, environmental xenoestrogens also have the potential to be metabolized into more reactive and inflammatory metabolites, thereby inducing increased ROS [19]. ROS are involved in apoptosis, activation of antigen presentation cells, and the initiation or amplification of diverse immunologic reactions that may be involved with the pathogenesis of autoimmune disease (Figure 2) [19].

Impairment of hepatic biotransformation of CYP expression may lead to ROS pathophysiology of autoimmunity. ROS have the ability to induce autoreactive molecules that may be involved with both the onset and the exacerbation of autoimmunity [20]. CYP enzymes are involved with metabolizing xenobiotics and producing ROS that may play a role in the pathophysiology of autoimmune disease.

In a study of mice offspring, BPA exposure to 15 and 300 mg/L of drinking water induced cytochrome CYP17 downregulation leading to potential proteomic alterations in immune function [21]. These mechanisms demonstrate the potential for BPA to disrupt proper CYP activity and potentially induce hepatotoxicity by promoting oxidative stress [17]. Increased production of ROS has demonstrated the ability to promote autoimmunity [22]. BPA activity has complex immune-activating reactions throughout the body. The impact of BPA on CYP enzyme expression may be a

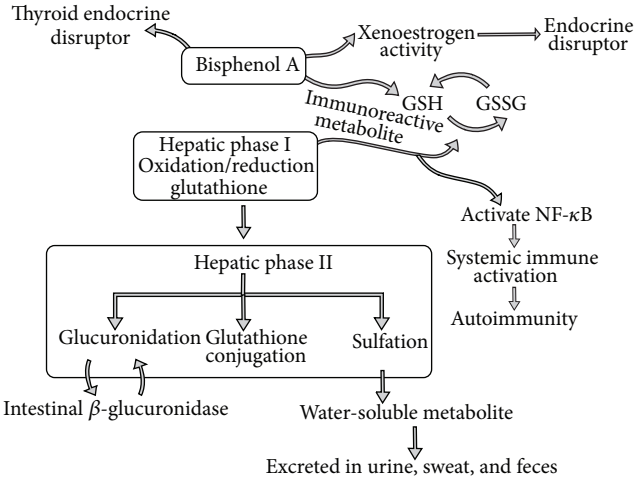

Figure 2: This diagram illustrates the hepatic biotransformation of bisphenol A. GSH: reduced glutathione; GSSG: oxidized glutathione.

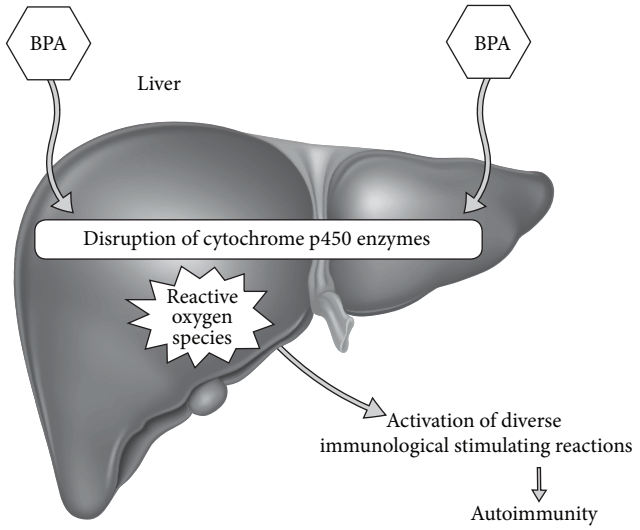

Figure 3: This diagram illustrates how bisphenol A can activate autoimmunity by disrupting cytochrome P450 enzymes. BPA: bisphenol A.

contributing mechanism to BPA autoimmune pathophysiology (Figure 3).

3. BPA Impact on Prolactin Synthesis and Autoimmunity

Although the peptide hormone prolactin is known primarily for its role in lactation, it also plays a critical role in modulating immune and inflammatory responses through various immune signaling pathways [23]. Prolactin has been shown to play significant roles in antigen presenting functions and in the initiation of the response against major histocompatibility complex (MHC) presenting self-antigens as found in autoimmunity [24].

A review of hyperprolactinemia and autoimmunity has found increased prolactin levels associated with production of anti-DNA antibodies, islet cell antibodies, thyroglobulin antibodies, thyroid peroxidase antibodies, adrenocortical antibodies, and transglutaminase antibodies with individuals suffering from systemic lupus erythematosus (SLE), diabetes mellitus type 1, Hashimoto's disease, Addison's disease, and celiac disease [25]. Prolactin has profound immunological stimulating, enhancing, and proliferative responses to antigens and mitogens by promoting increased cytokine activity and immunoglobin production. It also interferes with B cell tolerance and has autoimmune promoting effects [26].

BPA is an endocrine disruptor with powerful effects on the pituitary lactotroph cells, which are estrogen responsive and promote prolactin release. *In vitro* and *in vivo* studies have found that BPA mimics estradiol and induces hyperprolactinemia [27]. Therefore, BPA has potential impacts on autoimmune disease activation via its impact on increasing the immunostimulatory response of prolactin (Figure 4).

A link between BPA exposure and increased prolactin levels was found in women workers in occupational settings within one year. They demonstrated marked prolactin level increases and a multivariate analysis found BPA exposure was an independent risk factor for increased serum prolactin levels [28].

The correlation between hyperprolactinemia and autoimmune disease promotion has been reported in the literature in multiple papers during the past 20 years [29]. Additionally, recent evidence has found that BPA has major stimulatory impacts on prolactin release. These correlations strongly suggest that BPA may promote autoimmune pathophysiology by increasing prolactin release that then promotes immune-stimulating activity.

4. BPA and Estrogenic Activation of Immune Responses

In addition to the impact of BPA on prolactin release through its estrogenic influence on pituitary cells, BPA also appears to directly affect immune cell signaling pathways and thus immune responses [30]. BPA is classified as an endocrine disruptor in the form of a xenoestrogen and has the potential to mimic estrogen activity throughout the body [31]. This is important because increased circulating estrogens have demonstrated relationships with greater autoimmune activity [32]. Furthermore, epidemiological evidence suggests that the significant increase in the prevalence of autoimmune disease may in part be attributed to environmental estrogens (xenoestrogens). A review of the role of estrogens provides reasonable evidence of an association between xenoestrogen exposure and autoimmune disorders [33].

Various estrogen-promoted mechanisms have been found to trigger autoimmune reactivity. The reticulum transmembrane protein UNC93B1, which is essential for trafficking toll-like receptors (TLRs) from endoplasmic reticulum and is found to play a role in autoimmunity, has been shown to be upregulated by estrogenic signaling [34]. Estrogen activity has also been shown to directly and indirectly stimulate

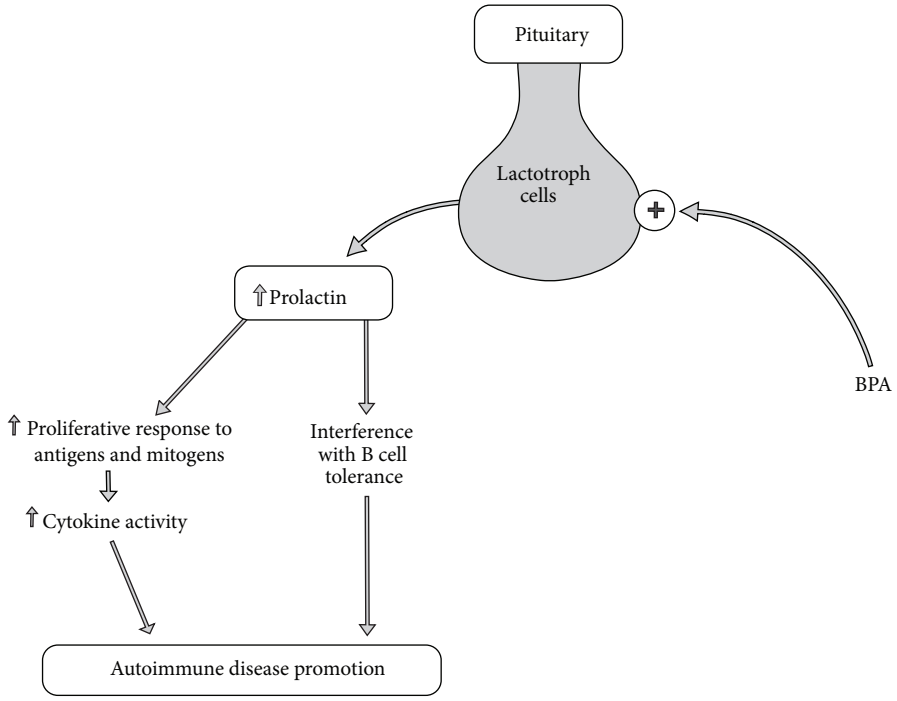

Figure 4: This diagram illustrates how bisphenol A can activate hyperprolactinemia and increase immunostimulatory responses, promoting autoimmunity. BPA: bisphenol A.

activation-induced deaminase (AID), leading to immune hyperstimulation. AID plays an important role in immune tolerance and the actual elimination of autoantibodies that may impact autoimmune reactivity [35]. Estrogen activity appears to promote signaling of T cell activation in autoimmunity [36]. Estradiol impacts macrophage production of tumor necrosis factor alpha [37]. Treatment of immune cells with estradiol has been shown to increase levels of B cell activating factor (BAFF) mRNA and protein that are associated with increasing severity of autoimmune disease expression [38]. Estrogen activity appears to impact dendritic cell differentiation and interferon production [39]. In summary, estrogen activity appears to have diverse and complex modulatory and stimulating roles in the immune system [40].

Many of these immune-stimulating responses that perpetuate chronic inflammation and autoimmunity may also be potentiated by the estrogenic activity of BPA [41]. BPA stimulates cell proliferation and induced expression of estrogen responsiveness. It also stimulates uterine, vaginal, and mammary growth and differentiation *in vivo* [42]. BPA treatment in mice induced splenocyte proliferation, a shift of cytokine profiles from Th-2 to Th-1 activity, and hyperstimulation of cellular immunity similar to patterns associated with Th-1 dominant autoimmune disease [43]. Overall, BPA has multiple estrogenic mechanisms in promoting abnormal immune responses that include altering T cell subsets, B cell functions, and dendritic cell activity and inducing abnormal immune signaling via its disruptive impact on estrogen receptor signaling, aryl hydrocarbon receptor signaling, and abnormal signaling of peroxisome proliferator-activated nuclear receptors [44]. These BPA estrogenic impacts on virtually all the major cells of the immune system and critical signaling pathways may be one way in which BPA promotes pathogenesis of autoimmunity (Figure 5).

5. BPA Impact on Immune Signaling Pathways

BPA has hapten and estrogenic activity, both of which play roles in activating hyperactive immune responses that may occur in autoimmune pathophysiology.

BPA exposure leads to aquatic animal hemocyte immune dysfunction, potentially increasing its role in induced autoimmunity through immune dysregulation. BPA injected into mussels leads to significant lysosomal membrane destabilization and a dramatic decrease in phosphorylation of the stress-activated p38 mitogen-activated protein kinases (MAPKs) and CREB-like transcription factor (cAMP-responsive element-binding protein) in mussels [45]. These results indicate BPA-induced alteration of hemocyte signal transducers and activator of transcription (STAT). These MAPK and STAT pathways are crucial in normal signaling to prevent upregulation of autoreactive T cells found to induce autoimmune inflammatory reactivity [46].

In addition to turning on gene expression of autoreactive T cells, alterations in these MAPK and STAT signaling pathways lead to chronic activation of antigen-presenting cells (APCs), loss of regulatory T cells (CD4+CD25+), apoptosis of APCs, and inhibition of innate and adaptive immunity wind-up found in the pathogenesis of autoimmunity [47]. The signaling pathways that are activated by BPA exposure have been shown to be the exact signaling pathways of molecular processes in autoimmune disease pathophysiology [48].

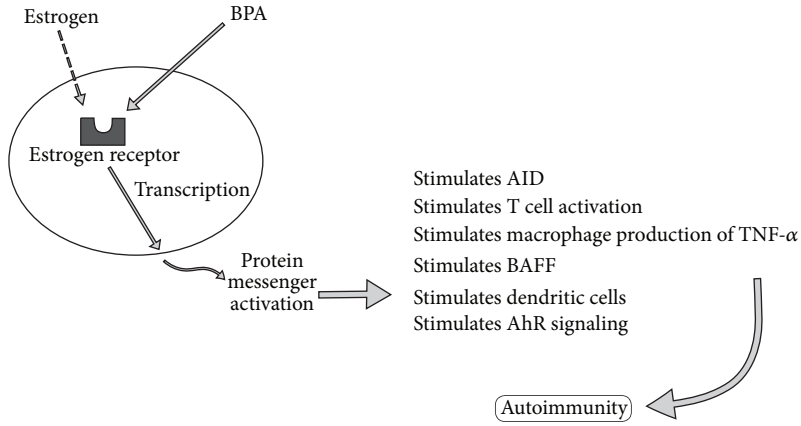

Figure 5: This diagram illustrates how bisphenol A can bind to estrogen receptors and promote estrogenic-mediated autoimmunity. AID: activation-induced deaminase; BAFF: B cell activating factor; BPA: bisphenol A; TNF-alpha: tumor necrosis factor alpha; AhR: aryl hydrocarbon.

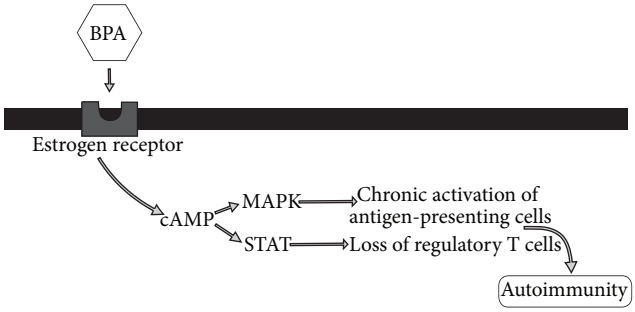

Figure 6: This diagram illustrates how bisphenol A can promote autoimmunity by cellular transcription activation. BPA: bisphenol A; cAMP: adenosine 3′5′-cyclic monophosphate; MARK: mitogen-activated protein kinase; STAT: signal transducer and activator of transcription.

Therefore, BPA activity as either an estrogenic endocrine disruptor or hapten-activating structure seems to specifically disrupt immune signaling pathways found in autoimmune disease (Figure 6).

6. BPA and Cytokine Expression

Cytokines have been shown to play a key role in the pathogenesis of autoimmune disease. The shift of cytokines into Th-1/Th-2 dominance and the IL-17/IL-23 (Th-17) axis has been shown to play pivotal roles in the model of autoimmunity and the breakdown of self-tolerance [49]. BPA has been shown to impact the differentiation processes of the dendritic cells that may cause unintended activation of the immune system in the absence of pathological conditions, thus promoting inappropriate polarization of T cells and cytokine profiles and shifting the immune system into an overzealous immunological state [50]. Additionally, BPA exposure prenatally to mice with oral feeding induced upregulation of Th-1 responses in adulthood [51].

The impact of BPA on naïve immune systems using T cell receptor transgenic mice followed by measurement of cytokine responses to antigens suggest that BPA can augment Th-1 reactions when administered orally in low doses (1.5 mg to 1.8 mg/kg weight) in water. Specifically BPA increased antigen-specific interferon gamma production leading to exaggerated T cell activation and polar Th-1 and Th-2 shifts [52]. These mechanisms associated with interferon have been shown to play powerful effector roles in the pathogenesis of autoimmunity, especially system autoimmunity such as systemic lupus erythematosus [53].

Animal studies have also shown that BPA exposure promotes cytokine inflammatory shifts associated with potential autoimmune development. BPA administered to mice in drinking water produced significant shifts of lymphocytes subpopulations. The production of inflammatory Th-1 type cytokines (IFN-gamma) was induced while Th-2 cytokine (IL-4) was suppressed with BPA treatment, promoting the transcription of IRF-1. The mRNA expression of GATA-3 was inhibited in BPA-treated groups in dosages of 0.015, 1.5, and 30 mg/mL for 4 weeks [54]. These responses indicated that BPA has the potential to induce Th-1 polar shifts of transcription factor that lead to exaggerated cellular immune responses leading to an exaggerated Th-1 immune response. The suppression of GATA-3 transcription factors and T cell polarization favoring a Th-1 bias has been shown to be an immune mechanism of multiple sclerosis autoimmunity in animals [55].

A study comparing the effect of BPA exposure on cytokine activity in adulthood and prenatally demonstrated that in adulthood exposure to BPA significantly promoted antigen-stimulated production of IL-4, IL-10, and IL-13, but not IFN-gamma. However, mice exposed prenatally to BPA showed increased production of not only IL-4 but also IFN-gamma. The percentages of T regulatory function (CD4+CD25+) were decreased in both groups exposed to BPA [56]. Loss of regulatory T cell function promotes abnormal cytokine shifts that occur in autoimmune diseases [57]. Suppression of regulatory T cell function leading to impaired

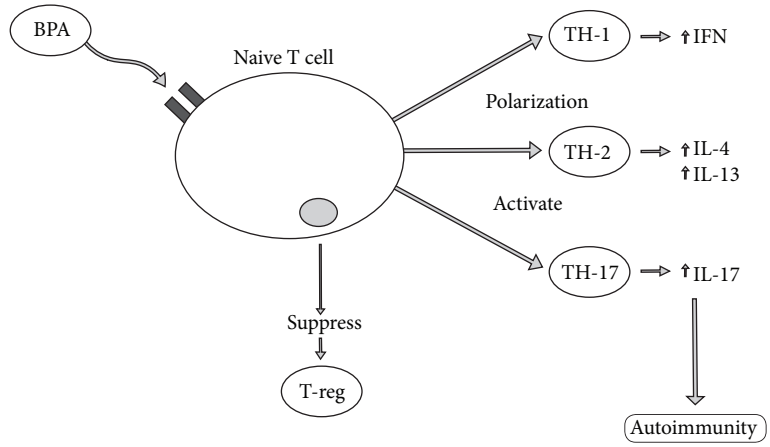

Figure 7: This diagram illustrates how bisphenol A can induce T cell shifts, promoting autoimmunity. BPA: bisphenol A; IFN: interferon; IL: interleukin; TH: T-helper; T-reg: regulatory T cell.

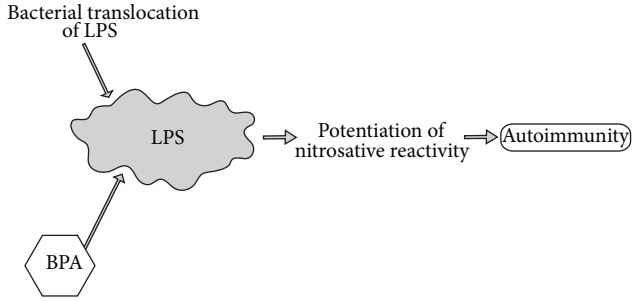

Figure 8: This diagram illustrates how bisphenol A can promote lipopolysaccharide inflammatory sequelae. BPA: bisphenol A; LPS: lipopolysaccharide.

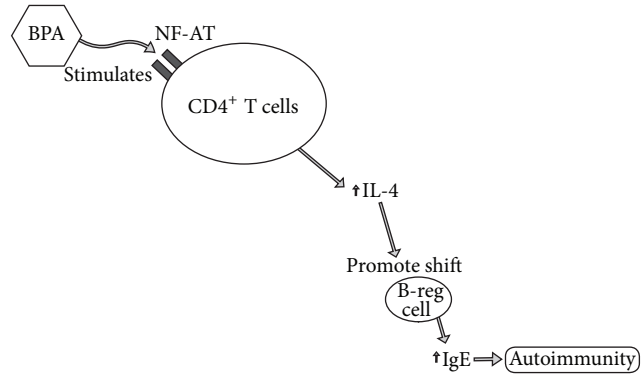

Figure 9: This diagram illustrates how bisphenol A can impact immunoglobulin-promoted autoimmunity. BPA: bisphenol A; B-reg cell: regulatory B cell; IL: interleukin; IgE: immunoglobulin E; NF-AT: $Ca2^{+}$/calcineurin-dependent nuclear factor binding sites.

cytokine modulation may be part of the immunopathology of BPA autoimmune development.

The delicate interplay between Th-1, Th-2, and Th-17 expression appear to be a key factor in autoimmune pathophysiology. Evidence indicates that BPA may induce polarity in this delicate balance and trigger inflammatory reactions, potentially leading to loss of self-tolerance as noted in subsequent paragraphs. The impact of BPA on the pathogenesis of abnormal cytokine shifts most likely occurs from complex web-like reactions. BPA's role as both a hapten and estrogenic endocrine disruptor appears to promote multiple interwoven pathways involved in adverse cytokine shifts that may play a role in autoimmune pathogenesis (Figure 7).

7. BPA and Lipopolysaccharide-Induced Nitric Oxide Production

Bacterial translocation of lipopolysaccharides (LPS) has the ability to activate oxidative and nitrosamine stress pathways associated with the inflammatory responses and pathophysiology of autoimmune responses [58]. BPA directly impacts LPS activation of these pathways, and the role of BPA on LPS activation could likewise play a role in abnormal immune reactivity [59].

Additionally, decreased activation of LPS-induced inflammatory reactions has also demonstrated a reduction in inflammatory sequelae of autoimmune cytokine and chemokine expression. Specifically, mice injected with BPA exhibited increased endotoxin-induced macrophage activation, suggesting that BPA may potentiate infectious autoimmune inflammatory reactions via enhanced tumor necrosis factor and nitric oxide reactivity [60]. Therefore, LPS-induced expression of nitrosative stress reactivity may be a key factor in BPA-promoted models of autoimmunity associated with infectious autoimmune reactions (Figure 8).

8. BPA Impacts on Antigen-Presenting Cell Reactivity

Antigen-presenting cells such as dendritic cells and macrophages appear to play a potential role with BPA and autoimmune reactivity. Dendritic cells (DCs) are important antigen-presenting cells that play a critical role in adaptive immunity due to their ability to activate naïve T cells, which, when

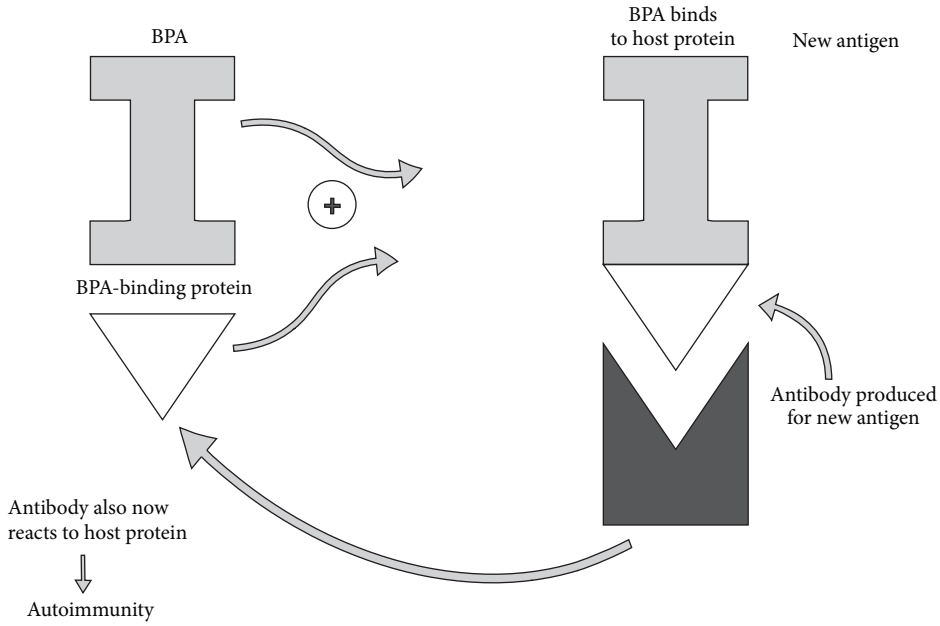

Figure 10: This diagram illustrates how bisphenol A can bind to the host protein, leading to a new epitope reaction against the host protein, resulting in autoimmunity. BPA: bisphenol A.

overzealous, could promote autoimmune activity [61]. DCs promote the expressions of Th-1, Th-2, or Th-17 cells that can be switched to express autoimmune inflammatory cascades [62]. DCs exposed to BPA in combination with tumor necrosis factor alpha promote CC chemokine ligand 1 (CCL1) signaling, a chemokine that is known to trigger chemotaxis of CCr8 expressing Th-2 and a subset of T regulatory cells, thereby promoting higher levels of IL-10 relative to those of IL-12p70 on CD40 ligation and preferentially inducing Th-2 deviation [63]. These variant responses from DCs exposed to BPA may play a role in autoimmunity.

Macrophage modulation of nitric oxide release is also critical for the regulation of apoptosis and differentiation of T cells that may lead to progression of autoimmune disease [64]. Additionally, BPA exposure has the ability to exert disruptive effects on macrophages by binding to estrogen receptors and leading to alteration of nitric oxide production and TNF-alpha synthesis in the homeostasis of TH-1 and TH-2 activity [65]. These macrophage expressions from BPA may promote immunological shifts that occur with autoimmunity, linking BPA's potential role to abnormal antigen-presenting cell responses.

9. BPA Effects on Immunoglobulin Activity

Increased immunoglobulin reactivity from endocrine disruptors such as BPA may raise concerns about immune hyperactivity associated with autoimmune immunopathology. The activation of immunoglobulins has a potential to promote inflammatory or anti-inflammatory activities through the activation of regulatory B (Breg) cells. Recent research in mice has shown that when B cell expression shifts into IL-10 production, there are suppressive effects on inflammatory responses. However, promotion of IgE-producing B cells plays a direct role in promoting inflammatory responses and the development of immune upregulation associated with most underlying inflammatory conditions, such as allergies and autoimmunity [66].

Recent research has shown that BPA has a direct impact on increasing immunoglobulin expression into the inflammatory IgE response, thereby potentially promoting an inflammatory cascade in autoimmunity. Specifically, exposure to BPA was shown to increase IL-4 production in $CD4^+$ T cells and antigen-specific IgE levels in sera via the stimulation of Ca^{2+}/calcineurin-dependent nuclear factor of activated T cells binding sites (NF-AT) [67]. These immune responses have the ability to potentiate allergies and autoimmune reactions in those with autoimmunity. Increased levels of IgE may play a direct role in promoting the inflammatory responses found in autoimmunity [68]. The potential for BPA to increase IL-4 and promote a shift of Breg cells into IgE production may be a mechanism for BPA autoimmune promotion (Figure 9).

In a murine model for SLE, animals implanted with BPA specifically demonstrated B cell activation and promotion of autoimmune disease such as lupus nephritis. BPA implantation enhanced autoantibody production by B1 cells both *in vitro* and *in vivo* in murine models of SLE. The study researchers suggested that BPA exacerbates preexisting autoimmune diseases such as SLE and that continued exposure to endocrine disruptors may potentiate the incidence and severity of autoimmune diseases [69].

Evidence of BPA on expressing B cell activity towards inflammatory expression and autoimmune development may partly explain the complex immune web reactions of this endocrine disruptor. Although inflammatory immunoglobulin reactivity may have a role to play in autoimmune expression, it is most likely part of a larger complex immune

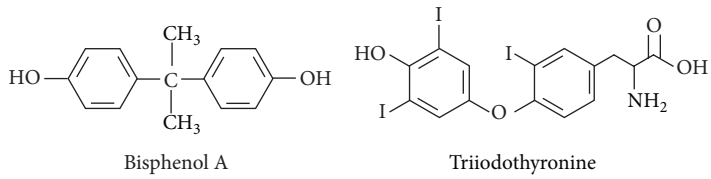

Figure 11: This diagram illustrates the structural similarity between bisphenol A and triiodothyronine, leading to potential cross-reactivity.

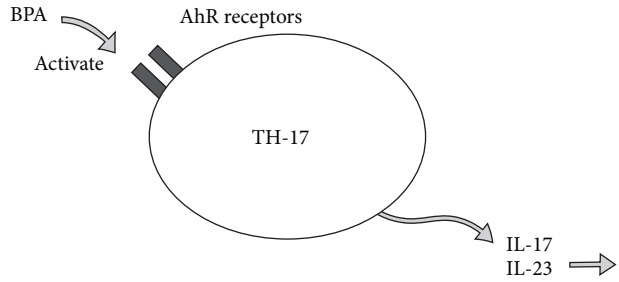

Figure 12: This diagram illustrates how bisphenol A can activate autoimmunity by inducing mRNA expression on aryl hydrocarbon receptors on TH-17 cells. AhR: aryl hydrocarbon; BPA: bisphenol A; IL: interleukin; TH: T-helper.

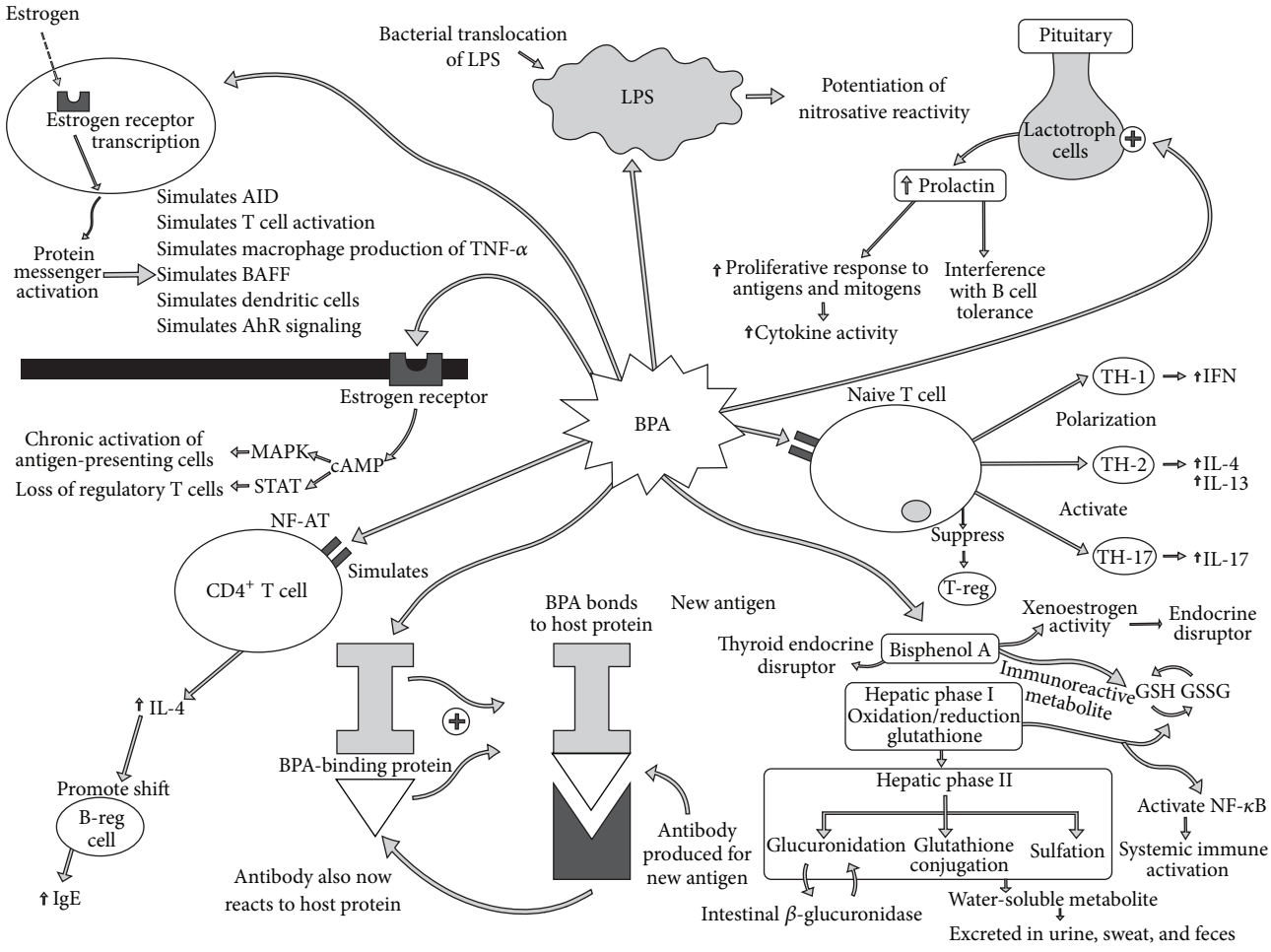

Figure 13: Potential of various autoimmune mechanisms from bisphenol A.

reaction that is linked to this very reactive endocrine disruptor.

10. BPA-Binding Protein: A Potential New Epitope

BPA binds to host protein, potentially creating a new epitope for immune reactivity. BPA binds to protein disulfide isomerase (PDI), also known as BPA-binding protein [70], a multifunctional protein involved in diverse cellular functions. This binding protein has been associated with endocrine disruptor mechanisms involving BPA [71]. The binding of environmental BPA to host protein may lead to self-tissue, antigen-antibody interactions associated with environmentally induced molecular mimicry. Autoimmune molecular mimicry requires the similarities of surface topologies leading to antigenic combining sites [72]. The binding of BPA to PDI in host has the potential to lead to new protein epitope activation of autoimmunity (Figure 10).

11. BPA and Autoimmune Molecular Mimicry

BPA and triiodothyronine (T3) possess such a degree of molecular structure similarity that BPA may act as an antagonist compound on T3 receptor sites [73]. When compounds have structural similarity, it may potentially lead to autoimmune cross-reactivity with antigen-antibody complexes [74]. In particular, environmental compounds such as hydrocarbon rings found both on BPA and T3 with anchor ring like similarities may induce mimicry [75]. A potential mechanism for the role of BPA in autoimmunity may be structural molecular mimicry, in particular with thyroid hormones (Figure 11).

12. BPA and TH-17 Aryl Hydrocarbon Receptors

Aryl hydrocarbon receptors (AhR) are involved with regulating immune responses and the development of TH-17 cells, which are key effector T cells in a variety of human autoimmune diseases. [76] Exposure to low dose BPA has been shown to upregulate mRNA expressions of AhR. AhR activation of TH-17 by BPA may potentiate autoimmunity. The role of chemical contamination and its ability to prompt AhR receptor activation of TH-17 have already been investigated in allergic and autoimmune diseases [77]. Although direct evidence has not been investigated for the role of BPA on AhR activation of TH-17 autoimmune reactivity, the potential mechanism may exist (Figure 12).

13. Conclusion

With the growing epidemic of autoimmune disease worldwide and the extensive use of consumer goods containing BPA, we must examine the risk of BPA as a potential triggering compound in autoimmune disease. Although no specific evidence has linked human or animal autoimmune disease development to BPA exposure, many of the mechanisms known to exist in autoimmune pathophysiology also appear to exist with immune reactivity from BPA exposure (Figure 13). Further investigation needs to be conducted correlating autoimmune disease development to BPA exposure. Additionally, the impact of BPA exposure on those already suffering from autoimmunity needs to be investigated further based on potential overlapping pathophysiology.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 613157, 8 pages
http://dx.doi.org/10.1155/2014/613157

Research Article

Chronic Exposure to Oral Pathogens and Autoimmune Reactivity in Acute Coronary Atherothrombosis

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Received 26 November 2013; Revised 18 January 2014; Accepted 20 January 2014; Published 25 February 2014

Academic Editor: K. Michael Pollard

Background. It has been hypothesized that various infective agents may activate immune reactions as part of the atherosclerotic process. We aimed to investigate the interrelationship between chronic exposure to oral pathogens and immune-inflammatory response in patients with acute coronary atherothrombosis. *Patients and Methods*. The study included 200 participants from Serbia: 100 patients with acute myocardial infarction (MI), and 100 age- and sex-matched controls. Antibodies to oral anaerobes and aerobes were determined as well as autoantibodies to endothelial cells, beta-2 glycoprotein I, platelet glycoprotein IIb/IIIa and anticardiolipin. Interleukin-6 (IL-6) and C-reactive protein (CRP) were measured. *Results*. The mean serum antibodies to oral anaerobes tended to be higher among subjects with MI (0.876 ± 0.303 versus 0.685 ± 0.172 OD, $P < 0.001$). Similarly, antibody levels against oral aerobes in patients were significantly different from controls. Antibodies against endothelial cell, beta-2 glycoprotein I, platelet glycoprotein IIb/IIIa, anticardiolipin along with CRP and IL-6 were highly elevated in patients. The levels of antibodies to oral bacteria showed linear correlation with tissue antibodies, CRP and IL-6. *Conclusion*. Antibody response to chronic oral bacterial infections and host immune response against them may be responsible for the elevation of tissue antibodies and biomarkers of inflammation which are involved in acute coronary thrombosis development.

1. Introduction

It has been recently hypothesized that various infectious diseases, both bacterial and viral, may activate vessel-associated leucocytes or immune reactions in the atherosclerotic process. Studies have also shown a strong association between poor dental health and cardiovascular diseases [1–3]. There are circumstances in which the presence or absence of teeth and the bacteria that reside on them could be the risk factors for the triggering of cerebrovascular and cardiovascular disorders, such as myocardial infarction. Several hypotheses can explain this scenario, among which asymptomatic bacteremia might play a role. Our body surface is colonized by over 10^{12} bacteria. A minuscule proportion of these bacteria gain access to our underlying tissue and are quickly dispatched by the body's immune response [4–7]. The bacteria, typically *Streptococcus sanguis, Streptococcus oralis,* and *Peptostreptococcus anaerobius,* arise in the oral cavity and are believed to enter the bloodstream as a result of trauma as bland as the manipulations of oral hygiene [8]. These bacteremias may infect sites of underlying pathologic changes of heart valves [9, 10]. On the damaged heart valves, adherent bacteria soon become embedded and protected in newly formed thrombi or platelet vegetation. Consequently, streptococci capable of initial adhesion and rapid induction of thrombosis are likely to be more virulent in clinical disease. As many as half of all cases of bacterial endocarditis have been attributed to viridans streptococci, with *S. sanguis* identified as the vector three to four times more frequently than *S. oralis* [11, 12]. This association may reflect the large proportion of these microorganisms in the oral flora and the frequency of these bacteremias in comparison with those that arise from other organs and tissues. The specificity of infection may also reflect special virulence traits of these bacteria. *Porphyromonas gingivalis, Prevotella intermedia,* and *Bacteroides forsythus* are Gram-negative small basil quality obligate anaerobic

bacteria and are held directly responsible for the formation of periodontitis. These bacteria usually secrete brown-black pigments and form colonies when they reproduce in blood agar plates used for their cultivation. These bacteria were classified in the *Bacteroides* genus until 1988 and 1990, when they were reclassified to the *Porphyromonas* and *Prevotella* genera, respectively, in accordance with new classification strategies made by Shah and Collins [13, 14].

These anaerobic bacteria, in conjunction with the facultative anaerobic bacteria such as *Streptococcus* mentioned above, can lead to mixed types of infections affecting various tissues, including the joints and the heart [15–20]. An extensive number of virulence factors include fimbriae, degradative enzymes, exopolysaccharide capsules, and atypical lipopolysaccharides; these factors, through various mechanisms of action, including mimicry or citrullination of self-peptide, can induce inflammation and autoimmunity against various tissue antigens [21–23].

For example, immunological mapping using a library of cyclic citrullinated α-enolase peptides led to the identification of a B-cell-dominant epitope comprising amino acids 5-21 of α-enolase (KIHAREIFDSRGNPTVE) where arginine-9 and arginine-15 are citrullinated, with an 82% sequence similarity with that of *P. gingivalis* [24]. Immunization with citrullinated human and *P. gingivalis* α-enolase and citrullinated fibrinogen causes similar pathology in humanized DR4 transgenic mice. This mechanism may be triggered by the release of different cytokines and prostanoids, such as interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor-alpha (TNF-α), prostaglandin E2, and different matrix metalloproteinases (MMP). These bacteria and released metabolites beyond this potential local pathogenicity may disseminate systemically and influence directly or indirectly the atheroma pathophysiology. Aside from increasing cytokine production, Gram-negative bacteria may also stimulate hypercoagulability, monocyte activation, and liver activation by releasing acute phase proteins, such as high-sensitivity C-reactive protein (hs-CRP) [4]. This repeated systemic exposure to orally derived bacteria, bacterial endotoxins, and systemic inflammation would eventually directly and/or indirectly affect the vascular walls, inducing a state of endothelial dysfunction.

The purpose of this study, then, is to investigate the potential interrelationship between chronic exposure to oral pathogens, the antibodies produced against them, and elevations in the levels of markers of immune-inflammatory response in patients with acute myocardial atherothrombosis.

2. Materials and Methods

2.1. Study Population. The study comprised 200 participants from Serbia, of whom 100 were patients admitted due to acute myocardial infarction. 100 were age- and sex-matched controls. In the patients group, the diagnosis of acute myocardial infarction was based on evidence of myocardial necrosis in a clinical setting consistent with acute myocardial ischemia [25]. The inclusion criteria for control groups were absence of known coronary artery diseases (previous stable or unstable angina as well as previous myocardial infarction) or carotid disease; initial electrocardiography (ECG) was recorded to confirm the absence of coronary artery disease. The exclusion criteria for the group of patients and also for controls were concomitant dilated cardiomyopathy, valvular heart disease, atrial fibrillation, major surgery, or trauma within previous months. All patients and controls with known or suspected thrombotic disorders, systemic illness, autoimmune diseases, sepsis, alcohol liver diseases, chronic obstructive pulmonary diseases, acute respiratory infections, current infections of any etiology or infections within previous 3 weeks, and malignancy and inflammatory diseases were also excluded. Study participants were asked about the risk factors for coronary artery disease (CAD), that is, smoking status, family history of CAD, hypertension, dyslipidemia, and diabetes.

The majority of the study participants were males (60% of patients and 58% of controls, P = n.s). The mean age of patients was 59.42 years and 59.03 years in controls (P = n.s).

The investigation conformed to the principles outlined in the Declaration of Helsinki. Signed informed consent or witnessed oral informed consent was obtained from all patients and healthy controls in accordance with the guidelines of the Ethical Review Committee of the Medical Faculty University of Nis, who approved the study protocol.

2.2. Preparation of Bacterial Antigens. Oral aerobes or facultative anaerobes (*Streptococcus sanguis*, *Streptococcus oralis*, and *Peptostreptococcus anaerobius*) and oral obligate anaerobes (*Porphyromonas gingivalis*, *Prevotella intermedia*, and *Bacteroides forsythus*) were purchased from American Type Culture Collection (Rockville, Maryland, USA) and cultivated according to the methods described earlier [8, 9]. In brief, bacteria were grown in different media and the cultures were incubated for 48–72 h at 35–37°C. Purity was assessed by colony morphology and gram stain. Bacteria were harvested at the late log phase by centrifugation at 10,000 g for 15 min and then washed twice with 0.15 M sodium chloride. The bacteria were lysed using a sonicator, and after separation of the lysate, the protein concentration was measured and used for coating ELISA plates and antibody measurement.

2.3. Serum Antibody Assay by Enzyme-Linked Immunosorbent Assay (ELISA). Pathogen-specific antibody was quantitated by enzyme-linked immunoassay. Wells of microtiter plates were coated with 100 μL of bacterial antigens (concentration of 10 μg/mL in 0.1 M of carbonate buffer, pH 9.6). Plates were incubated overnight at 4°C and then washed three times with 200 μL Tris-buffered saline (TBS) containing 0.05% Tween 20, pH 7.4. The nonspecific binding of immunoglobulins was prevented by adding 2% bovine serum albumin (BSA) to phosphate-buffered saline (PBS) and incubated overnight at 4°C.

Plates were washed as described above, and then serum samples diluted 1 : 200 in 0.1 M PBS Tween containing 2% BSA were added to duplicate wells and incubated for 1 h at room temperature. Plates were washed, and then alkaline phosphatase goat anti-human IgG F(ab′)2 fragments (KPI, Gaithersburg, MD) with optimal dilution of 1 : 400 in serum

diluent were added to each well; plates were incubated for an additional 1 h at room temperature. After washing five times with TBS-Tween buffer, the enzyme reaction was started by adding 100 μL of paranitrophenylphosphate in 0.1 mL diethanolamine buffer 1 mg/mL containing 1 mM $MgCl_2$ and sodium azide pH 9.8. The reaction was stopped 45 min later with 50 μL of 1 N NaOH. The optical density (OD) was read at 405 nm by means of a microtiter reader. To detect nonspecific binding, several control wells contained all reagents except human serum, or wells were coated with HSA followed by the addition of human serum and all other reagents to be used for specificity of the antigen-antibody reaction.

2.4. Autoimmunity and Immunity Markers. Antibodies against beta-2 glycoprotein I (IgG) were determined by using ELISA method (Calbiochem, La Jolla, CA, USA, Cat# B59407). Anticardiolipin antibodies were determined by using a test kit from Sigma, Cat# P1867. Antiendothelial cell, beta-2 glycoprotein I, and antiplatelet glycoprotein IIb/IIIa (IgG) antibodies were measured by coating each ELISA well plate with one μg of pure antigen followed by the addition of serum. All additional steps are described in the ELISA section.

2.5. Markers of Inflammation. Levels of IL-6 and hs-CRP were measured using kits manufactured by Diagnostic Products Corporation, Los Angeles, CA, on an IMMULITE Automated Immunoassay Analyzer. The IMMULITE system utilizes assay-specific, antibody- or antigen-coated plastic beads as the solid phase, alkaline phosphatase labeled reagent, and a chemiluminescent substrate. The IMMULITE system automates the entire assay process. Light emission was measured by a photomultiplier tube, and the results were calculated for each sample using different calibrators and controls.

The established reference ranges of the lab performing the tests were from 0.7 to 4.6 pg/mL for IL-6 and from 0 to 1 mg/dL for hs-CRP. Values above the established reference ranges were marked as positive.

2.6. Statistical Analysis. Results of normally distributed continuous variables are expressed as the mean value ± standard deviation. Analysis of normality of the continuous variables was performed with the Kolmogorov-Smirnov test. Differences between examined groups were assessed by unpaired t-test and Mann-Whitney U test and χ^2 testing was used for discrete variables. Relative Risk (RR), odds ratio (OR), and 95% Confidence Interval (CI) for the RR and OR were calculated.

Correlations between continuous variables were analyzed with the two-way Pearson correlation tests. Hs-CRP levels were not of linear nature. Therefore, in order to fulfill the statistical requirement, hs-CRP was logarithmically transformed before entering the analysis. Differences were considered to be significantly important if the null hypothesis could be rejected with > 95% confidence. All P values were two-tailed. The PASW 18.0 statistical software package was used for all calculations.

Table 1: Characteristics of patients with acute myocardial infarction.

Characteristics	(%)
ECG abnormalities at entry	
ST segment elevation	45
Without ST segment elevation	55
Systolic BP, mmHg	
<120	32.4
120–139	21.6
140–159	15.4
>160	30.6
Mean (SD)	132 ± 35.98
Diastolic BP, mean (SD), mmHg	79 ± 23.33
Heart rate, heartbeats/min	
<70	16.3
70–89	38.7
90–109	36.9
>110	8.1
Mean (SD), mmHg	22.73
Previous disease	
Previous MI	29.7
Previous CABG	17.1
Aspirin before admission	34.2
Duration of staying in hospital, mean (SD)	10.8 (5.4)
LVEF, mean (SD)	54.4 (13.30)
LVEF < 40	16 (16.2)
New event	19.6

ECG: electrocardiogram; BP: blood pressure; MI: myocardial infarction; CABG: coronary artery bypass grafting; LVEF: left ventricle ejection fraction. Values are % unless otherwise indicated.

3. Results

3.1. Clinical Characteristics of Patients with Acute Myocardial Infarction. In this study, we measured the levels of antibodies against oral pathogens as well as antibodies against endothelial cells, beta-2 glycoprotein I, platelet glycoprotein IIb/IIIa, anticardiolipin antibodies, and inflammatory markers such as hs-CRP and interleukin 6, in blood samples of patients with myocardial infarction and compared them to the levels of the same antibodies and markers in samples from control subjects.

Table 1 outlines clinical characteristics of patients with acute myocardial infarction. Mean value of systolic blood pressure was 132 ± 35.98 mm Hg. Patients spent in hospital a period of 10.8 ± 5.4 days to fulfill medical treatment.

3.2. Antibodies against Oral Pathogens. IgG antibodies to oral anaerobes were highly present in patients with acute coronary atherothrombosis. A total of 88% of patients with cardiovascular disease had elevated antibodies above the mean of controls, as shown in Figure 1; RR was 1.33 (1.13 to 1.56) 95% CI.

Overall, the mean OD of serum IgG antibodies to oral anaerobes tends to be higher among subjects with coronary

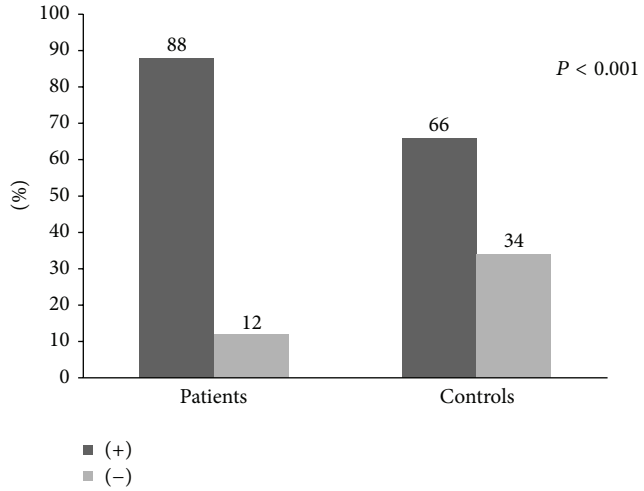

Figure 1: Comparison of the levels of IgG antibodies against oral anaerobes within the study participants.

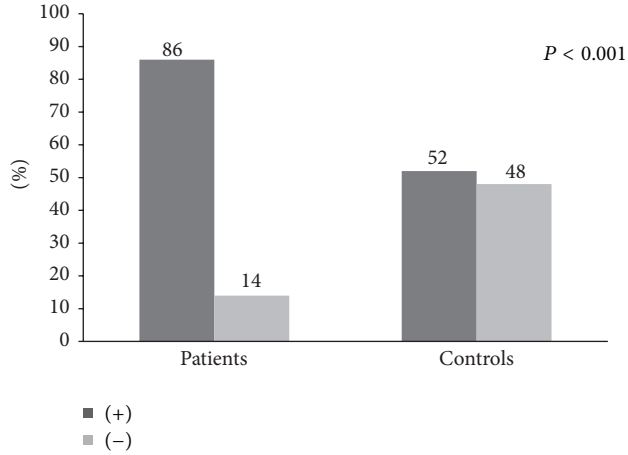

Figure 2: Comparison of the levels of IgG antibodies against oral aerobes within the study participants.

artery disease than those without (0.876 ± 0.303 OD versus 0.685 ± 0.172, $P < 0.001$) (Table 2).

IgG antibodies to oral aerobes were highly present in patients with acute coronary atherothrombosis. A total of 86% of patients had antibodies detectable compared to 52% of controls, RR 1.65 (1.34 to 2.02; 95% CI) (Figure 2).

The mean OD of serum IgG antibodies to oral aerobes tends to be higher among subjects with coronary artery disease than those without (0.996 ± 0.323 OD versus 0.769 ± 0.239 OD and $P < 0.001$) (Table 2).

3.3. Autoimmunity and Inflammation. Subjects with acute coronary artery atherothrombosis showed very strong autoimmune response with elevation in antiendothelial cell IgG antibodies in the group (45% versus 23%, O.R. 2.73, 95% CI for OR 1.48–5.04, RR 1.95, 95% CI for RR 1.28–2.97, $\chi^2 = 3.14$, $P = 0.001$, Figure 3.

The mean serum antiendothelial cells IgG antibodies were 0.684 ± 0.211 OD in patients versus 0.598 ± 0.193 OD in controls, $P = 0.004$ (Table 3).

Also, anti-beta-2 glycoprotein I antibodies IgG were detected in 25% of patients with acute coronary atherothrombosis compared to 8% of controls (OR 3.91, 95% CI 1.67–9.18, RR 3.12, 95% CI for RR 1.48–6.59, $\chi^2 = 2.992$, $P < 0.001$); see Figure 3. The mean serum titers are shown in Table 3.

Antibodies to platelet glycoprotein IIb/IIIa were detected in 53% of patients and 12% of controls (OR 8.26, 95% CI 4.02–16.98, RR 4.41, 95% CI for RR 2.51–7.74, $\chi^2 = 5.18$, $P < 0.001$), Figure 3.

Anticardiolipin antibodies were detected in 45% of patients and 28% of controls (OR 2.10, 95% CI for OR 1.16–3.78; RR 1.6, 95% CI for RR 1.09–2.35, $\chi^2 = 2.43$, $P < 0.001$). The titers were significantly different between groups, as shown in Table 3.

Our study showed that 46% of patients had elevated levels of circulating IL-6. Statistically, this proportion is significantly higher compared to controls (only 4%), ($\chi^2 = 5.53$, $P < 0.001$; odds ratio (OR), 20.44; (95% CI, 6.57–59.88); RR 11.5 (4.3 to 30.7). Concentrations of IL-6 were significantly higher in patients compared to controls (9.38 pg/mL (2.00–18.85) versus 1.5 pg/mL (1.2–1.8), $P < 0.001$).

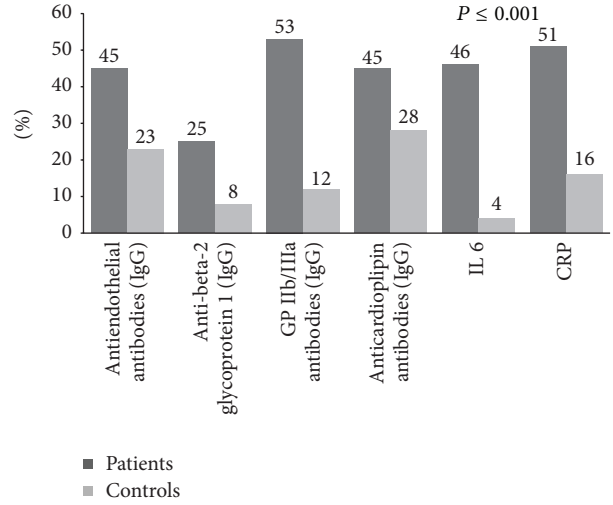

Figure 3: Comparison of the levels of IgG antibodies against antigens associated with autoimmunity activation and markers of inflammation in the study participants.

There was a significant difference between patients and controls in regard to CRP; 51% of patients had CRP above reference range compared to 16% of controls, $\chi^2 = 4.65$, $P < 0.001$, RR 3.18 (1.95–5.49): OR 5.46 (2.81–10.63) (Figure 3). The median of this marker of inflammation was 2.67 mg/dL (0.384–20.895) in patients and 0.225 mg/dL (0.075–0.623) in controls, $P < 0.001$.

Antibodies to both oral anaerobes and aerobes showed strong and significant correlation with different parameters of autoimmunity, immunity, and inflammation. Pearson's linear correlation, coefficient of correlation (r), and P values are shown in Tables 4 and 5.

Table 2: Antibodies against oral pathogens in the study participants.

ORAL pathogen (Bacterial agent)	Study participants	Mean	SD	95% CI		P
				Lower bound	Upper bound	
Oral anaerobes (OD)	Patients	0.876	0.303	0.662	1.035	<0.001
	Controls	0.685	0.172	0.569	0.715	
Oral aerobes (OD)	Patients	0.996	0.323	0.768	1.226	<0.001
	Controls	0.769	0.239	0.622	0.873	

Table 3: Autoantibodies in the study participants.

Autoantibodies	Group	Mean	S.d.	95% CI		Minimum	Maximum	P
				Lower bound	Upper bound			
Antiendothelial cells (OD)	Patients	0.684	0.211	0.509	0.579	0.229	0.955	0.004
	Controls	0.598	0.193	0.444	0.494	0.215	0.775	
Beta 2- glycoprotein I (OD)	Patients	0.665	0.344	0.595	0.1735	0.242	2.266	0.003
	Controls	0.540	0.205	0.499	0.582	0.280	1.437	
Platelet glycoprotein IIb/IIIa (OD)	Patients	0.351	0.100	0.331	0.372	0.225	0.718	0.001
	Controls	0.306	0.074	0.290	0.321	0.176	0.499	
Anticardiolipin (OD)	Patients	0.552	0.180	0.515	0.589	0.228	1.161	0.001
	Controls	0.415	0.097	0.396	0.435	0.237	0.704	

Table 4: Correlations of oral anaerobes IgG with different parameters of autoimmunity and inflammation.

Autoantibodies and inflammation	Antibodies to oral anaerobes (IgG)	
	r	P
Antiendothelial cells	0.541	0.01
Anti-beta 2 glycoprotein I	0.459	0.01
Antiplatelets glycoprotein IIb/IIIa	0.499	0.01
Anticardiolipin	0.647	0.01
Interleukin 6	0.199	0.01
hs C-reactive protein	0.229	0.01

r—coefficient of correlation.

Table 5: Correlations of oral aerobes IgG with different parameters of autoimmunity and inflammation.

Autoantibodies and inflammation	Antibodies to oral aerobes (IgG)	
	r	P
Antiendothelial cells	0.547	0.01
Anti-beta-2 glycoprotein I	0.443	0.01
Antiplatelets glycoprotein IIb/IIIa	0.546	0.01
Anticardiolipin	0.686	0.01
Interleukin 6	0.180	0.01
hs C-reactive protein	0.149	0.01

r—coefficient of correlation.

4. Discussion

Our understanding of the pathogenesis of the acute thrombotic complications of the atherosclerosis has burgeoned in recent years. We now understand that many acute thrombotic coronary occlusions do not necessarily result from critically stenosed sites in the arteries. This distinction between lesions versus lumen diameter challenges our traditional reliance upon coronary anatomy [26–29]. Atherothrombosis is the major determinant of acute ischemic cardiovascular events, such as myocardial infarction and stroke. Thus, its understanding is essential to enable the development of targeted and more effective therapies. Although related in part to alterations in lipid metabolism, atherosclerosis is now considered a primarily immune-mediated disease [30].

The role of the immune system and autoimmune reactions in atherosclerosis appears to be a double edged-sword, with some of them being proatherogenic, while others can be antiatherogenic depending on what stage in the long-lasting process of atherosclerosis.

The purpose of our study, then, was to investigate the potential interrelationship between chronic exposure to oral pathogens, the antibodies produced against them, and elevations in the levels of markers of immune-inflammatory response in acute, urgent, and lifesaving clinical settings in patients with acute myocardial atherothrombosis.

Our results indicated that IgG antibodies against oral pathogens (oral aerobes/facultative anaerobes) and oral obligate anaerobes were highly present in the patients with acute myocardial infarction, suggesting high exposure to chronic infection. Upon searching the literature, we found that it is has been recently proposed that chronic infections (bacterial *Helicobacter pylori*, *Chlamydia pneumonia,* and periodontitis among many others) can contribute to the development of atheromas either directly (endothelial injury, invasion of endothelial cells, and platelet aggregation) or indirectly (production of antibodies to lipopolysaccharide, cytokines and dysfunction of the immune system) [31].

In response to infection (e.g., oral bacteria among others), the immune system jumps into action, deploying cells as well as antibodies in order to recognize and destroy the invaders. Antibodies are molecules produced by plasma cells and B cells against the "enemy"—the infectious agent. However, owing to molecular mimicry or antigenic similarity between these infectious agents and human tissue structure, in a genetically susceptible individual, components of the body's immune system target one or more types of the person's own tissue, which may result in autoimmunity [18, 32, 33].

Taking these together, evidence indicates that infectious agents play a pivotal role in the induction of autoimmunities. The question of how infectious agents contribute to autoimmunity has continued to be of interest to clinical and basic researchers and immunologists in general [18].

In many cases, it is not a single infection but rather the "burden of infections" from childhood that is responsible for the induction of autoimmunity [18]. Thus, oral pathogens can also give their contribution towards autoimmunity.

An example of this is the case of anti-phospholipid (aPL) syndrome, in which anticardiolipin and anti-beta-2 glycoprotein I pathogenic antibodies are detected. In patients with systemic lupus erythematosus (SLE) or antiphospholipid syndrome, serum complexes and anti-beta-2 glycoprotein-I-oxidized-LDL complex autoantibodies are elevated [34, 35]. Similarly, such complexes and antibodies found in the bloodstream of patients with vascular complications, such as myocardial infarction and unstable angina, strongly associate with arterial thrombosis.

Our results indicated that anti-beta-2 glycoprotein antibodies and anticardiolipin antibodies (aCLs) can be detected in patients with myocardial infarction.

The data is similar from case-control studies that demonstrate the association of aCLs with stroke and acute myocardial infarction [32, 36]. Also, the authors found that IgG/IgM/IgA aCL and IgA for anti-beta-2 glycoprotein I associated with increased risk of ischemic stroke, arterial thrombosis, atherosclerotic immune process, acute myocardial infarction, and peripheral vascular diseases [36]. Although the exact mechanisms remain unknown, anti-beta-2 glycoprotein I was thought to interact with beta 2-glycoprotein I on the endothelial membrane and induce inflammatory reactions [37].

Artenjak et al. [38] reported on the correlation between aPL and cardiovascular risk in nonautoimmune settings. Taken together, these results did not demonstrate a clear association between aPL and acute cardiovascular events.

Beta-2-glycoprotein I is present at high concentrations in the blood stream and is expressed by many cell populations, including endothelial cells, lymphocytes, and monocytes. It binds negatively not only charged molecules, including phospholipids, heparin, and oxLDL, but also the surface of activated platelets and the membrane of apoptotic cells [39–43]. In our study population, those autoantibodies were highly present in patients with acute myocardial infarction. One should have in mind that antiendothelial cell antibodies may cause vasculitis as part of an autoimmune response. This is a heterogeneous family of antibodies. The IgG antibodies are highly present also in the blood sera of SLE patients and may mediate immunologic injury to blood vessel walls.

Finally, significant elevation in the levels of IL-6, CRP and endothelial cell antibody indicates that inflammation driven by oral pathogens plays a significant role in the development of atherothrombosis [44–48].

5. Conclusion

Taking together the above presented data, it appears that oral pathogens, through the release of toxins, seem to be capable of inducing changes in the host proteins. These can be recognized by the immune system, triggering an inflammatory process associated with the clinical manifestation of atherosclerosis—acute myocardial infarction. This and other immunopathogenic mechanisms need to be further elucidated.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The presented results are part of the study protocol designed for a Ph.D. thesis with the title "The role of infectious agents in acute coronary syndromes" which was presented at the Medical Faculty University of Nis and Clinical Center of Nis, Serbia, which were Dr. Burazor's employers at the time. The Ph.D. thesis was successfully defended. Dr. Burazor wants to thank Professor Aristo Vojdani and his employees for all their help and support.

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 982073, 12 pages
http://dx.doi.org/10.1155/2014/982073

Research Article

Differential Immunotoxicity Induced by Two Different Windows of Developmental Trichloroethylene Exposure

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Received 1 October 2013; Revised 19 November 2013; Accepted 20 November 2013; Published 20 February 2014

Academic Editor: Aristo Vojdani

Developmental exposure to environmental toxicants may induce immune system alterations that contribute to adult stage autoimmune disease. We have shown that continuous exposure of MRL+/+ mice to trichloroethylene (TCE) from gestational day (GD) 0 to postnatal day (PND) 49 alters several aspects of $CD4^+$ T cell function. This window of exposure corresponds to conception-adolescence/young adulthood in humans. More narrowly defining the window of TCE developmental exposure causes immunotoxicity that would establish the stage at which avoidance and/or intervention would be most effective. The current study divided continuous TCE exposure into two separate windows, namely, gestation only (GD0 to birth (PND0)) and early-life only (PND0-PND49). The mice were examined for specific alterations in $CD4^+$ T cell function at PND49. One potentially long-lasting effect of developmental exposure, alterations in retrotransposon expression indicative of epigenetic alterations, was found in peripheral $CD4^+$ T cells from both sets of developmentally exposed mice. Interestingly, certain other effects, such as alterations in thymus cellularity, were only found in mice exposed to TCE during gestation. In contrast, expansion of memory/activation cell subset of peripheral $CD4^+$ T cells were only found in mice exposed to TCE during early life. Different windows of developmental TCE exposure can have different functional consequences.

1. Introduction

The chlorinated hydrocarbon and industrial solvent trichloroethylene (TCE) is a widespread environmental contaminant. As noted in a 2011 IRIS report, the EPA has concluded that "there is substantial potential for environmental exposure to TCE as its improper disposal has resulted in the widespread contamination of groundwater and soil" [1]. Contact with TCE may be elevated for people living near waste facilities, where TCE is released, residents of some urban or industrialized areas, or individuals using TCE-containing products. Regardless of whether TCE exposure is oral, dermal, or inhalation-based, the chemical is rabidly absorbed and distributed. Based on the likelihood of exposure together with likely negative health impact TCE is consistently ranked 16th out of 275 chemicals on the CERCLA list of hazardous chemicals.

One of the predominant human health effects associated with TCE exposure is immunotoxicity, most notably the development of autoimmunity and other types of hypersensitivity diseases. Chronic TCE exposure in adults (both occupational and environmental) has been linked to a variety of autoimmune diseases including systemic lupus erythematosus, scleroderma, hepatitis, and diabetes [2–10]. In addition, there are many cases in recent years of a TCE-induced hypersensitivity disorder that targets the skin and liver [11, 12]. Alterations in $CD4^+$ T cells are often found to be an effect biomarker in patients suffering from TCE-induced immunotoxicity [2, 13–15].

Most studies of TCE-induced autoimmunity or hypersensitivity in humans have focused on adult exposure to the higher concentrations of TCE that are most commonly found in the workplace. The type of low-level TCE exposure

people may experience through drinking water contamination is generally thought to be risk-free. However, there is evidence that the developing immune system is especially sensitive to even low-level immunotoxicants. A recent review compared early versus adult exposure to several immunosuppressive toxins including lead and tributyltin in animal models [16]. In all cases, sensitivity was greater if exposure occurred during development. In fact, immune suppression in developmentally-exposed offspring often occurred at doses that were ineffective in adults. Developmental sensitivity to toxicants has also been found in humans [17, 18]. This includes evidence that adult onset autoimmune disease can be triggered by pre- and early postnatal toxicant exposure [19, 20].

Developmental exposure to TCE in humans is not uncommon; one study showed that 100% of breast milk samples from 4 US urban areas had detectable levels of TCE [21]. Gestational and early life TCE exposure has primarily been examined for its neurotoxicity [22]. However, children continuously exposed for 3–19 years beginning *in utero* to a water supply contaminated with solvents [with TCE being the predominant toxicant (267 ppb)] had altered ratios of T cell subsets and increased levels of autoantibodies [2].

We have studied a direct cause and effect relationship between TCE exposure and immunotoxicity in a mouse model. Adult exposure to TCE primarily altered effector $CD4^+$ T cells, with little effect on $CD8^+$ T cells, B cells, or T_{reg} cells. The effects of TCE on $CD4^+$ T cells were seen after only 4 weeks and included expansion of activated/memory ($CD44^{hi}$ $CD62L^{lo}$) $CD4^+$ T cells and altered cytokine production [23–25]. Adult TCE exposure seemed to have minimal effect on thymus cellularity. After chronic adult exposure (26–32 weeks) the TCE-induced alterations in $CD4^+$ T cells led to T cell-mediated liver inflammation identical to that seen in idiopathic autoimmune hepatitis (AIH) in humans [24, 26].

We have also examined mice following continuous TCE exposure beginning *in utero* and then encompassing lactation as well as an additional 4 weeks of direct exposure. This continuous exposure to TCE at concentrations lower than that used for adult exposure altered thymocyte cellularity and modified the phenotype and function of peripheral $CD4^+$ T cells [27].

Although informative, studies of continuous toxicant exposure do not reveal which windows of exposure have the most impact on the developing immune system. For example, it has been shown that early gestational exposure to certain toxicants such as lead are not as likely to suppress Th1 function as late gestational exposure [28]. This suggests that within the developmental period there exists particular windows of relative susceptibility and resistance [29]. Thus, knowing more about the functional outcomes of particular developmental windows of TCE exposure is needed to accurately estimate risk and to plan interventions. This study was designed to compare central and peripheral immune system alterations in mice exposed to TCE during two crucial windows of immune development and toxicant sensitivity, namely, gestation [GD0 to birth (PND0)] and early life (PND0 to PND49).

2. Materials and Methods

2.1. Mice and TCE Exposure. Developmental exposure to TCE has been described [30]. Basically, breeding pairs of 8-week-old MRL+/+ mice (Jackson Laboratories, Bar Harbor, ME) were established. In one experiment immediately following detection of pregnancy, GD0, the females were divided (following stratified randomization) into 3 groups and given water with 0, 0.01, or 0.1 mg/mL TCE. Controls were given water containing only 1% Alkamuls EL-620, the reagent used to solubilize the TCE. All the drinking water was Ultrapure unchlorinated to assure that chlorination by-products do not confound the results. The TCE-containing drinking water was changed 3 times/week to offset degradation of TCE. Maternal exposure to TCE-containing drinking water ended at birth in this experiment. The female pups that underwent the gestation only exposure to TCE were examined at PND49. As observed previously maternal toxicity was not noted at the level of body weight, mating index, fertility index, sex ratio of litters, gestational length, and food and water consumption.

In the second experiment, the female breeders were not exposed to TCE during pregnancy, but were given TCE-containing drinking water immediately after giving birth. Thus, the pups were exposed to TCE from PND0 to PND21 via lactation. Once the female pups were weaned at PND21 they were exposed to TCE directly in their drinking water for the duration of the experiment. The female pups from this early life exposure were sacrificed at PND49.

Both female breeders and resulting pups in both experiments were weighed weekly and water consumption was monitored. All studies were approved by the Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

When the female pups were sacrificed at PND49 pooled spleen cell suspensions and thymus cell suspensions from paired mice from each litter (n = 5–7 litters/treatment group) were examined by flow cytometry. In addition, $CD4^+$ T cells were isolated from the pooled spleen cell suspensions and stimulated with immobilized anti-CD3 antibody and anti-CD28 antibody for 24 hours as described [25]. Culture supernatants were then collected for cytokine evaluation, and the activated $CD4^+$ T cells were frozen for subsequent qRT-PCR analysis. In addition, adherent macrophages isolated from pooled peritoneal exudates from 2-3 mice/litter were incubated for 20 hours in medium alone or in the presence of LPS (1 μg/mL) and IFN-γ (100 units/mL). Approximately 80% of adherent peritoneal exudate cells (PEC), regardless of treatment group, expressed the transmembrane protein F4/80, a marker of mature macrophages (data not shown). Culture supernatants from the peritoneal macrophages were then collected for cytokine evaluation. RLT Lysis Buffer (Qiagen Sciences, Germantown, MD) was then added directly to the remaining adherent cells before freezing for subsequent qRT-PCR analysis.

2.2. qRT-PCR. Fluorescence-based quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was conducted using RNA isolated from activated $CD4^+$ T cells,

Table 1: Primer sequences used for qRT-PCR.

Gene		Primer sequences 5′ to 3′	Tm (°C)
Aire	Sense	AGATCGCGGTGGCCATAG	57.4
	Antisense	TCGTGGTCGGCTAGAGCAT	58.4
Bax	Sense	TTGCTGATGGCAACTTCAACTGGG	60.2
	Antisense	TGTCCAGCCCATGATGGTTCTGAT	60.4
Bim	Sense	CTGTGTAATGTGCCCTACTGTTTC	55.8
	Antisense	GGAAGAGAACCAGCCACTACC	57.3
CD70	Sense	TGCTGGTGGTGTTTATTACTGTG	55.4
	Antisense	CTCTGGTCCGTGTGTGAAGG	57.7
Cdkn1a	Sense	AATCCTGGTGATGTCCGACCTGTT	60.2
	Antisense	GTGACGAAGTCAAAGTTCCACCGT	59.4
Dnmt1	Sense	TGATAAGGAGGACAAGGAGAATGC	56.4
	Antisense	CACCGCCAAGTTAGGACACC	58.3
Dnmt3a	Sense	CAGCACCATTCCTGGTCATGCAAA	60.2
	Antisense	TCCTGTGTGGTAGGCACCTGAAAT	60.2
Fas	Sense	CGCCCGCTGTTTTCCC	57.6
	Antisense	GCAAGCACCAGAGGCAGG	59.4
FasL	Sense	GGCTGGGTGCCATGCA	59.4
	Antisense	GGCACTGCTGTCTACCCAGAA	59.2
IAP	Sense	GCACCCTCAAAGCCTATCTTAT	54.6
	Antisense	TCCCTTGGTCAGTCTGGATTT	55.8
Ifng	Sense	AGCTCATCCGAGTGGTCCAC	59.1
	Antisense	AGCAGCGACTCCTTTTCCG	57.8
Il2	Sense	CCCAAGCAGGCCACAGAATTGAAA	60.2
	Antisense	AGTCAAATCCAGAACATGCCGCAG	59.9
Il4	Sense	AGCCATATCCACGGATGCGACAAA	60.8
	Antisense	AATATGCGAAGCACCTTGGAAGCC	60.0
Il6	Sense	AGAGGAGACTTCACAGAGGATACC	57.1
	Antisense	CATTTCCACGATTTCCCAGAGAAC	56.1
Muerv	Sense	TGGTGGTCGAGATGGAGGTTA	57.5
	Antisense	CCGTGAATGGTGGTTTTAGCA	55.8

peritoneal macrophages or thymocytes. The details for conducting qRT-PCR, including quality control and reference gene selection, have been described previously [31]. The primers used are described in Table 1.

2.3. Phenotypic Analysis of Spleen and Thymus Cells. The phenotypic analysis of 30,000 events per sample was carried out using a CyFlow ML (Partec GmbH, Munster, Germany) as described previously using monoclonal antibodies from BD Biosciences or eBioscience [32], and the data were presented as mean percentage ± standard error. Fluorescence Minus One controls and isotype Ig controls were included.

2.4. Cytokine Analysis. The culture supernatants from the activated $CD4^+$ T cells or peritoneal macrophages were examined using READY-SET-GO ELISA kits for mouse IL-2, IL-6, or TNF-α from eBioscience, San Diego, CA.

2.5. Statistics. The data are presented as means and standard deviations. Assays were conducted using samples from 5–7 individual litters per treatment group. Comparisons between values obtained from controls and different treatment were made using a Student's *t*-test. The threshold for statistical significance was set at $\alpha = 0.05$.

3. Results

3.1. Gross Changes Induced by TCE Exposure. Two windows of TCE developmental exposure were examined in female MRL+/+ mice. The mice were either exposed to TCE during gestation (GD0-PND0) or during early life (PND0-PND49). All of the mice were assessed at 7 weeks of age (PND49) for a variety of immune parameters. TCE was added at 0.01 or 0.1 mg/mL to the drinking water of the dams and/or the pups. Based on water consumption, the resulting TCE exposures were described in Figure 1. Many of the alterations in $CD4^+$ T

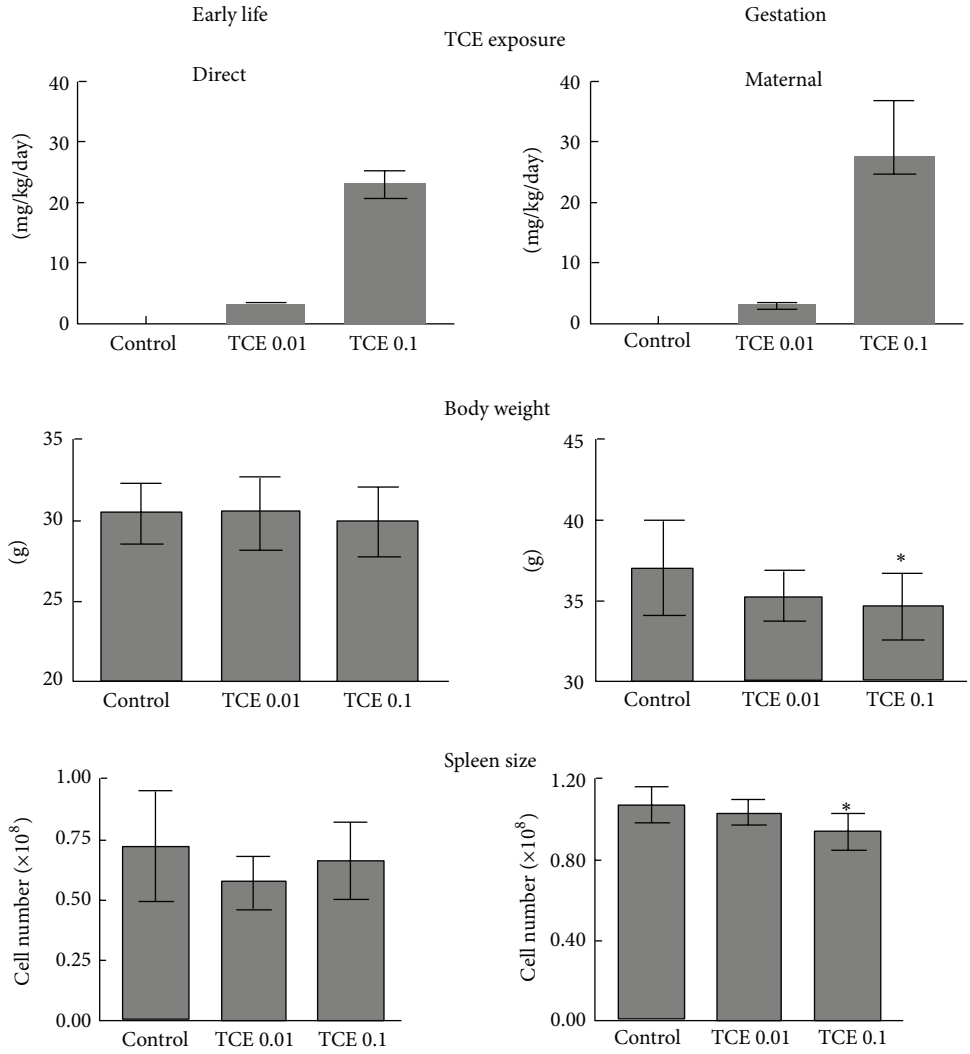

Figure 1: Characteristics of two windows of developmental TCE exposure. Female mice were exposed to TCE (0.01 or 0.1 mg/mL) during gestation only, or during early life only (lactation and 4 weeks of direct exposure). All of the developmentally-exposed mice were examined at PND49. For the gestation only exposure, maternal TCE ingestion based on water consumption was presented. For the early life exposure direct TCE ingestion of the pups postweaning based on water consumption was presented. Body weight and spleen cell numbers (mean ± SD) were determined at PND49. *Significantly different ($\alpha < 0.05$) compared to control values.

cells associated with adult-only direct exposure to TCE were found after 4 weeks [23, 25, 33]. Thus, the PND49 end point can be used to test how the effects of gestational or early life exposure compared to the effects of a direct 4-week adult-only exposure.

Similar to adult exposure to TCE, the early life exposure in the current study did not alter the weight of the female pups at PND49 (Figure 1). In contrast, female pups exposed to the highest concentration of TCE during gestation were significantly smaller than controls. Spleen cell numbers in the same mice were also lower than those of controls. Spleen cell numbers were not altered in mice exposed to TCE during early life only. Thus, TCE exposure during gestation rather than early life, did induce some gross changes in body weight and spleen size.

3.2. Developmental TCE Exposure and Changes in Macrophage Function. Potential TCE-induced alterations in peripheral immune function were examined. These included spleen cellularity and functional activity of key cellular components of chronic inflammation, namely, macrophages and $CD4^+$ T cells. IL-6 is a pleiotropic cytokine that has proinflammatory, anti-inflammatory and growth factor properties. Adult exposure to TCE has been shown to suppress macrophage production of IL-6 [34]. In the current study early life exposure to TCE at 0.1 mg/mL similarly inhibited IL-6 production at both the protein and gene expression level (Figure 2). In contrast, gestational exposure to TCE did not suppress IL-6 production by peritoneal macrophages.

3.3. Developmental TCE Exposure and Changes in $CD4^+$ T Cell Gene Expression. Early-occurring changes in peripheral

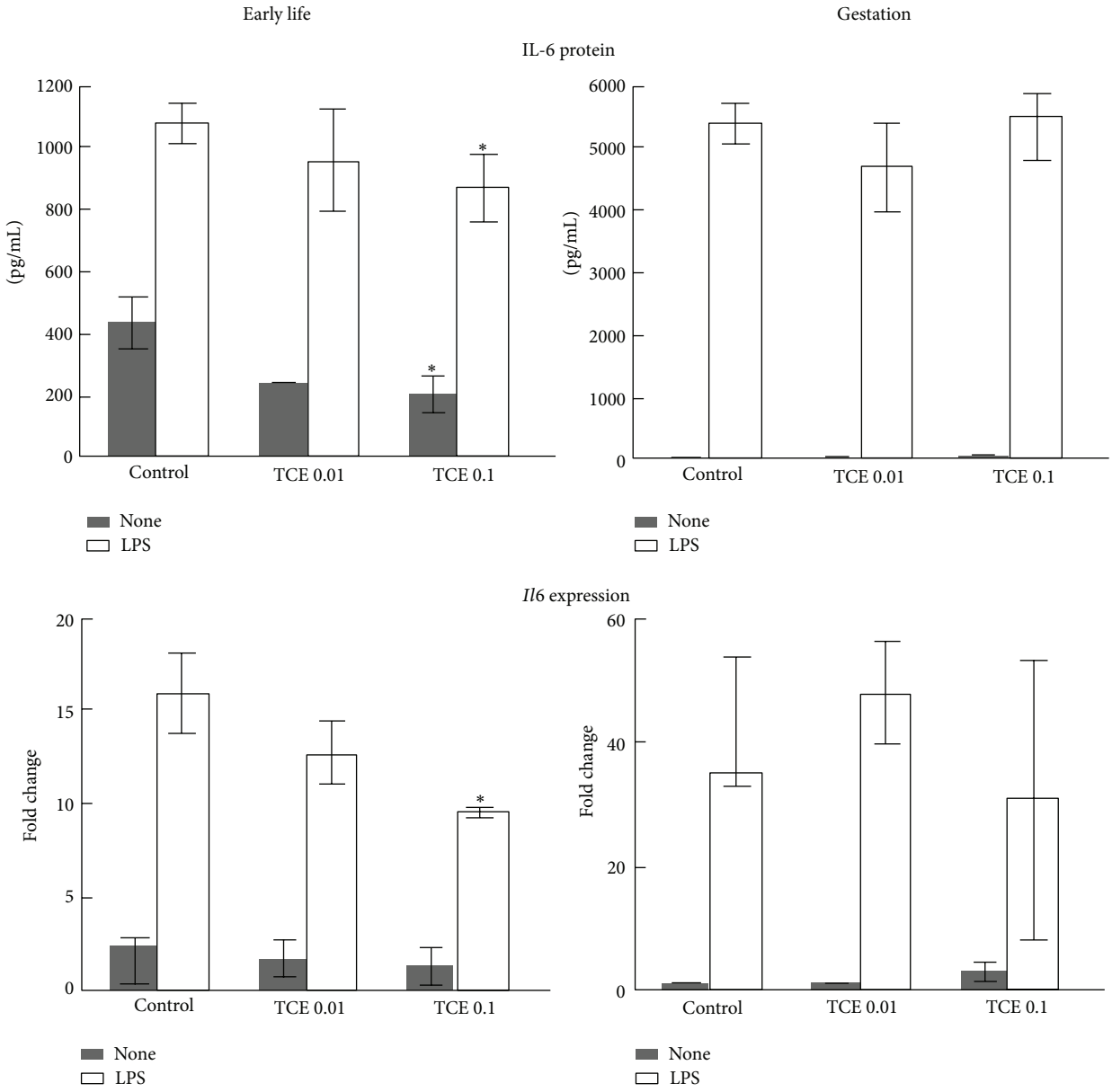

Figure 2: TCE inhibited IL-6 production and *Il6* expression in macrophages. Peritoneal macrophages were incubated with LPS and IFN-γ following isolation from untreated control mice or from mice exposed to TCE (0.01 or 0.1 mg/mL) during gestation only or during early life only. Culture supernatants were examined for IL-6 (mean ± SD). *Il6* gene expression was examined in the same peritoneal macrophages. The data represents the mean ± SD. *Significantly different ($\alpha < 0.05$) compared to control values.

$CD4^+$ T cells have been observed following adult and continuous exposure to TCE. These include expansion of the activation/memory subset of peripheral $CD4^+$ T cells and increases in the production of T cell-derived cytokines [24]. These potential alterations were assessed in the current study. TCE did not alter the percentages of total splenic $CD4^+$ T cells, $CD8^+$ T cells, or B cells, regardless of concentration or window of exposure (data not shown). However, similar to both adult and continuous TCE exposure, early life exposure to TCE at 0.01 or 0.1 mg/mL did increase the percentage of $CD44^{hi}$ $CD62L^{lo}$ $CD4^+$ T cells, the population of $CD4^+$ T cells considered to represent a memory/activated $CD4^+$ T cell phenotype (Figure 3). Gestational exposure did not increase the percentage of activated/memory $CD4^+$ T cells in the periphery at PND49.

Splenic $CD4^+$ T cells isolated from control and TCE-treated mice were activated *in vitro* prior to an examination of gene expression.Compared to baseline gene expression in unstimulated $CD4^+$ T cells, activation *in vitro* increased expression of almost all genes in all $CD4^+$ T cells (data not shown). To simplify comparison the gene expression of *in vitro* stimulated $CD4^+$ T cells from TCE-treated mice was compared to that of similarly activated $CD4^+$ T cells from control mice. This comparison yielded some subtle but

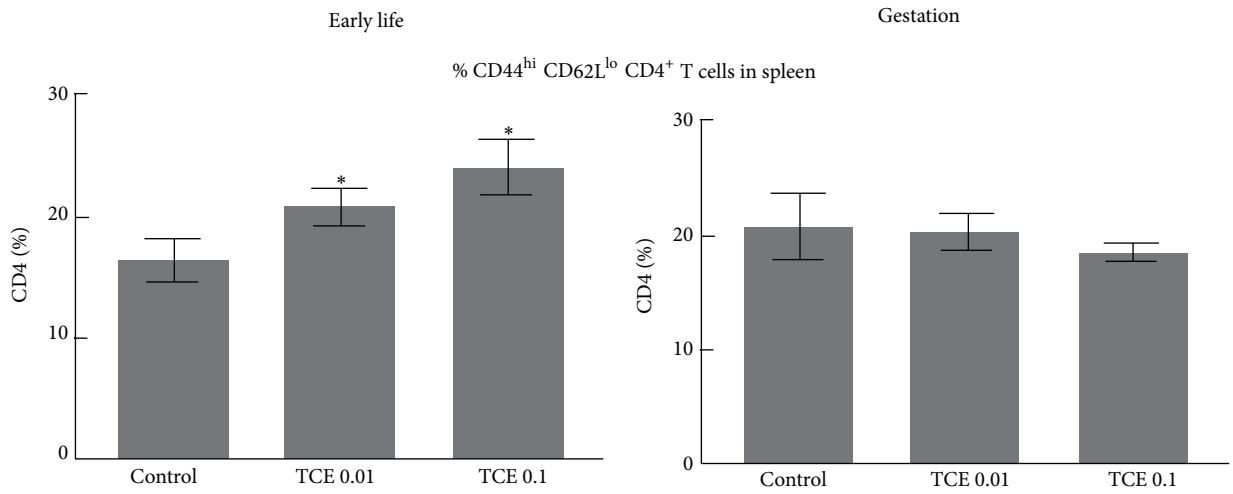

Figure 3: TCE enhanced the percentage of memory/activated $CD4^+$ T cells following early life exposure. Spleen cell suspensions from PND49 mice that had been exposed to TCE during gestation only or during early life only were examined by flow cytometry to determine the percentage of $CD4^+$ T cells that expressed high levels of CD44 and low levels of CD62L. *Significantly different ($\alpha < 0.05$) compared to control values.

significant differences. As observed previously following adult exposure early life exposure to TCE increased expression of *Ifng* and *Il2* but had little effect on *Il4* (Table 2). Gestational exposure, unlike early life exposure, did not increase the expression of these cytokine genes in activated peripheral $CD4^+$ T cells. Thus, once again, early life exposure, unlike gestational exposure, mirrored what has been observed in adult and continuous TCE exposure.

Although $CD4^+$ T cells from the two windows of developmental TCE exposure differed in their expression of cytokine genes, they demonstrated remarkably similar expression profiles for *Iap* and *Muerv*. These two genes encode for retrotransposons Intracisternal A particle and murine endogenous retrovirus. The expression of these two retrotransposons is kept in check by epigenetic processes, predominantly DNA methylation [35]. Events that cause global DNA hypomethylation tend to increase expression of *Iap* and *Muerv*, while DNA hypermethylation tends to suppress baseline expression of the retrotransposons. In this study both gestational and early life exposure to the lower concentration of TCE inhibited expression of Iap and MuERV in peripheral $CD4^+$ T cells.

DNA methylation is regulated by DNA methyltransferases, a family of enzymes that catalyze the transfer of a methyl group to DNA. This family encompasses *Dnmt1* that is thought to participate in maintenance DNA methylation, and *Dnmt3a*, involved in *de novo* DNA methylation in response to external stimuli [36]. At PND49, neither TCE exposure appreciably altered expression of *Dnmt1* or *Dnmt3a* in $CD4^+$ T cells. Thus, the downregulation of *Iap* and *Muerv* in $CD4^+$ T cells from mice developmentally exposed to TCE, regardless of gestation or early life, could not be explained by an increase in the expression of *Dnmt1* or *Dnmt3a*.

3.4. Developmental TCE Exposure and Changes in $CD4^+$ T Cell Function. In addition to gene expression, cytokine production was examined as a potential marker of TCE-induced alterations in $CD4^+$ T cell function. Early life exposure to TCE, similar to adult exposure, increased the production of certain cytokines including IL-2 by $CD4^+$ T cells (Figure 4). Gestational exposure alone was not sufficient to increase cytokine production in the peripheral $CD4^+$ T cells.

3.5. Developmental TCE Exposure and Thymus Cellularity. The effects of developmental TCE exposure on central immune function were also examined. The number of total thymocytes was not altered by TCE exposure during gestation or early life (data not shown). However, the composition of thymic subsets was altered in one set of the TCE-exposed mice. Exposure to the lower concentration of TCE during gestation increased the percentage of thymic single positive $CD4^+$ T cells (Figure 5). The same mice also demonstrated an increase in the percentage of double negative population 1 (DN1) thymocytes as well as a decrease in percentage of DN4 thymocytes. Unlike gestational exposure early life exposure to TCE did not change the percentages of the different thymocyte subsets measured. Thus, in terms of thymocyte cellularity early life exposure generated results that resembled that of adult exposure, while gestational exposure generated results that most resembled that of previously documented continuous exposure [27]. The thymocytes were then incubated for 18 hours to promote gene expression. Interestingly, neither gestational nor early life exposure to TCE altered *Iap* expression in the thymus, unlike peripheral $CD4^+$ T cells (Figure 6). This decreases the likelihood that the altered thymus cellularity of the gestationally exposed mice could be attributed to global changes in DNA methylation.

3.6. Developmental TCE Exposure and Thymus Gene Expression. The expression of genes that regulate thymic apoptosis and development were examined in an attempt to explain

Table 2: Gene expression in $CD4^+$ T cells from mice exposed to TCE during gestation or early life.

	Early life			Gestation		
	Control	TCE 0.01	TCE0.1	Control	TCE 0.01	TCE 0.1
Iap	1.0 ± 0.17	**0.12 ± 0.02**	0.42 ± 0.36	1.00 ± 0.1	**0.43 ± 0.05**	**0.72 ± 0.19**
Muerv	0.95 ± 0.26	**0.6 ± 0.11**	0.76 ± 0.23	1.0 ± 0.15	**0.44 ± 0.04**	0.95 ± 0.26
Il2	1.0 ± 0.1	**1.49 ± 0.2**	**1.31 ± 0.1**	0.91 ± 0.1	0.97 ± 0.29	**0.66 ± 0.1**
Il4	1.0 ± 0.17	1.1 ± 0.35	1.4 ± 0.34	1.1 ± 0.43	1.47 ± 0.75	1.44 ± 0.93
Cdkn1a	1.0 ± 0.03	0.9 ± 0.07	0.92 ± 0.07	1.05 ± 0.38	1.62 ± 0.64	1.41 ± 1.1
Ifng	0.91 ± 0.16	0.95 ± 0.11	**1.31 + 0.22**	1.32 ± 1.0	1.0 ± 0.81	1.42 ± 1.11
Dnmt1	1.0 ± 0.24	1.05 ± 0.12	0.89 ± 0.22	1.11 ± 0.51	1.58 ± 0.36	1.73 ± 0.71
Dnmt3a	0.98 ± 0.02	1.08 ± 0.07	1.09 ± 0.07	0.98 ± 0.51	1.12 ± 0.35	1.2 ± 0.87

Gene expression was examined in splenic $CD4^+$ T cells incubated with anti-CD3 and anti-CD28 Abs for 24 hours following isolation from untreated control mice or from mice exposed to TCE during gestation only, or during early life only. The data represents the mean ± SD. *Significantly different ($\alpha < 0.05$) compared to control values.
The bolded text represents values that are significantly different from control values ($\alpha < 0.05$).

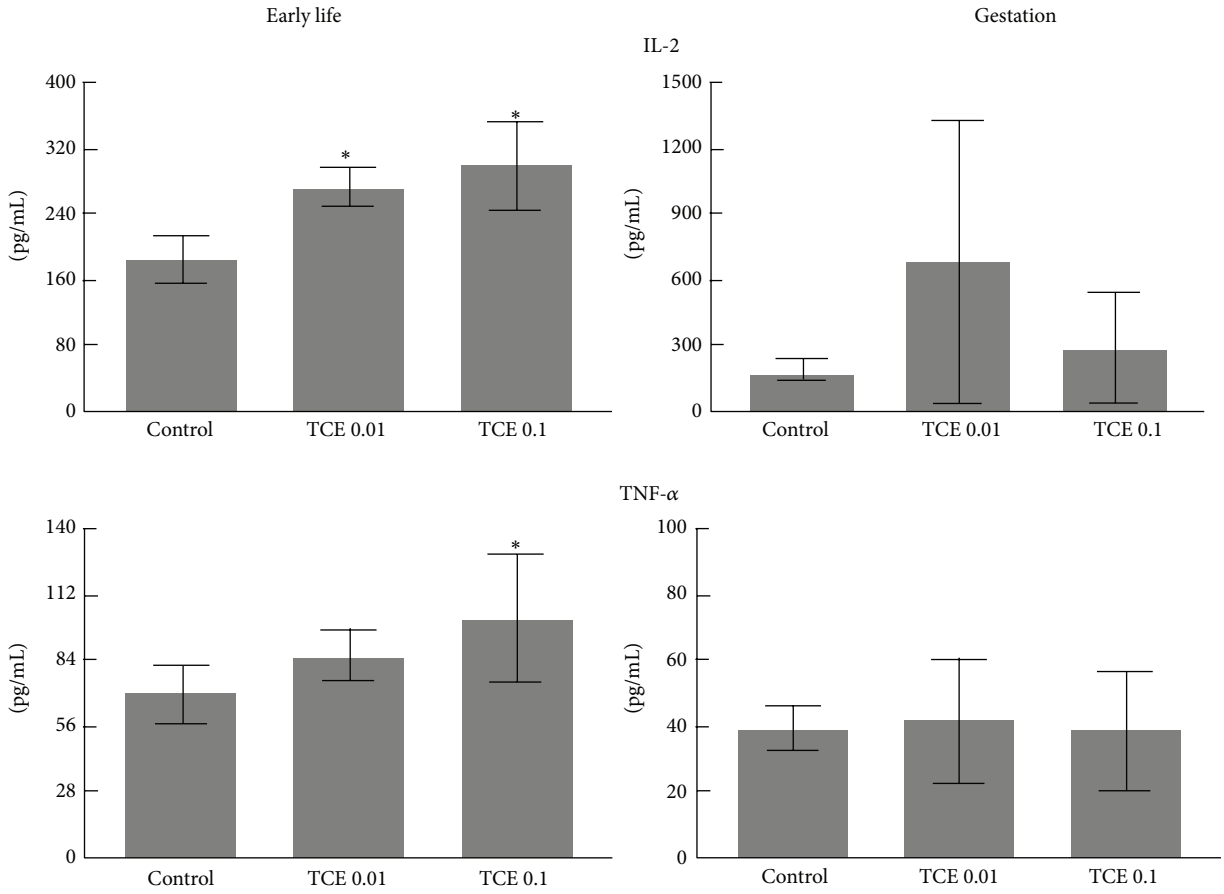

Figure 4: TCE altered cytokines in $CD4^+$ T cells from mice exposed to TCE during early life. Cytokine levels in culture supernatants from $CD4^+$ T cells prepared as described in Figure 4 were examined. *Significantly different ($\alpha < 0.05$) compared to control values.

the altered cellularity in the thymi of mice gestationally exposed to the lower concentration of TCE. In line with the unaltered *Iap* expression in the thymi of mice gestationally exposed to TCE, the expression of enzymes that regulate DNA methylation, namely, *Dnmt1* and *Dnmt3a*, were also unaltered by TCE in the thymus (Table 3). Deficiencies in thymic expression of the gene for the proapoptotic protein Bim or its downstream protein Bax can cause a defect in the negative selection process [37]. Neither of these genes was altered by TCE exposure in the thymus. Survivin, a protein originally described as an inhibitor of apoptosis, has more recently been shown to be required for maturation of DN thymocytes to DP thymocytes [38]. Even though gestational TCE exposure did seem to block an early stage

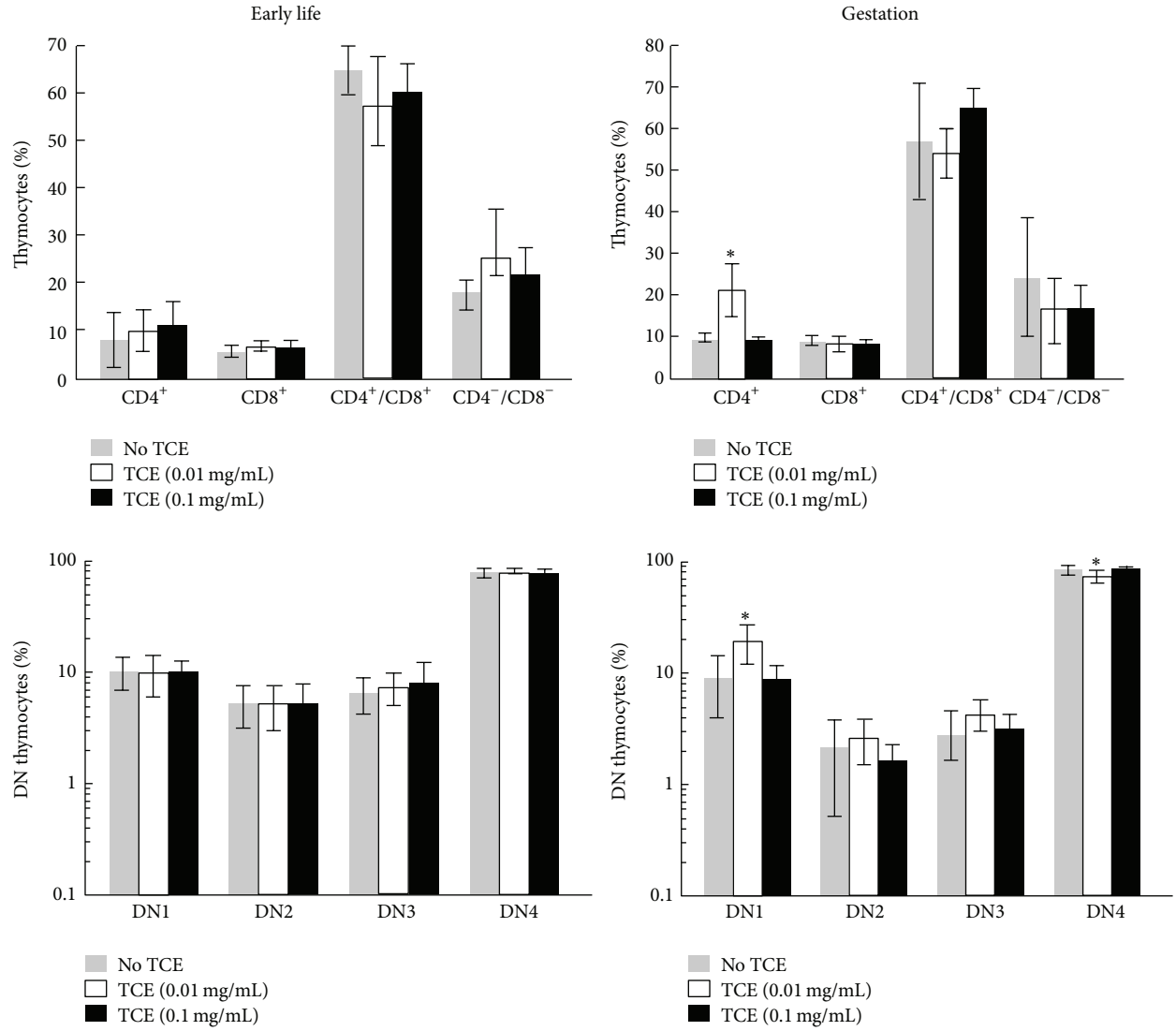

Figure 5: TCE altered thymus cellularity in mice exposed to TCE during gestation. Thymocyte suspensions from PND49 mice that had been exposed to TCE during gestation only or during early life only were incubated for 18 hours and then examined by flow cytometry to determine the percentage of single positive, double negative, and double positive cells. In addition, after gating on the double negative cell population, the cells were stained with anti-CD44 and anti-CD25 Abs and labeled as DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). *Significantly different ($\alpha < 0.05$) compared to control values.

of DN thymocyte differentiation, it did not alter expression of *Survivin*. Gestational exposure to TCE did not alter expression of *Fas*. However, both concentrations of TCE did suppress expression of *Fasl*, the gene that encodes for the ligand for *Fas*, and an important mediator of activation-induced apoptosis. Thus, the increase in SP $CD4^+$ thymocytes and DN1 thymocytes may be attributable to a decrease in apoptosis mediated by a TCE-induced decrease in the expression of *Fasl*. However, since the altered cellularity was only found in thymi of mice gestationally exposed to 0.01 mg/mL TCE, while the decrease in *Fasl* was found in mice exposed to either concentration of TCE, the role of *Fasl* in the altered cellularity needs to be clarified.

4. Discussion

The concordance rate for developing an autoimmune disease in identical twins demonstrates the involvement of an ill-defined genetic susceptibility [39]. To mimic this requirement "autoimmune-prone", MRL+/+ mice were used in our studies. Late in life, MRL+/+ mice can spontaneously develop a relatively mild lupus-like disease, as well as other autoimmune disorders such as Sjogren's syndrome and T cell-infiltrating pancreatitis [40, 41]. Young female MRL+/+ mice, with their propensity for autoimmunity but absence of overt disease, make a good model to test the immunostimulatory capacity of developmental TCE exposure. Others have similarly used

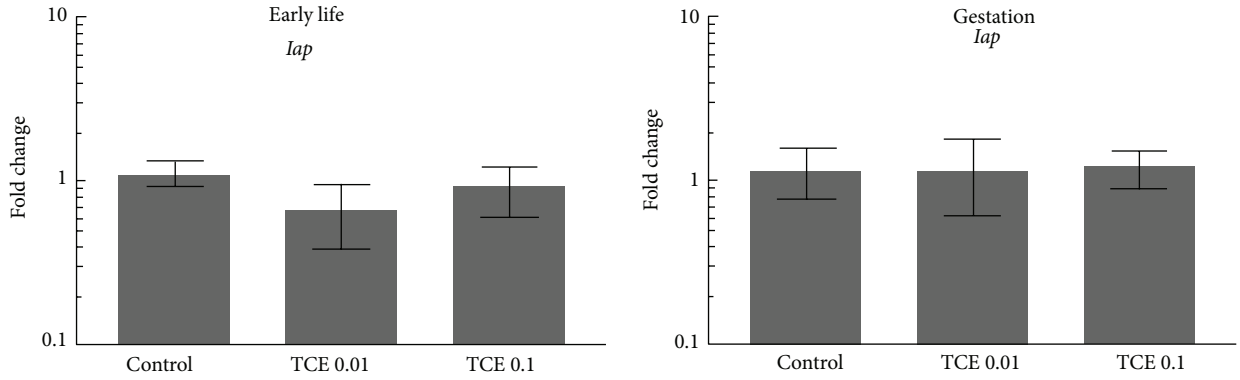

Figure 6: TCE did not inhibit *Iap* in thymocytes from either window of developmental TCE exposure. Gene expression was examined in thymocytes incubated for 18 hours following isolation from untreated control mice or from mice exposed to TCE during gestation only, or during early life only. The data represents the mean ± SD. *Significantly different ($\alpha < 0.05$) compared to control values.

Table 3: Gene expression in thymi of mice exposed to TCE during gestation.

	Gestation		
	Control	TCE 0.01	TCE 0.1
Bax	1.04 ± 0.14	0.78 ± 0.24	1.06 ± 0.75
Bim	1.02 ± 0.15	0.82 ± 0.3	0.87 ± 0.21
Dnmt1	1.0 ± 0.3	1.16 ± 0.5	1.03 ± 0.25
Dnmt3a	1.14 ± 0.56	1.17 ± 0.46	0.93 ± 0.26
Survivin	1.3 ± 0.97	0.53 ± 0.47	0.68 ± 0.42
Aire	1.1 ± 0.58	0.71 ± 0.09	0.76 ± 0.51
Fas	0.96 ± 0.49	0.83 ± 0.46	0.78 ± 0.28
Fasl	1.06 ± 0.42	**0.4 ± 0.25**	**0.45 ± 0.28**

Gene expression was examined in thymocytes incubated for 18 hours following isolation from untreated control mice or from mice exposed to TCE during gestation only.
The data represents the mean ± SD. *Significantly different ($\alpha < 0.05$) compared to control values.
The bolded text represents values that are significantly different from control values ($\alpha < 0.05$).

"autoimmune-prone" mouse strains to test the developmental immunotoxicity of chemicals such as dioxin, bisphenol A, and mercury [42–44].

The exposure associated with the highest TCE concentration of 0.01 mg/mL (10 ppm) is considerably lower than acceptable human occupational exposure. The EPA's Maximum Contaminant Level for public drinking water is 5 ppb, but TCE has been found at levels up to 1.4 ppm [45] In humans exposed to TCE-contaminated water the amount ingested is only a fraction of the TCE absorbed via inhalation and dermal contact [46]. Since TCE exposure in the mice will be limited to ingestion it could be argued that mouse exposure to 10 ppm is within the range of possible human environmental exposure.

For essentially all of the parameters tested the early life exposure to TCE induced immune system changes similar to adult exposure, albeit at much lower concentrations. On the other hand, the results obtained from gestational only exposure to TCE were similar to those documented after continuous exposure to TCE. Gestational exposure to TCE caused alterations in thymic cellularity and effects on body weight and spleen size. In contrast, gestational TCE exposure had little effect on peripheral $CD4^+$ T cells, at least the parameters measured at PND 49. Unlike gestational exposure, early life exposure to TCE had no effect on thymic cellularity but was able to increase the percentage of activated/memory $CD4^+$ T cells in association with increased production of cytokines. This implies that exposure to TCE even as early as PND0 had more of an impact on deployed peripheral $CD4^+$ T cells than on the thymus. It is possible that lactational exposure to TCE at the concentrations used was not sufficient to impact the still-developing thymus, and that the effects observed following early life exposure were primarily attributable to the direct TCE exposure begun at PND21. An additional study of lactational only exposure to TCE is needed to resolve this question. Immune development in mice correlates well with immune development in humans, albeit with different kinetics [47]. One difference is that the perinatal immune system in mice is even more immature than that of humans. This means that the immune maturation that occurs during human gestation is commensurate with that found in mice at weaning at PND21. Thus, lactation only exposure in mice would correspond with late gestation in humans.

Taken together, it would seem that the individuals most susceptible to the most wide-ranging effects of TCE on the central and peripheral immune system are those exposed to the chemical during gestation as well as early life. Such a scenario in humans could occur when contaminated drinking water for both mother and child results in indirect TCE exposure during gestation and lactation and then direct exposure after weaning. However, it should be noted that, although the two different windows of TCE developmental exposure had distinct effects on peripheral and central T cells, both windows of exposure inhibited expression of retrotransposons in peripheral $CD4^+$ T cells. This implies that both gestation and early life are times during which epigenetic changes can be induced. Whether these changes manifest themselves in functional alterations that promote

TCE-induced hypersensitivity later in life needs to be determined. Epigenetic processes can regulate several aspects of normal $CD4^+$ T cell function including Th1/Th2 differentiation [48], cytokine production [49], and maintenance of Treg cells [50]. There are also several pieces of evidence that epigenetic processes regulate the activity of self-reactive $CD4^+$ T cells that mediate autoimmune disease [51–53]. In view of the connection between epigenetic alterations and $CD4^+$ T cell autoreactivity, the need to further investigate the capacity of TCE to induce these alterations becomes even more important.

How TCE impacts $CD4^+$ T cells is still being defined. Earlier studies showed that the $CD4^+$ T cell-altering effects of TCE were most likely mediated by its primary oxidative metabolite trichloroacetaldehyde hydrate (TCAH) [32, 33]. As an aldehyde TCAH can form a functionally active chemical interaction known as a Schiff base with amines on the surface of the $CD4^+$ T cells [54]. The possibility that TCAH forms a Schiff base with a costimulator receptor such as CD28, and thus provides bystander costimulation for antigen-activated $CD4^+$ T cells is being investigated. How this mechanism would impact thymocytes during development is unclear. In adults, TCE metabolism to TCAH is primarily mediated by cytochrome P450 2E1 (CYP2E1), and to a lesser extent by CYP1A1, and occurs primarily in the liver. However, since CYP1A1, unlike CYP2E1, can be detected in GD7 in the thymus [55], it is possible that CYP1A1 generates TCAH in the thymus of mice developmentally exposed to TCE.

Characterizing TCE-induced developmental immunotoxicity may be part of an important big picture. Immune dysfunction in the form of hypersensitivity disorders are among the most common medical conditions affecting children in the USA. Many of these appear to be increasing. For example, more children are being diagnosed with type I diabetes [56], childhood Graves' disease [57], and lupus nephritis [58] than ever before. Similarly, the CDC reports that the prevalence of food allergies increased from 3.4% in 1997–1999 to 5.1% in 2009–2011 [59]. The prevalence of skin allergies also increased from 7.4% in 1997–1999 to 12.5% 2009–2011. It is possible that the increase in childhood hypersensitivity is linked to increased exposure to environmental chemicals with potential developmental toxicity. More than 85,000 synthetic chemicals have been developed in the past 75 years, and only about 20% of the 3,000 most widely used chemicals have ever been tested for general developmental toxicity let alone developmental immunotoxicity [60]. As part of NHANES 2003-2004, it was found that after adjusting for covariates levels of many chemicals in pregnant women were increased compared to nonpregnant women [61]. Certain polychlorinated biphenyls, organochlorine pesticides, polycyclic aromatic hydrocarbons and many other chemicals were detected in 99-100% of pregnant women. Some of these chemicals cross the placenta and mediate developmental immunotoxicity. Developmental immunotoxicity is based on the premise that during the maturation of the immune system toxicant exposure results in a qualitative or quantitative difference in the effect or a greater persistence of effect. This increased sensitivity may be attributed to (i) a greater chemical exposure per pound of body weight, (ii) immature metabolic systems unable efficiently clear toxicants, (iii) developing immune system that is easily disrupted, and (iv) more time to develop chemical-induced immune-mediated diseases. Studying developmental immunotoxicity of chemicals such as TCE with proinflammatory effects may provide important clues to the etiology of idiopathic autoimmune disease.

5. Conclusion

Early life exposure to TCE induced changes in peripheral $CD4^+$ T cell commensurate with those found in mice exposed to TCE as an adult. Gestational TCE exposure induced changes in central immune function similar to that observed following continuous exposure. However, the two windows of exposure did have one effect in common, namely, the down-regulation of retrotransposon expression in $CD4^+$ T cells. Since the effects induced by the two developmental windows of TCE exposure were associated with TCE concentrations lower than those effective in adult, exposure the differential responses are functionally important.

Conflict of Interests

There is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors would like to gratefully acknowledge the excellent technical assistance of Brannon Broadfoot, Kirk West, and Rachel Lee and the support of the UAMS Translational Research Institute (National Institutes of Health UL1RR029884). This work was supported by Grants from the Arkansas Biosciences Institute, the National Institutes of Health (1R01ES017286; 1R01ES021484, R21ES017311), and the Organic Compounds Property Contamination class action settlement (CV 1992-002603).

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 437231, 18 pages
http://dx.doi.org/10.1155/2014/437231

Review Article

A Potential Link between Environmental Triggers and Autoimmunity

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Received 26 September 2013; Revised 19 November 2013; Accepted 21 November 2013; Published 12 February 2014

Academic Editor: K. Michael Pollard

Autoimmune diseases have registered an alarming rise worldwide in recent years. Accumulated evidence indicates that the immune system's ability to distinguish self from nonself is negatively impacted by genetic factors and environmental triggers. Genetics is certainly a factor, but since it normally takes a very long time for the human genetic pattern to change enough to register on a worldwide scale, increasingly the attention of studies has been focused on the environmental factors of a rapidly changing and evolving civilization. New technology, new industries, new inventions, new chemicals and drugs, and new foods and diets are constantly and rapidly being introduced in this fast-paced ever-changing world. Toxicants, infections, epitope spreading, dysfunctions of immune homeostasis, and dietary components can all have an impact on the body's delicate immune recognition system. Although the precise etiology and pathogenesis of many autoimmune diseases are still unknown, it would appear from the collated studies that there are common mechanisms in the immunopathogenesis of multiple autoimmune reactivities. Of particular interest is the citrullination of host proteins and their conversion to autoantigens by the aforementioned environmental triggers. The identification of these specific triggers of autoimmune reactivity is essential then for the development of new therapies for autoimmune diseases.

1. Introduction

The immune system walks a fine line to distinguish self from nonself in preserving the integrity of the host [1]. Interference with this fine line can result in overactivity to self-antigens, leading to autoimmunity. During the past 20 years a significant increase has been observed in the incidence of autoimmune disease worldwide. The etiology and pathogenesis of many autoimmune diseases remain unknown. It does appear that a close interplay between environmental triggers and genetic factors is responsible for the loss of immunological tolerance and autoimmunities [2, 3] (Figure 1). Therefore, in relation to the role of heritability in autoimmunity, genome-wide association studies reported that genetics only accounted for a minority of autoimmunity cases, and in many cases disease discordance exists in monozygotic twins [4]. For this reason, research and publications dedicated to environmental factors in autoimmunity have grown by an average of 7% every year since 1997 [2]. This includes toxic chemicals, infections, and dietary components. Indeed, detection of reactive antibodies to various citrullinated peptides and proteins in autoimmune disease is the best indication for gene-environment interactions [5].

2. Dysregulation of Immune Homeostasis

The full collaboration of both the innate and adaptive arms of the immune system plays a crucial role in the promotion or inhibition of autoimmune disease. Generally, to clear infections the innate immune cells can upregulate costimulatory molecules and produce a mixture of pro- and anti-inflammatory cytokines such as interleukin-1-beta (IL-1β), IL-12, transforming growth factor-beta (TGF-β), IL-23, tumor necrosis factor-alpha (TNF-α), and IL-6 that regulate the adaptive arm of the immune system. However,

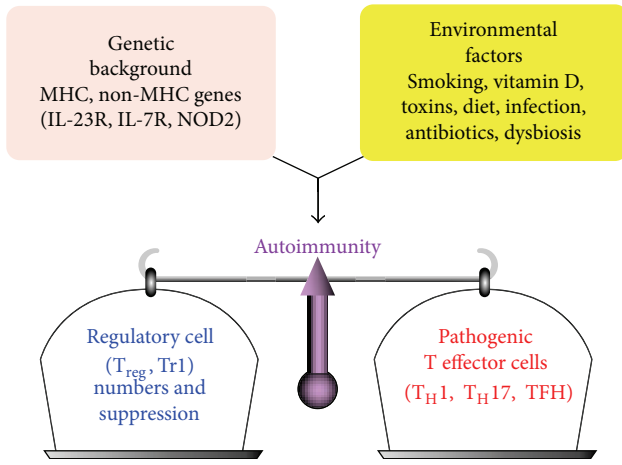

Figure 1: The balance of immunity. A combination of host genetic factors and exposure to environmental triggers promote the development of autoimmune disease. A balance must be maintained between the regulatory T cells and the pathogenic T effector cells.

a dysregulated immune response to environmental triggers, such as pathogens, microbiota, or toxins, can initiate a chronic inflammatory response through activation of T-helper-1 (Th1), Th17, and TNF-α and the production of IL-17, IL-22, interferon-gamma (IFN-γ), and IL-21, resulting in inflammation, antibody production and tissue injury [6].

Therefore, a dysregulated adaptive immune system is at the core of the pathogenesis of autoimmune and other immune-mediated diseases. Hyperactivation of innate immune response affects the adaptive immune response as well as development effector T and B cells. Paired with defects in the regulatory T cells, this results in the breakdown of immune homeostasis and the development of autoimmunity [7].

To induce an autoimmune response in the lymph nodes, effector T cells first have to acquire a defined cytokine fingerprint and then must migrate to the appropriate target organs where they initiate tissue inflammation. The effector cells that participate in the induction of autoimmunities are IFN-γ-producing Th1 cells, IL-17- and IL-22-producing Th17 cells, and IL-21-producing follicular Th cells or TFH cells. It has been shown that overactivation or expansion of these newly discovered TFH cells causes antibody production and the development of lupus-like disease in an animal model [8]. In fact, high concentrations of circulating T cells that resemble TFH cells have been detected in a subgroup of patients with lupus. This increased frequency of TFH cells correlated with both disease severity and end-organ damage [9]. Unfortunately, a decrease in frequency and function of $FOXP3^+T_{REG}$ cell is often seen in autoimmune diseases. This decrease seems to be associated with the inflammatory environment that contributes to the dysregulation of T_{REG} cells [7].

In the environment of immune homeostasis, the actions of autoreactive Th1, Th17, and TFH cells are countered by $FOXP3^+$ regulatory T cells that produce TGF-β and IL-10. But in an inflammatory milieu the deletion of different transcription factors results in the generation of T_{REG} cells that are unable to suppress the autoreactive T cells (Figure 2).

Thus, tight control of autoreactive T cells, in particular TFH cells, by T_{REG} cells is necessary to suppress the development of autoimmune lupus-like disease.

In order to induce long-lasting remission of immune-mediated diseases, two important factors have to be in place: controlling the inflammatory environment and boosting the frequency and function of $FOXP3^+$ regulatory T cells.

3. Toxicants and Autoimmunity

A number of experimental studies and clinical reports have shown that autoimmune reactivity and/or autoimmune diseases are induced in humans and chronic exposure to various chemicals in animal models. These were summarized by Bigazzi in 1997 [10]. Furthermore, very recently, this role of environmental chemicals, in particular, the induction of autoimmunities by toxicants, was summarized by Pollard et al. [11] in his paper, "Toxicology of autoimmune diseases." The mechanism of toxicant-induced autoimmunity is described by either toxicant induction of aberrant cell death making the hidden cellular material available to anti-gen presenting cells [12, 13] or by immune reactions to xenobiotics through covalent binding of chemicals or haptens to human tissue proteins and formation of neoantigens [14] (Figure 3). This is due to the fact that reactive organic compounds most often bind covalently; that is, their electrophilic properties enable them to react with protein nucleophilic groups such as thiol, amino, and hydroxyl groups. Examples of such reactive, haptenic compounds that frequently lead to sensitization after dermal contact or inhalation are toluene diisocyanate, trimellitic anhydride, phthalic anhydride, benzoquinone, formaldehyde, ethylene oxide, dinitrochlorobenzene, picryl chloride, penicillins, and D-penicillinamine. Sensitizing metal ions react somewhat differently in that they oxidize proteins or

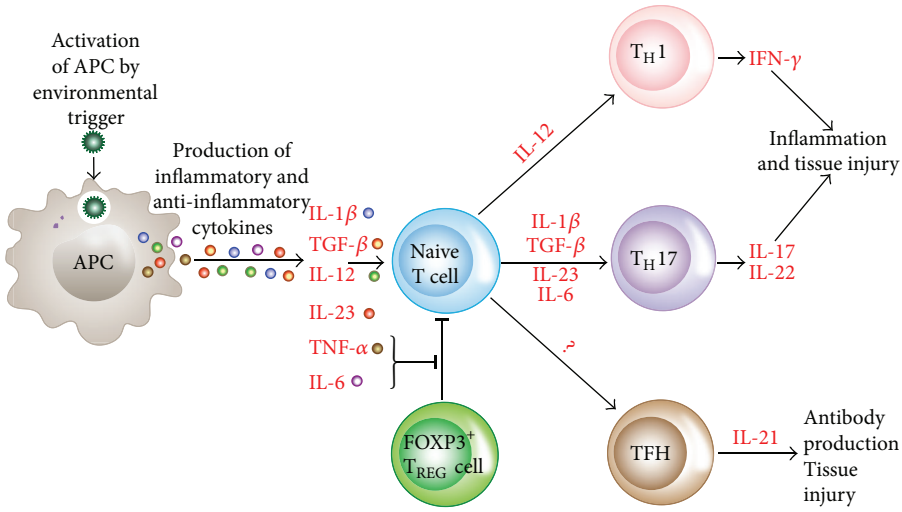

FIGURE 2: Differentiation of naïve T cells into pathogenic effector T cells. APCs can be activated by numerous factors, resulting in the release of cytokines that promote the differentiation of naïve T cells into various subsets of pathogenic effector T cells that drive inflammation, tissue injury, and autoantibody production. Segmented filamentous bacteria (SFB) can also promote the development of Th17 cells and autoimmune responses *in vivo*. Proinflammatory cytokines derived from both innate and adaptive immune cells attenuate T_{REG} cell-mediated suppression of effector T cells.

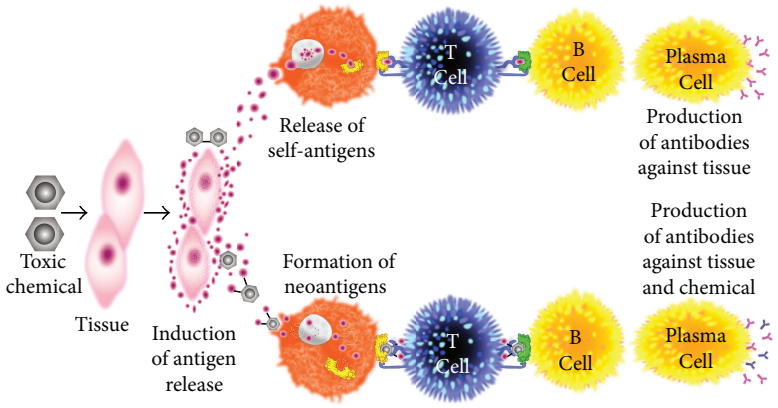

FIGURE 3: Putative mechanism of chemical-induced autoimmunity.

form stable protein-metal chelate complexes by undergoing multipoint binding with several amino acid side-chains [12]. For example, in regard to nail polish and its association with primary biliary cirrhosis (PBC), halogenated compounds could bind to mitochondrial proteins, changing their immunogenicity and inducing antimitochondrial antibodies [10, 15, 16].

In contrast to haptenic compounds, most xenobiotics eliciting adverse immune reaction are unable to bind to proteins when entering the body; however, they can do so after conversion to reactive metabolites. These xenobiotics can be considered as prohaptens, which, after metabolization, manage to bind to human tissue proteins and induce antibody production against both the haptenic chemicals as well as tissue proteins.

Another mechanism is the activation of toll-like receptors by xenobiotics. This predisposes individuals to toxicant-induced inflammatory cytokine production, which exacerbates autoimmune diseases [17].

These and other mechanisms of action were explored in relation to exposure to organic solvents as a risk factor for autoimmune disease in a very extensive systemic review of literature and meta-analysis [18]. After reviewing a total of 103 articles and the inclusion of 33 in the meta-analysis, it was concluded that (1) exposure to organic solvents was associated with systemic sclerosis, primary systemic vasculitis, and multiple sclerosis (MS) and (2) individuals who carry genetic factors for autoimmunities should avoid any exposure to organic solvents in order to avoid increasing their risk for autoimmune diseases.

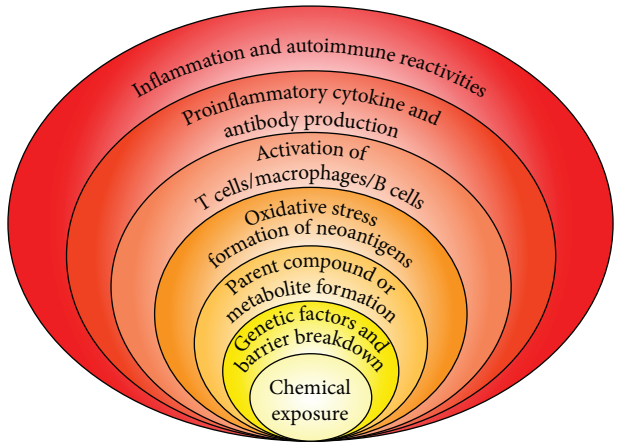

Figure 4: Potential molecular mechanisms implicated in chemical-induced autoimmune reactivities.

In addition to the above mechanisms of actions (as shown in Figures 3 and 4), these autoimmune responses and diseases can be induced by solvents and other environmental chemicals through a variety of effects at the biochemical and cellular levels.

(i) Chemicals are capable of altering cellular proliferation, Th1, Th2, Th3, Th17, apoptosis, and tissue-specific function.

(ii) Chemicals are capable of inducing protein or lipid adducts which activate Th17 cells and induce the production of IL-17 and IL-21.

(iii) Chemicals can activate HSP90 and induce production of anti-HSP90 autoantibodies.

(iv) Chemicals are capable of inducing DNA-hypermethylation and change in cellular functions.

(v) Chemicals can increase ROS production and the induction of DNA-fragmentation.

(vi) Chemicals may compete with thyroid hormones or interfere with iodine transportation and induce oxidative stress that leads to an inflammatory response to the thyroid gland.

(vii) Chemicals not only stimulate the release of reactive oxygen species but also stimulate the synthesis of nitric oxide by nitric oxide synthase [18].

Finally, modification of DNA methylation is an additional mechanism by which environmental triggers induce changes in gene expression. For example, environmental pollutants, cigarette smoke and alcohol consumption have been advocated for autoimmunity incidence due to their links with the induction of DNA methylation [10, 19].

Overall, the precise mechanisms responsible for the development of environmentally induced autoimmune disorders are unknown. Additionally, mechanisms involved in the initiation of a disease process might differ from mechanisms responsible for exacerbation of the established illness. Therefore, one or more of these mechanisms either individually or jointly can have strong effects on the development of autoimmune reactivity, which may then be followed by autoimmune disease (Figure 4).

4. Induction of Autoimmunities by Infection

Although some infections can protect individuals from specific autoimmune diseases, infectious agents play a pivotal role in the induction of autoimmune disorders. The question of how infectious agents contribute to autoimmunity has continued to be of interest to clinical and basic researchers and immunologists in general [20].

An autoimmune disease can be induced or triggered by infectious agents, which can also determine its clinical manifestations. Most infectious agents, such as viruses, bacteria, fungi, and parasites, can induce autoimmunity via different mechanisms. In many cases, it is not a single infection but rather the "burden of infections" from childhood that is responsible for the induction of autoimmunity [20].

Almost every autoimmune disease is linked to one or more infectious agent. During the past 50 years molecular techniques have been utilized to explore the interaction between infections and autoimmunities [20–23]. One of the classical examples of this relationship is rheumatic fever, which presents several weeks after infection with beta hemolytic streptococcus. Molecular resemblance between the bacterial M5 protein and human α-myosin results in a breakdown of immunological tolerance and antibody production against α-myosin in genetically susceptible individuals [21, 24]. In the case of antiphospholipid syndrome (APS), anticardiolipin and anti-β_2-glycoprotein I pathogenic (β_2GPI) antibodies are detected. Although there is molecular mimicry between β_2GPI and infections, such as cytomegalovirus, *Haemophilus influenza, Neisseria gonorrhoeae*, rubella, toxoplasma, and tetanus toxoid, and IgM antibodies against them have been detected, the direct connection between these infections and APS has not been established [24]. Another example of associating infection with autoimmune disease is type 1 diabetes. Type 1 diabetes is an autoimmune disease resulting from the destruction of β-islet cells by autoreactive

T cells and the concomitant release of various islet cell antigens [21, 25]. The appearance of antibodies against glutamic acid decarboxylase 65 (GAD-65) and tyrosine phosphatase precedes the onset of the disease by 5–10 years [26, 27]. Several lines of evidence link infections with type 1 diabetes.

(1) A search on PubMed using the keywords "association of viruses with type 1 diabetes" produces close to 1,400 manuscripts.

(2) Enteroviruses such as coxsackie B4 virus and rotavirus, the most common cause of childhood gastroenteritis, not only share homology with GAD-65, but can cause the precipitation of type 1 diabetes when introduced. Higher levels of anti-coxsackievirus and rotavirus antibodies are detected in sera from patients with recent onset of type 1 diabetes [28, 29].

(3) Using PCR technology, coxsackie B4 virus was detected in islet cells of 65% of patients versus only 6% of controls [30].

(4) Inoculation of the virus to genetically susceptible strains of mice resulted in insulitis and diabetes, fulfilling Koch's postulates [21, 31, 32].

(5) Both DNA and RNA viruses are capable of initiating antiviral responses that cross-react with insulin, GAD-65, and other islet cell antigens [21, 33, 34].

Altering the balance of gut microbiota toward either a tolerogenic or nontolerogenic state using antibiotics or probiotics may influence the development of type 1 diabetes [35, 36]. Therefore, just as with their viral counterparts, there is sufficient indirect evidence that gut and other microbial agents, for example, *Mycobacterium avium,* are potential triggers for type 1 diabetes [37].

This multifaceted interaction between genetics, immune dysregulation, various infections, and autoimmune diseases such as rheumatoid arthritis (RA) and thyroid disease reveals many possibilities for pathogenic relationships between different species of infectious agents and autoimmunity [20, 38, 39]. These infectious agents and their association with RA and thyroid autoimmunity are shown in Tables 1 and 2.

4.1. Mechanisms Responsible for the Induction of Autoimmunity by Infection. Autoimmunity can be induced by infectious agents through the following mechanisms: molecular mimicry, epitope spreading, standard activation, viral persistence, polyclonal activation, dysregulation of immune homeostasis, and autoinflammatory activation of innate immunity [20]. In some cases, even if infections are not directly responsible for the induction of autoimmunities, they can often target the site of autoimmune inflammation and amplify the autoimmune disease [68]. In this case, infections can have one of three effects: first, it can exacerbate ongoing disease, leading to greater severity and duration; second, it can induce a relapse; and, third, it can lead to chronic progressive disease.

4.1.1. Molecular Mimicry. In the most likely mechanism by which infection induces autoimmunity, foreign antigens very often may bear sufficient structural similarity to self-antigens.

Table 1: Infectious agents associated with rheumatoid arthritis.

Infection	Reference
Porphyromonas gingivalis	Farquharson et al. 2012 [40]
Segmented filamentous bacteria	Wu et al. 2010 [41]
Yersinia enterocolitica	Gaston and Lillicrap 2003 [42]
Salmonella typhi	McColl et al. 2000 [43]
Shigella flexneri	Hannu et al. 2005 [44]
Proteus mirabilis	Ebringer and Rashid 2006 [45]
Campylobacter jejuni	Pope et al. 2007 [46]
Klebsiella pneumoniae	Domínguez-López et al. 2000 [47]
Clostridium difficile	Cope et al. 1992 [48]
Staphylococcus aureus	Liu et al. 2001 [49]
Streptococcus pyogenes	Faé et al. 2006 [50]
Candida albicans	Hermann et al. 1991 [51]
Leptospira pomona	Sutliff et al. 1953 [52]
Chlamydia	Carter et al. 2010 [53]
Mycoplasma arthritidis	Cole and Ward 1979 [54]
Mycobacterium tuberculosis	Kim et al. 2006 [55]
Borrelia burgdorferi	Imai et al. 2013 [56]
Parvovirus	Kerr et al. 1995 [57]
Epstein-Barr virus	Pratesi et al. 2006 [58]

Table 2: Infectious agents associated with thyroid autoimmunity.

Infection	Reference
Yersinia enterocolitica	Bech et al. 1978 [59]
Epstein-Barr virus	Shimon et al. 2003 [60]
Parvovirus	Mori et al. 2007 [61]
Hepatitis C	Fernandez-Soto et al. 1998 [62]
Mumps	Parmar et al. 2001 [63]
Rubella	Ziring et al. 1977 [64]
Coxsackievirus	Brouqui et al. 1991 [65]
HTLV-1	Kawai et al. 1992 [66]
Human herpes virus types 6 and 7	Leite et al. 2008 [67]

This is called antigenic mimicry or molecular mimicry. Immune response to microbial antigens could result in activation of T cells that are cross-reactive with self-antigens. This is due to the fact that a single T cell can respond to various peptides with similar charge distribution and overall shape [20, 69]. Examples of bacterial or viral antigens, their cross-reactivity with various tissue antigens, and potential ensuing autoimmune diseases are shown in Table 3.

Mechanisms of infection-induced autoimmunity through molecular mimicry are shown in Figure 5.

Initiation of immune response to the foreign antigens such as coxsackievirus that share identical amino acid residues with self-proteins such as GAD-65 may generate a cross-reactive antibody response that incorrectly recognizes the self-protein as a foreign antigen. When the self-antigen is

Table 3: Examples of bacterial and viral antigens that can cross-react with self-antigens with potentially resultant diseases.

Pathogen antigen	Cross-reactive self-antigen	Autoimmune disease
Herpes simplex virus	Corneal antigen	Stromal keratitis
Campylobacter jejuni	Ganglioside in peripheral nerve	Guillain-Barré syndrome
Coxsackievirus	Glutamic acid decarboxylase	Type 1 diabetes
Theiler's murine encephalomyelitis virus	Proteolipid protein	Multiple sclerosis
Yersinia enterocolitica	Thyrotropin receptor	Thyroid autoimmunity
Borrelia burgdorferi	Leukocyte function associated antigen	Lyme arthritis
Salmonella typhi and *Yersinia enterocolitica*	HLA-B27	Reactive arthritis
HHV-6, EBV, Rubeolla, influenza virus, and HPV	Myelin basic protein	Multiple sclerosis
Streptococcal M protein	Myosin and other heart valve proteins	Rheumatic fever
Trypanosoma cruzi	Cardiac myosis	Chagas heart disease

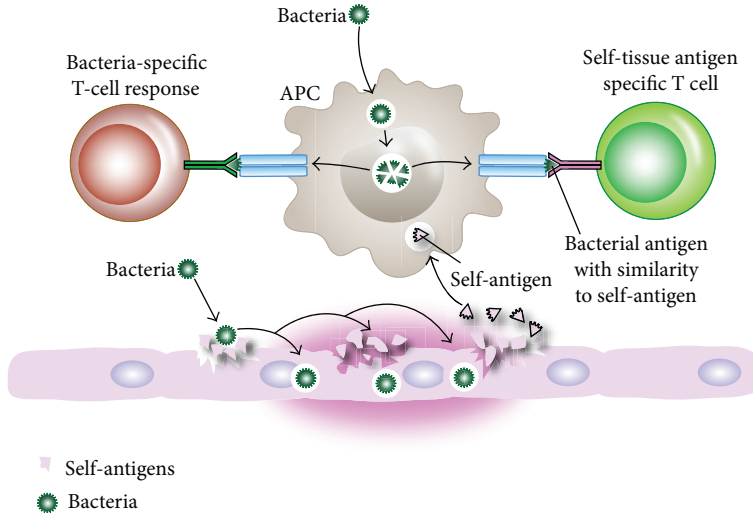

Figure 5: Mechanisms of infection-induced autoimmunity through molecular mimicry. Bacterial induction of self-tissue antigen release and simultaneous presentation of bacterial and self-tissue antigens to T cells; activated T cells can produce antibodies against both bacterial and self-tissue antigens.

a cell surface molecule such as GAD-65, the antibody- and cell-mediated immune response can lead to tissue damage [69].

Given the vast numbers of microbial proteins and their cross-reaction with human proteins, immune response against microbial antigens will not always result in autoimmunity. However, such an initial immune response could result in epitope spreading or exposure of other regions of the same self-protein and production of more antibodies [69]. The criteria for the mechanism of autoimmunity induction were reviewed and summarized by Kivity et al. 2009 [20]. In the classical examples of autoimmunities induced by infections summarized in Table 3, all these criteria are present.

4.1.2. Epitope Spreading. Epitope spreading is a phenomenon in which the immune system expands its response beyond the original epitope recognized by T or B cells to induce the release of non-cross-reactive epitopes that are recognized by the immune system later [70]. Epitope spreading can result from a change in protein structure. One such example is protein citrullination, the changing of an amino acid from arginine to citrulline. This can result not only in immune reaction against the original protein or its citrullinated form, but also against other citrullinated proteins.

Epitope spreading is demonstrated in rheumatic fever, in which a chronic autoimmune response against streptococcal M protein and heart valve tissue can result in immune response against collagen or laminin. This immune response against collagen or laminin is no longer specific to the bacterial M protein or its cross-reactive tissue protein. In pemphigus, blistering of the mouth precedes blistering of the skin, and blisters in the mouth are associated with the presence of antibodies against desmoglein-3 protein, which is specific to the mouth epithelial cell antigens. It is only later on when T cells attack skin desmoglein-1 that autoantibodies are produced against skin-specific antigens, and skin blistering develops [71]. In a mouse model of encephalomyelitis, Theiler's murine encephalomyelitis virus T-cell response to myelin develops first against dominant myelin proteolipid (PLP) peptide 139–151. As the disease progresses, response to the different and less dominant epitope PLP peptide 178–191 emerges. This mechanism of

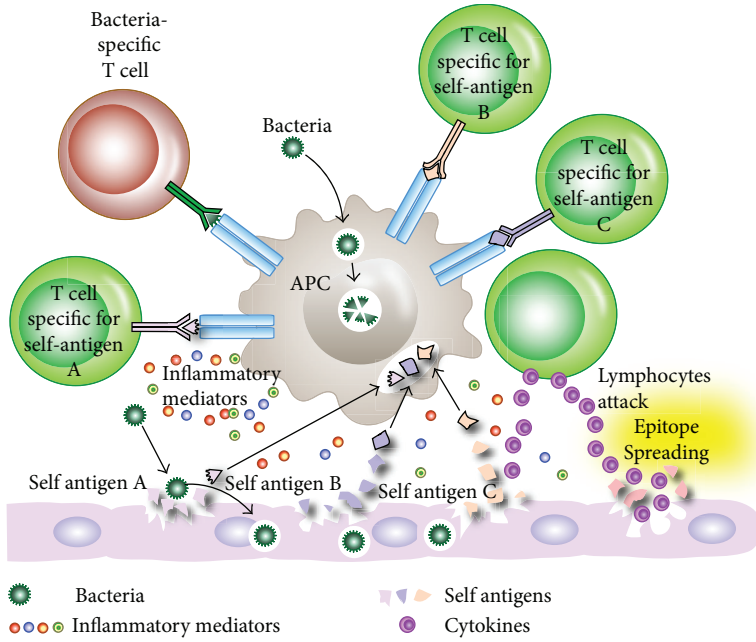

Figure 6: Bacterial infection induces release of tissue antigen and presentation of bacterial and self-tissue antigens resulting in the induction of autoreactive T cells. T cells and inflammatory mediators cause the release of more self-antigens which differ from the original antigens. T-cell responses can then spread to involve T cells specific to other self-antigens. This T-cell response against different epitopes results in antibody production against multiple tissue antigens.

infection-induced autoimmunity through epitope spreading is shown in Figure 6.

4.1.3. Bystander Activation and Stimulation of Pattern Recognition Receptors. Bystander activation occurs when viral antigens stimulate toll-like receptors and other pattern recognition receptors become activated in the inflammatory environment [72]. This activation of receptors on an antigen-presenting cell (APC) causes the release of proinflammatory cytokines which can induce tissue damage and the release of hidden antigens (Figure 7). The release of tissue antigens can activate autoreactive T cells that initially were not involved in the immune reactivity against the original infection [20]. Additionally, virally infected APCs and the concomitantly released mediators are able to activate autoreactive Th1 or Th17 cells in a bystander manner. Upon recognition of virally infected tissue cells, viral-specific T cells then release cytotoxic granules such as granzymes and cytokines such as TNF-α, IL-17, lymphotoxin, and nitric oxide. This inflammatory environment can lead to the bystander killing of uninfected neighboring cells. Microbial superantigens can induce a broader form of bystander activation by cross-linking MHC class II molecules to TCRs on APCs and T-cell activation (Figure 8). T cells that are stimulated in this manner may contain a subset recognizing specific tissue antigen [73]. Examples of superantigens are staphylococcal antigens, mycoplasma antigens, enteric-microbiota LPS, EBV, retrovirus, and many heat shock proteins. Some of these superantigens do not cause autoimmune disease but are involved in the exacerbation of EAE, arthritis, IBD, and other disorders [1].

4.1.4. Persistent Infection and Polyclonal Activation of B Cells. In many autoimmune diseases, such as lupus, RA, type 1 diabetes, and MS, B-cell functions are closely correlated with disease activity. Antibodies produced by B-cell-derived plasma cells contribute significantly to disease pathogenesis [69]. In these and other disorders, prolonged infectivity with a virus such as EBV, viral proteins, or viral genomes can lead to autoimmunity by the constant activation and proliferation of B cells.

After a long period of polyclonal B-cell activation, sometimes monospecific clones can emerge, accompanied by very high levels of antibody production and the formation of circulating immune complexes. Finally, this mixture of polyclonal antibodies and immune complexes may cause the autoimmune disease [74], as shown in Figure 9.

5. Dietary Components and Autoimmunities

It is undeniable that the diet of the industrialized and urbanized parts of the world today is vastly different from what it was even two or three decades ago, with a whole new range of novel food experiences that come from new food component sources, new breeds of food plants and food animals, genetic modifications, chemical ingredients, flavors, and preservatives. Over recent decades, a significant increase in the incidence of autoimmune diseases such as diabetes and MS in industrialized countries has led to the postulation that diet is a potential environmental risk factor

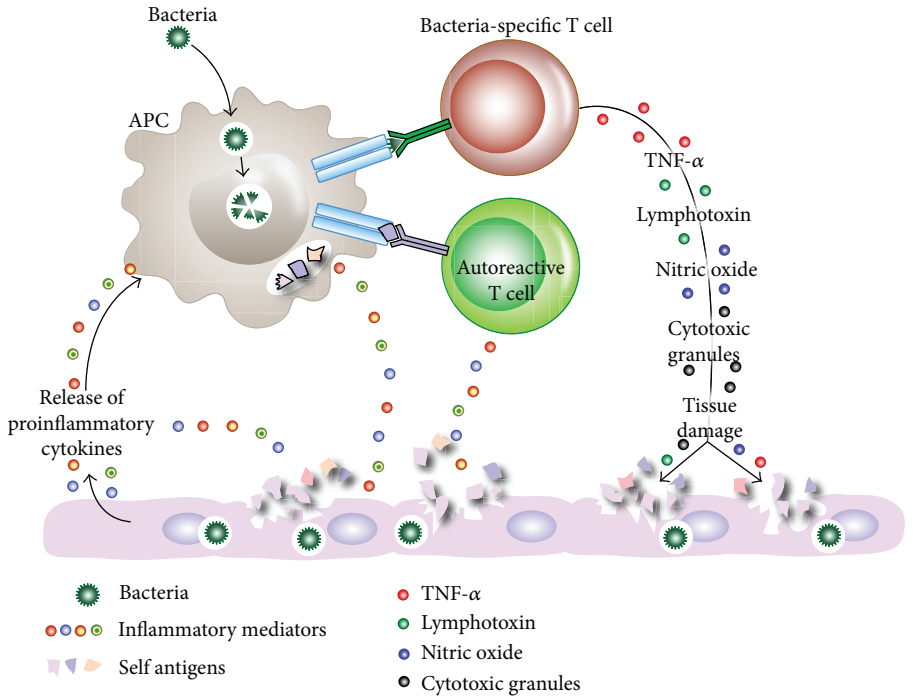

Figure 7: Microbial infection stimulates toll-like receptors (TLRs) and other pattern recognition receptors on antigen-presenting cells (APCs), leading to the production of proinflammatory mediators, which in turn can lead to tissue damage. The release of both tissue antigens and bacterial antigens results in bacterial-specific T cells and autoreactive T cells in the process called bystander activation, which contributes to autoimmunity.

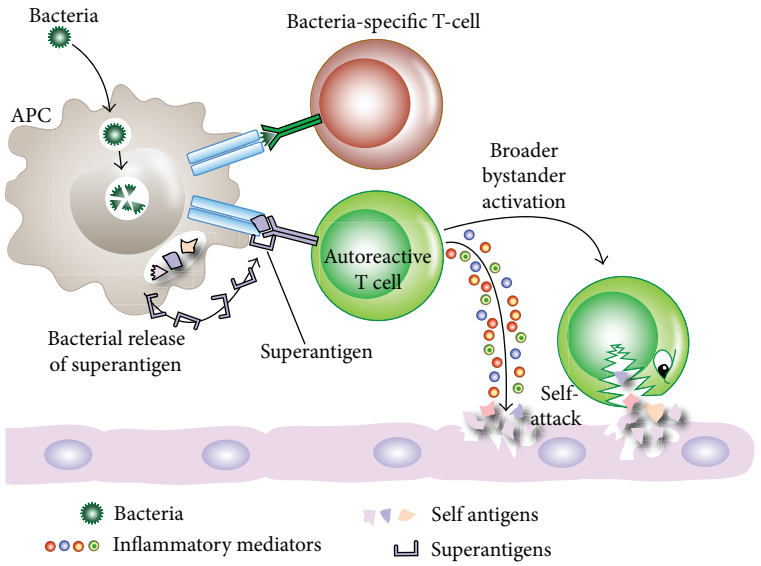

Figure 8: Superantigens and autoimmunity. Infection can lead to the release of superantigens, which can cross-link between MHCII and TCR, causing broader bystander activation, some of which may be specific for self-antigens, leading to attack on self-tissues.

for such disorders. The link between gluten ingestion and gluten sensitive enteropathies is already well established and accepted [3]. High levels of dietary sodium are associated with raised blood pressure and adverse cardiovascular health [75] and have been shown to affect the immune system [76]. Low levels of vitamin D have been linked with MS, systemic lupus erythematosus (SLE), RA, and other autoimmune disorders [3]. Lactose intolerance is no laughing matter for those afflicted with it or other milk-related disorders. The pleasures of a modern diet unfortunately come with caveats and unexpected catches that urgently need investigation.

5.1. Sodium Chloride in Diet and Autoimmune Diseases. For the past five decades various studies have been conducted on

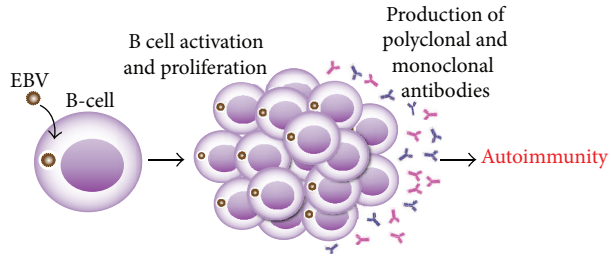

Figure 9: Infections, B cells, and autoimmunity. Prolonged infection with a virus, such as EBV, can lead to constant activation and proliferation of B cells, resulting in the production of monoclonal and polyclonal antibodies as well as immune complexes, causing autoimmune disease.

the comparative sodium intake levels in different countries [75, 76]. Animal experiments, epidemiological studies, and clinical trials have provided convincing evidence for the detrimental effect of sodium intake on blood pressure (BP), coronary heart disease, and stroke, as well as noncardiovascular diseases [77–81]. These comparative studies have shown that generally the simpler and less modernized a society and culture are, the lower the sodium intake is, with a concomitant lessening of the associated disorders. Understandably, the high salt content of the modern Asian diet is known worldwide, particularly the use of soy sauce as a seasoning [75, 82]. Indeed, in comparison to home-made meals, the salt content of fast foods can be many times higher [75]. The concentration of Na^+ in plasma similar to standard culture medium is about 149 mM. The consumption of high-salt processed foods may increase this concentration to a higher level and result in a change in physiological conditions. It has been theorized that the consumption of processed foods containing high amounts of salt may in part be responsible for the increasing incidence of autoimmune diseases. In a recent study it was demonstrated that an excess uptake of salt can affect the innate immune system, in particular, macrophage function [83]. However, until very recently little was known about increased NaCl intake, its direct effect on the T-helper cell populations, and the connection of all this to autoimmune diseases. Upon stimulation of the T-cell receptor and the cytokine environment, the naïve $CD4^+$ T cell can differentiate into functionally distinct effector cell subsets. This differentiation is also driven by key transcriptional regulators such as T-bet for Th1, GATA binding protein-3 for Th2, FOXP3 for Th3, retinoid acid receptor-related orphan receptor gamma t (RORγt) for Th17, and transcriptional regulator B-cell lymphoma 6 (BCL6) for T-follicular-helper (TFH) cells [84].

Among these $CD4^+$ T-cell subsets, the IL-23-dependent IL-17-producing $CD4^+$ helper T cells play a pivotal role in autoimmune disease [85]. Adding salt to the wound of complex autoimmune diseases, it has been shown that sodium chloride can drive autoimmune disease by the activation or induction of pathogenic Th17 cells [86]. These elegant experiments were conducted in a culture medium containing an additional 10–40 mM concentration of salt, mimicking animals fed a high-salt diet. Increased NaCl concentrations markedly induced the conversion of naïve $CD4^+$ T cells to $CD4^+$ T cells expressing IL-17A (Figure 10). This effect was dose dependent, and the optimum IL-17A induction was achieved by increasing the concentration of NaCl by 40 mM. Moreover, the authors demonstrated that a high-salt diet could accelerate neuropathology in a mouse model of multiple sclerosis through cellular signaling pathways involving transcription factor NFAT5, the protein kinase enzyme P38, and salt-sensing kinase SGK1. In comparison with the controls, mice on the high-salt diet not only displayed a much higher number of infiltrating $CD3^+$ and $MAC3^+$ cells but also almost doubled the number of $CD4^+$ T cells expressing IL-17A or pathogenic Th17 cells [86, 87]. This effect of the high-salt diet was specific for Th17 conditions, since the high salt levels did not significantly alter cell death, lymphocyte proliferation, or enhancement of Th1 or Th2 differentiations. The mechanism by which a high-salt diet enhances the differentiation of naïve $CD4^+$ cells to pathogenic Th17 cells is shown in Figure 11.

Extracellular NaCl concentration through the activation of IL-23 receptor and its binding by IL-23 influences the activity of SGK1 and NFAT5 which drives the expression of transcription factor RORγt, IL-23R, IL-17A, and IL-17F resulting in the phenotype switch from naïve $CD4^+$ T cells to pathogenic Th17 cells in MS, psoriasis, and other autoimmune disorders (Figure 12). The data presented in these manuscripts [86, 87] clearly indicate that high intake of sodium potentiates pathogenic Th17 cell generation in *in vitro* and *in vivo* systems in an SGK1-dependent manner and, therefore, has the potential of increasing the risk of promoting autoimmune diseases. Moreover, the elevated *in vivo* Th17 resulting from a high-salt diet raises the important question of whether or not increased salt in westernized diets and in processed foods contributes to an increased generation of pathogenic Th17 cells and towards an unprecedented increase in autoimmune diseases [87].

Thus, as indicated, dietary salt is just one of many dietary components that can influence T-helper cell differentiation and the development of autoimmune disease. The effect of other dietary nutrients, for example, vitamins, and other diverse environmental factors on metabolism and microbiota should also be investigated [88].

When this information is taken together with the strong and consistent evidence that implicates high salt intake with high BP and other cardiovascular disorders, it is alarming to note as laid out in all these studies that most adult populations have daily salt intakes well over the recommended US daily

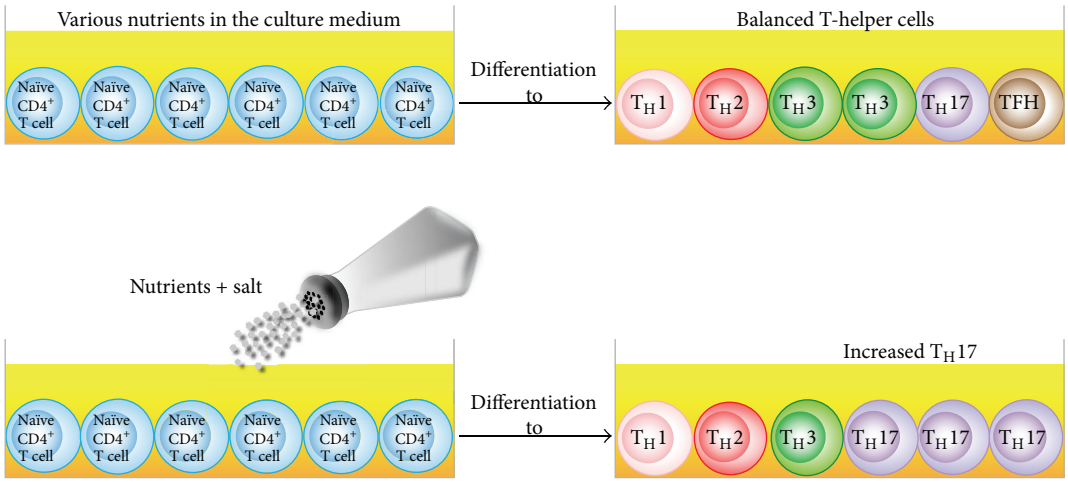

FIGURE 10: Salt affects the differentiation of naïve $CD4^+$ cells. Increased concentrations of salt resulted in the differentiation of naïve $CD4^+$ T cells into a greater number of T_H17 cells.

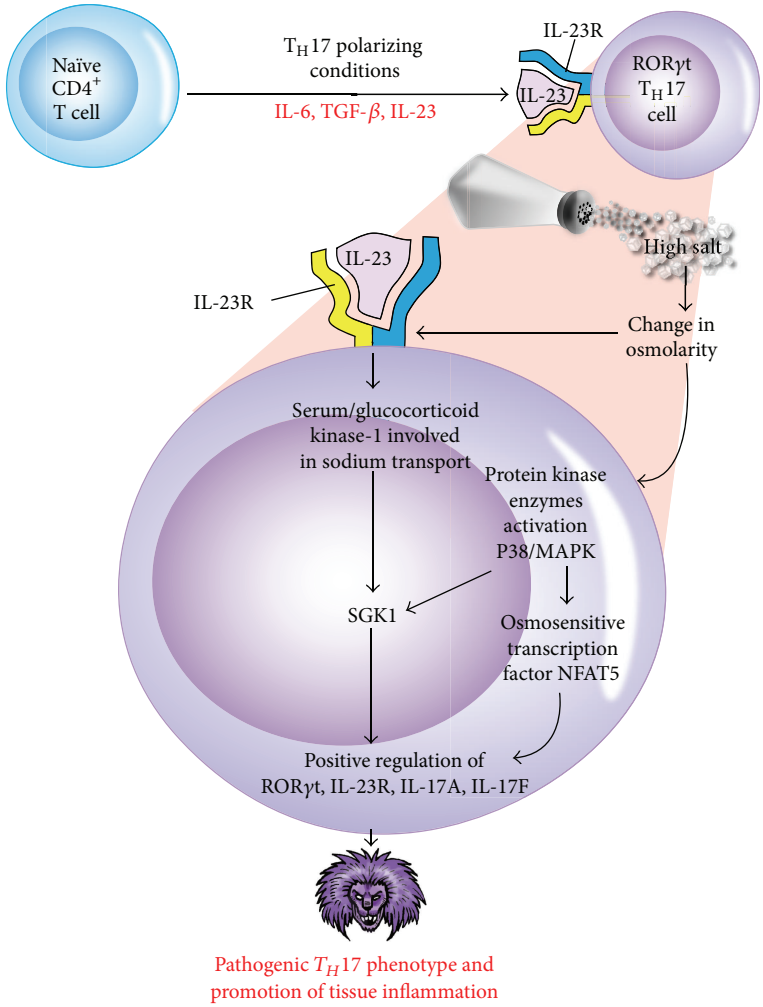

FIGURE 11: Mechanism by which a high-salt diet enhances the differentiation of naïve $CD4^+$ cells to pathogenic T_H17 cells that may exacerbate experimental autoimmune encephalitis. High salt concentration, change in osmolarity, the influence of IL-23 and IL-23 receptor signaling, and the activation of various enzymes drive the expression of T_H17-associated cytokines and the formation of pathogenic T_H17 phenotype.

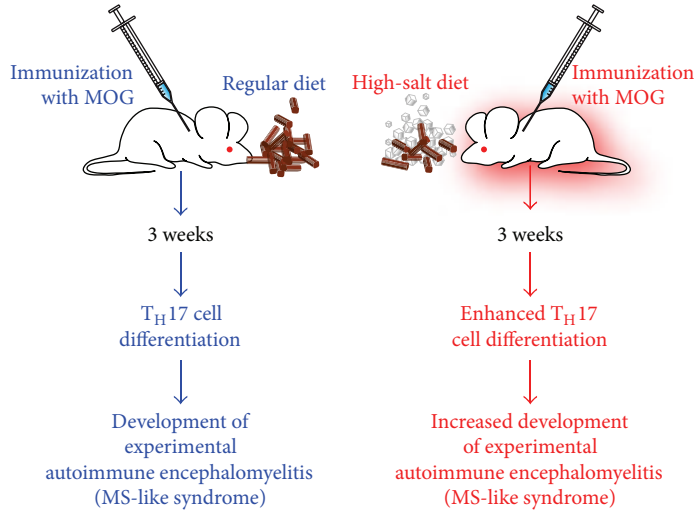

Figure 12: High-salt diet increases risk of autoimmune disease. In two groups of mice, both of which were immunized with MOG to induce EAE, the mice that had been given a high-salt diet (HSD) showed enhanced differentiation of naïve T cells into pathogenic T_H17 cells and a subsequent increased, more profligate development of EAE.

level of 1.5 g/day for middle-aged and older adults [89]. On the face of it a voluntary decrease in salt consumption seems to be an easy policy to implement, but good sense and good health face the formidable opposing forces of flavor, habit, and culture.

5.2. The Role of Milk and Wheat Components in Autoimmune Diseases. In relation to dietary proteins it has been well established that different proteins and peptides in milk and wheat are involved in autoimmune diseases [90–93]. Milk contains more than 400 different proteins, most of which have over 150 amino acids (AA). AA that mimic collagen may induce RA, while those that mimic neural cell antigens may induce multiple sclerosis or other neuroimmune disorders.

For example, a study reports that, as a consequence of immunological cross-reactivity or molecular mimicry between the extracellular IV-like domain of the milk protein butyrophilin and myelin oligodendrocyte glycoprotein (MOG), butyrophilin can modulate the encephalitogenic T-cell response to MOG in experimental autoimmune encephalitis [92]. Epidemiological and ecological investigations suggest that early infant nutrition, particularly drinking cow's milk, may induce autoimmunity, leading to type 1 diabetes. This autoimmune reactivity is due to cross-reactivity of cow's milk, particularly its albumin component, with islet cell antigen-1 and beta cell surface protein. These studies suggest that dysregulation of oral tolerance triggers a cellular and humoral immune response against various components of milk proteins, and cross-reaction with B-cell molecules may result in autoimmunity [94–97]. In association with various autoimmune disorders, wheat proteins and, more specifically, gluten, have received significant attention [98–100]. Indeed, it has been demonstrated that a wheat-based diet induces not only Th1-type cytokine bias in the gut but also increased T-cell reactivity to gluten, with a higher frequency of diabetes [99–101]. In addition to diabetes, it has been shown that celiac disease (CD) is associated with various extraintestinal autoimmune disorders that involve the thyroid, joints, heart, skin, pancreas, bone, liver, reproductive organs, and the nervous system [102–112].

Although the exact mechanisms for the induction of these autoimmunities are not definitely known, there is a growing body of evidence indicating that these diseases may result from molecular mimicry between gliadin or transglutaminase and various tissue antigens, including nervous system proteins [33–35, 68]. Interestingly, the celiac peptide VVKVGGSSSLGW shares more than 30% homology with the trangslutaminase peptide 476–487 (RIRVGQSMNMGS) [113]. Therefore, antibodies generated against transglutaminase in the intestine can bind to extraintestinal tissues such as those of the liver, pancreas, lymph nodes, muscle, heart, and brain [114–117]. Very recently, we used both affinity-purified and, monoclonal antibodies against α-gliadin 33-mer peptide to examine the cross-reaction between gliadin with different food and tissue antigens [91]. We observed significant immune reactivity when these antibodies were applied to cow's milk, milk chocolate, milk butyrophilin, whey protein, casein, yeast, oats, corn, millet, instant coffee, and rice. With regard to the reaction of α-gliadin antibody with various tested tissue antigens, the most significant binding occurred with asialoganglioside, hepatocyte, glutamic acid decarboxylase 65, adrenal 21-hydroxylase, and various neural antigens [92].

These studies collectively indicate that circulating antibodies present in patients with nonceliac gluten sensitivity (NCGS) and CD interact with different food antigens and transglutaminases in various tissues, which may induce the formation of antigen-antibody aggregates that can trigger the activation of the inflammatory cascade.

While most studies about the implications of cross-reactivity with various autoimmunities are limited to milk

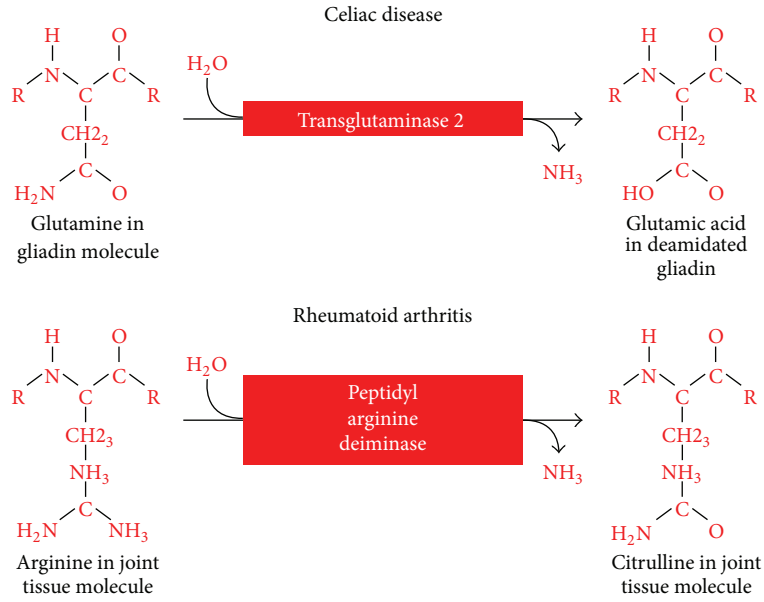

FIGURE 13: The central role of catalytic enzymes in celiac disease and rheumatoid arthritis. Key enzymes that catalyze the modification of glutamine to glutamic acid or arginine to citrulline as new epitopes have a central role in CD and RA.

and wheat, a thorough investigation and understanding of the immunologic cross-reactivity of other food proteins and peptides are essential for advancing our knowledge about the involvement of these dietary components in the development of many autoimmune disorders. Finally, the identification of triggers of autoimmunity can be used in the development of new therapies for autoimmune diseases.

6. Using Gluten Sensitivity, Celiac Disease, and Oral Pathogens to Understand Autoimmunities

There is a lot that clinicians can learn about autoimmune diseases from looking at gluten sensitivity and celiac disease. Some of the features of CD HLA-DQ2/DQ8 association, target organ (villi) T-cell infiltration, and disease-specific autoantibodies produced against modified antigens such as deamidated gliadin and deamidated gliadin-transglutaminase complex [99, 116] are paralleled in chronic joint disorder [94, 118]. These observations suggest that it might be feasible to use CD to identify disease-relevant epitopes in RA and other autoimmune disorders, such as type 1 diabetes and multiple sclerosis [100]. For example, the key enzymes that catalyze the modification of glutamine to glutamic acid or arginine to citrulline as new epitopes have a central role in CD and RA (Figure 13).

It is interesting to note that this process of arginine deamination and the formation of citrullinated proteins and peptides in the joint or other tissues could be potentiated by oral pathogens such as *Porphyromonas gingivalis* [40, 119–124] (Figure 14). That is why RA can also cause inflammation in other organs, including the skin, lungs, heart, and peripheral nerves, often with serious consequences.

This is only one observation suggesting that environmental triggers can change self-tissue antigens to become disease-associated T-cell epitope, resulting in antibody production against the citrulline-containing new epitope. Therefore, if CD as an autoimmune disorder is driven by transglutaminase-2 and deamidated gliadin, then we may state that RA is caused by environmental factors, such as *P. gingivalis* or EBV. These environmental factors, by causing the formation of various citrullinated self-epitopes such as collagen type II, fibrin, vimentin, keratin, α-enolase, and filaggrin, are involved in the induction of RA [119–125]. Experience with CD has taught scientists that genes, environmental factors, and target tissue antigens are all important issues for consideration in understanding the molecular structure of epitopes recognized by T cells and B cells within the inflamed target organ [100, 124].

6.1. Mechanism Involved in the Induction of Autoimmunity by Oral Pathogen. A similar mechanism applies to the autopathogenic correlation of periodontitis induced by *P. gingivalis* and RA [40, 119, 124]. It is possible that both diseases share a common aetiopathogenic background [124]. This mechanism includes the posttranslation modification or citrullination of bacterial proteins and self-antigens simultaneously, generating neoepitope structure. This can result in a breakdown in self-tolerance and antibody production against citrullinated bacterial antigens as well as citrullinated host proteins [125]. One such antigen is α-enolase, which features significant homology between human and bacterial α-enolase. Therefore, antibodies produced against citrullinated bacterial α-enolase will react with human α-enolase, and antibodies produced against human citrullinated α-enolase will react strongly against bacterial α-enolase. For this

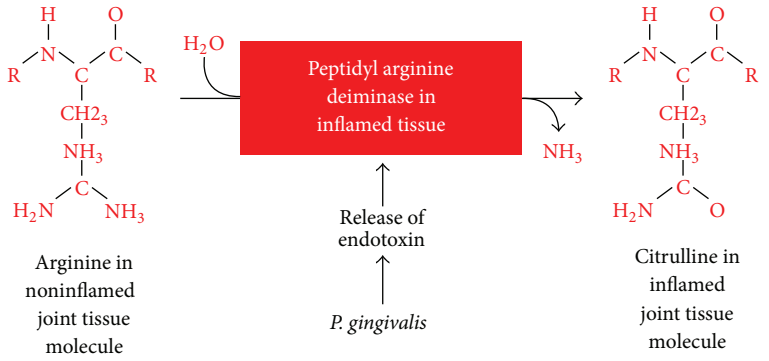

Figure 14: Potentiation by oral pathogens. Oral pathogens such as *P. gingivalis* can potentiate the deamination of arginine or formation of citrullinated proteins and peptides in joint and other tissues.

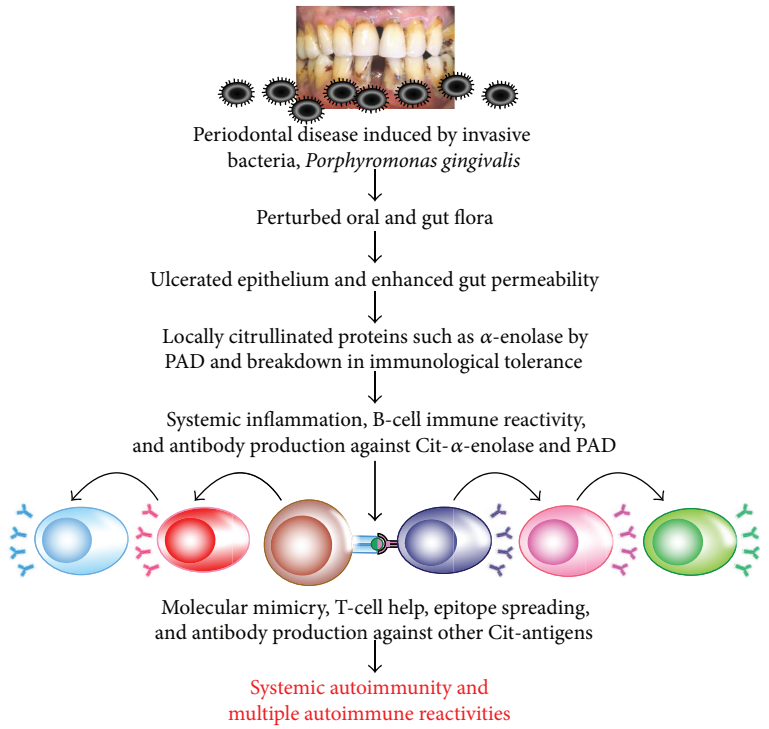

Figure 15: Proposed model for the pathogenesis of multiple autoimmune reactivities by infection. Bacterial generation of autoantigens, local inflammation generating autoantigens by PAD, antibody production against one autoantigen, epitope spreading, antibody production against multiple antigens, systemic inflammation, and multiple autoimmune reactivities.

reason, elevated levels of α-enolase antibodies are detected in the synovium of 60% of patients with RA [122]. Indeed, immunological mapping using a library of cyclic citrullinated α-enolase peptides led to the identification of a B-cell-dominant epitope comprising amino acids 5–21 of α-enolase (KIHAREIFDSRGNPTVE) where arginine-9 and arginine-15 are citrullinated, with an 82% sequence similarity with that of *P. gingivalis* [126, 127]. Immunization with citrullinated human and *P. gingivalis* α-enolase and citrullinated fibrinogen causes similar pathology in humanized DR4 transgenic mice. This mechanism may be the common denominator between autoimmunity and cardiovascular disease. These findings suggest that, by mimicking the molecular structure of host-citrullinated proteins, *P. gingivalis* peptidylarginine deiminase-citrullinated bacterial α-enolase could trigger a loss of tolerance to structurally similar host proteins, resulting in expression of anti-citrullinated protein antibodies and the development of RA [128, 129].

These antibodies can be detected up to 10 years before the clinical onset of RA and the production of IgM antibodies against IgG (called rheumatoid factor) in the majority of patients.

In the joint, the specificity of anti-citrullinated peptide is enhanced through epitope spreading to other citrullinated autoantigens such as fibrinogen, collagen, filaggrin, and vimentin (see Figure 15).

7. Conclusion

Putting all this information together, it appears that there are common mechanisms in the immunopathogenesis of multiple autoimmune reactivities. In genetically susceptible individuals, environmental triggers such as xenobiotics and *P. gingivalis* can, respectively, induce the formation of neoantigens, or be capable of inducing the citrullination of host proteins and converting them to autoantigens. These modified proteins can be recognized by the immune system, triggering antibody production and the inflammatory process involved in the clinical manifestations of autoimmune diseases.

To optimize the chances of therapeutic success it is essential to identify the environmental triggers first and then attempt to remove them from the patient's environment (e.g., toxic chemicals and food associated with autoimmunities). In the case of infections, this also helps to guide the clinical use of various medications which are now often used for prophylaxis. Therefore, careful monitoring for the presence of infections in the patient's blood or tissue will be desirable for monitoring the effects of the drug therapy [40, 119–124].

Manipulation of environment triggers and the host immune system during the clinical and in particular preclinical stages of autoimmune disease will offer significant insight and guide early intervention for many autoimmune disorders that, according to the American Autoimmune Related Disease Association, Inc. (http://www.aarda.org/), affects approximately 10% of the world's population [130], while others put it as high as 20%. Finally, identification of triggers of autoimmunities could be used in the development of new therapies for autoimmune diseases.

Conflict of Interests

Aristo Vojdani, PhD, MSc, CLS is the owner, CEO and technical director of Immunosciences Lab., Inc., a clinical reference testing laboratory in Los Angeles, CA, USA.

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Hindawi Publishing Corporation
Autoimmune Diseases
Volume 2014, Article ID 452853, 12 pages
http://dx.doi.org/10.1155/2014/452853

Review Article

The Role of Decay Accelerating Factor in Environmentally Induced and Idiopathic Systemic Autoimmune Disease

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Received 10 October 2013; Accepted 19 November 2013; Published 27 January 2014

Academic Editor: Aristo Vojdani

Decay accelerating factor (DAF) plays a complex role in the immune system through complement-dependent and -independent regulation of innate and adaptive immunity. Over the past five years there has been accumulating evidence for a significant role of DAF in negatively regulating adaptive T-cell responses and autoimmunity in both humans and experimental models. This review discusses the relationship between DAF and the complement system and highlights major advances in our understanding of the biology of DAF in human disease, particularly systemic lupus erythematosus. The role of DAF in regulation of idiopathic and environmentally induced systemic autoimmunity is discussed including studies showing that reduction or absence of DAF is associated with autoimmunity. In contrast, DAF-mediated T cell activation leads to cytokine expression consistent with T regulatory cells. This is supported by studies showing that interaction between DAF and its molecular partner, CD97, modifies expression of autoimmunity promoting cytokines. These observations are used to develop a hypothetical model to explain how DAF expression may impact T cell differentiation via interaction with CD97 leading to T regulatory cells, increased production of IL-10, and immune tolerance.

1. Introduction

Decay accelerating factor (DAF) was first described in 1969 in human erythrocytes that inhibited complement activation *in vitro* [1]. (The gene and protein designations used for decay accelerating factor in this paper are *DAF* for the human gene and DAF for the human protein. The mouse genes are *Daf1* and *Daf2* and the protein is DAF1.) However, its biological significance was not appreciated until 1982 when the human protein was isolated and deficiency of DAF was found in patients with paroxysmal nocturnal hemoglobinuria (PNH) [1–3]. The major function of DAF is to protect self-cells from complement-mediated attack by inhibiting the cleavage of C3 and C5, blocking the formation of C3 and C5 convertases, and accelerating their decay [4]. In humans, DAF is expressed as a posttranslationally modified glycosylphosphatidylinositol-(GPI-) anchored molecule [5, 6]. In mice, functionally equivalent, GPI-anchored, and transmembrane-anchored DAF proteins are produced, which are derived from two different genes, *Daf1* and *Daf2*, respectively [7]. *Daf1* is ubiquitously expressed, whereas, *Daf2* is mostly present in the testis and splenic dendritic cells [8]. DAF is also found in soluble form in plasma, cerebrospinal fluid, saliva, synovial fluid, and urine [9]. In humans, *DAF* is encoded by a single gene which maps to q32 on chromosome 1 [10]. It is widely expressed on the surface of all major circulating blood cells as well as epithelial and endothelial cells [9, 11]. Constitutive expression can vary depending on tissue and cell type [8, 12]. In human cells, *DAF* expression is modulated by cytokines such as IL-1, IL-6, TNF-α, TGF-β1, and IFN-γ, prostaglandins, and tissue-specific factors and controlled by the transcription factor SP1 [13–17]. In 1988, DAF was found to be rapidly expressed in T-cells

activated by mitogens and *in vitro* stimulation with anti-DAF antibodies led to phosphatidylinositol-specific phospholipase C dependent T-cell proliferation [18]. This led to the hypothesis that an alternative function of DAF may be to regulate T-cell tolerance. Subsequently, DAF has been shown to negatively regulate a variety of autoimmune diseases including animal models of antiglomerular basement membrane glomerulonephritis, experimental autoimmune myasthenia gravis (EAMG), experimental autoimmune encephalomyelitis (EAE), cardiac allograft rejection, and idiopathic and induced models of systemic lupus erythematosus (SLE) [19–24].

2. Complement System and DAF

The complement system is among the oldest evolutionary components of the immune system. It was discovered in 1896 as a heat-labile fraction of serum that led to opsonization of bacteria. Biochemical characterization showed that the complement system is composed of over 30 proteins that function to mediate removal of apoptotic cells and eliminate pathogens. Three separate pathways (i.e., classical, alternative, and lectin pathways) converge to convert C3 to C3 convertase, an enzyme capable of initiating a cascade that results in cell membrane pore formation and subsequent cell lysis known as the membrane attack complex (MAC) (Figure 1). To protect host cells from complement activation four plasma membrane complement regulatory proteins are expressed, CD59 (membrane inhibitor of reactive lysis (MIRL)), CD35 (type 1 complement receptor (CR1)), CD46 (membrane cofactor protein (MCP)), and CD55 (decay accelerating factor (DAF)), that interrupt the complement cascade on self-cells. CD59 blocks MAC complex formation [25], CD35 acts as a cofactor to inactivate C3b and C4b by factor I and interacts with C3b and C4b to promote immune-complex removal [9], and CD46 acts as a cofactor to inactivate C3b and C4b through factor I [9]. DAF inhibits the cleavage of C3 and C5 by blocking the formation of C3 and C5 convertases and accelerating their decay [4]. The original premise of the complement system as a member of the innate immune system, however, was redefined three decades ago when it was shown that complement participates in B- and T-cell responses especially the induction and regulation of type I helper (T_h1) $CD4^+$ T-cell responses [26].

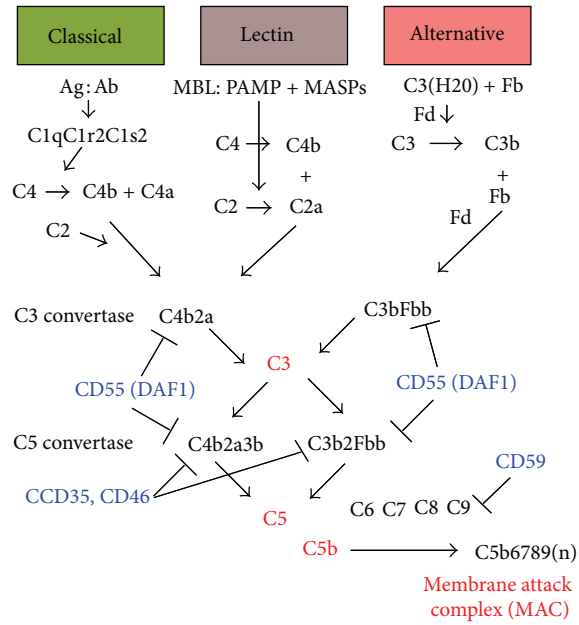

Figure 1: Overview of the complement system. Stimulated by antigen : antibody complexes, bacterial cell surfaces, and spontaneous hydrolysis, respectively, the classical, lectin, and alternative pathways converge to convert C3 to C3 convertase, an enzyme capable of initiating a cascade that results in cell membrane pore formation and subsequent cell lysis known as the membrane attack complex (MAC). To protect host cells from complement activation four main plasma membrane complement regulatory proteins are expressed, CD59 (membrane inhibitor of reactive lysis (MIRL)), CD35 (type 1 complement receptor (CR1)), CD46 (membrane cofactor protein, (MCP)), and CD55 (decay accelerating factor (DAF)), that interrupt the complement cascade on self-cells. CD59 blocks MAC complex formation, CD35 acts as a cofactor to inactivate C3b and C4b by factor I, and interacts with C3b and C4b to promote immune-complex removal, CD46 acts as a cofactor to inactivate C3b and C4b through factor I and DAF inhibits the cleavage of C3 and C5 by blocking the formation of C3 and C5 convertases and accelerating their decay. MBL: mannose-binding lectin; MASPs: MBL-associated serine proteases; PAMP: pathogen-associated molecular pattern; Fb: factor B.

3. DAF in Human Autoimmune Diseases

Deficiency of DAF is found in PNH patients, a GPI linked protein deficiency, which leads to systemic complications particularly through intravascular hemolysis and platelet activation [27–30]. Interestingly, however, it has been reported that DAF (CD55) deletion, also known as the Inab phenotype, hardly alters the sensitivity of cells to lysis by complement and only with inhibition of CD59 does hemolysis occur *in vitro* [31–34]. Deficient expression of the complement regulatory proteins CD55 and CD59 has been found in a variety of human diseases (Table 1) [35–61], however, most prevalently in autoimmune hemocytopenia, autoimmune vasculitis, and other diseases involving dysregulated immune responses [41, 44, 62–68]. It is important to note that deficient expression seen in these diseases does not always mimic the PNH pattern of deficiency on specific cell populations but instead shows decreased expression of all cells, suggesting the nonclonal nature of the deficiency [44].

A paradox exists in SLE where complement activation is associated with tissue injury, yet deficiencies in the early classical complement component pathways predispose to SLE [69, 70]. C1q deficiency due to either gene deletion or anti-C1q autoantibodies has been shown to be related to disease activity; in fact between 53 and 93% of SLE patients have been reported to have low C1q during active disease [71]. This has been reconciled by studies demonstrating the protective role of classical complement pathway components (C1, C2, and C4) in facilitating the clearance of immune complexes as well as autoantigens in apoptotic debris [70, 72]. However,

Table 1: Decreased DAF expression in human disease.

Disease	Cell surface DAF deficiency	Citation
Immune dysregulation		
Sjogren's Syndrome	T-lymphocytes	[36]
SLE with lymphopenia and anemia	T-lymphocytes (CD8+), endothelium, lymphocytes, and anemia	[38, 41, 73]
Psoriatic skin	Epithelium and endothelium	[39]
Systemic sclerosis	Endothelium	[37]
Vasculitic skin lesions	Endothelium	[35]
Recurrent pregnancy loss in aPL	Endometrium	[42]
Autoimmune hemocytopenia	Platelets, lymphocytes, and RBC	[43, 44]
Rheumatoid arthritis	Neutrophils and RBC	[45, 46]
Myasthenia gravis	SNP with decreased expression	[47]
Vitiligo	Whole epidermis	[48]
Asthma	Bronchial epithelial cells and SNP	[49, 50]
Proliferative disorders		
Myelodysplastic syndrome	Granulocytes and RBC	[51, 52]
Plasma cell dyscrasias	RBC	[53]
Lymphoproliferative disorders	RBC	[54]
Anemias		
Anemia of malaria	RBC	[55–59]
Aplastic anemia	RBC	[51]
HIV	RBC, lymphocytes, and PBMC	[60, 61]

RBC: red blood cell; SNP: single-nucleotide polymorphism; aPL: antiphospholipid; PBMC: peripheral blood mononuclear cell.

decreased complement regulatory components on lymphocytes and erythrocytes, including CD55 and CD59, have been shown to be associated with lymphopenia and autoimmune hemolytic anemia (AHIA). In 2003, it was shown that AIHA patients with SLE had decreased levels of DAF compared to SLE patients without AIHA but similar levels to patients with AIHA but without SLE [73]. No correlation was seen, however, between IgG or IgM antiphospholipid antibodies [73]. In a follow-up study an analysis of 40 SLE patients with and without lymphopenia showed that mean fluorescence intensities (MFI) of CD55 and CD59 were diminished on T- and B-cells in lymphopenic patients compared to nonlymphopenic patients [41]. These results were unrelated to disease activity [41]. In two recent studies Alegretti et al. conducted a peripheral blood flow cytometric analysis of SLE patients and healthy controls [67, 68]. They found a decrease in the percent of DAF high peripheral lymphocytes and decreased DAF MFI in peripheral lymphocytes in patients with lymphopenia; however, no difference was seen in non-lymphopenic SLE patients compared to controls [67, 68]. Mean DAF levels were also shown to be decreased on red blood cells and granulocytes but not on monocytes; however, no relationship between disease activity and DAF lymphocyte levels was seen [67, 68]. Collectively, these studies suggest a role for complement regulatory proteins in the pathophysiology of a subset of SLE patients with lymphopenia and AIHA. The most plausible explanation is antibody-dependent cellular cytotoxicity and complement-mediated cell lysis which would explain the correlation between reduced CD55 and CD59 in AIHA and lymphopenic SLE populations. An alternative hypothesis that decreased CD55 lymphocyte levels predispose a subset of SLE patients to lymphopenia should not be ignored.

Not nearly as many studies have investigated the relationship between DAF and other systemic autoimmune diseases. One study found that percentages of DAF negative $CD4^+$ and $CD8^+$ T-cell subsets were higher in Sjogren's Syndrome (SS) patients [36]. The DAF expression observed in other circulating blood cells was not changed [36]. However, these results were not thought to be due to increases in DAF low cells but rather a decrease in DAF high cells [36]. In patients with systemic sclerosis (Scleroderma or SSc), DAF was found to be decreased or undetectable in endothelium of both lesional skin and nonlesional skin compared to controls [37]. Interestingly this result was later reproduced in morphea lesions, suggesting a link between systemic autoimmune disease skin lesions and low DAF levels, although low DAF levels are also seen in psoriatic skin lesions [38, 39]. These studies, however, are limited by their power and lack of supporting follow-up studies.

4. DAF in Animal Models of Systemic Autoimmune Disease

DAF1 has been shown to play a role in the maintenance of immune tolerance in mouse models of autoimmune disease. Deletion of *Daf1* was shown to increase susceptibility to antiglomerular basement membrane disease and to markedly

enhance susceptibility in a mouse model of myasthenia gravis [20, 23]. This increased susceptibility was later shown to result from the influence of DAF1 on T-cell hypersensitivity, when it was demonstrated that *Daf1*$^{-/-}$ T-cells displayed a C3 dependent enhanced response to antigen restimulation resulting in increased IFN-γ [24, 74, 75]. Similar results were found in MRL-*Fas*^{lpr} mice, an idiopathic SLE model, where deletion of *Daf1* resulted in exacerbated lymphadenopathy and splenomegaly, increased serum antichromatin autoantibody, and aggravated dermatitis [21]. In a follow-up study it was shown that, aside from local skin inflammation, these effects were largely complement independent [76].

These observations stimulated us to investigate the role of DAF1 in murine mercury-induced autoimmunity (mHgIA). Mice exposed to mercury develop lymphadenopathy, hypergammaglobulinemia, humoral autoimmunity, and immune-complex disease, which are consistent with the systemic features observed in SLE [77, 78]. All forms of inorganic mercury tested, including $HgCl_2$, vapor, or dental amalgam, elicit the same disease [79–81] as do different routes of administration [82, 83]. Sensitivity to mHgIA is influenced by both MHC and non-MHC genes and covers the spectrum from nonresponsiveness to overt systemic autoimmunity [82, 84–86]. Disease expression is influenced by TCR costimulatory molecules including CD28 and CD40L [87, 88], proinflammatory cytokines, including IFN-γ [80, 89] and IL-6 [90], and modulators of innate immunity, including endosomal Toll-like receptors (TLR) [91, 92], demonstrating that multiple checkpoints and pathways are implicated in the regulation of the disease. In addition lupus prone strains exhibit accelerated and more severe systemic autoimmunity following mercury exposure [93–95]. Environmental factors have been associated with systemic autoimmune diseases in humans [96, 97]. Exposure to mercury has been implicated as an environmental trigger in the induction of autoimmunity [98–100] including production of autoantibodies and proinflammatory cytokines, such as IL-1β, TNF-α, and IFN-γ [101], and membranous nephropathy [102].

We found that autoimmune-prone NZB mice had low endogenous levels of DAF1 while mHgIA-resistant DBA/2 animals have high endogenous levels [103]. Furthermore, we showed that induction of mHgIA in B10.S mice was associated with reduction of DAF1 on activated CD4$^+$ T cells [103]. Both these observations, reduction of DAF1 in autoimmune-prone mice and reduction upon induction of mHgIA, support the argument that DAF1 is required to maintain immune tolerance. In a follow-up study the absence of *Daf1* in C57BL/6 mice was shown to cause increased serum autoantibodies and exacerbated hypergammaglobulemia following mercury exposure (Table 2) [19]. This response, however, could not be explained by increased T-cell activation but rather was explained by increased levels of IFN-γ, IL-2, IL-4, and IL-10 (Table 2) [19]. Furthermore, depletion of C3 was found to have no major effects on development of mHgIA suggesting that the role of DAF1 in mHgIA is independent of an intact complement system [104]. This is supported by results showing that C3 levels are not affected following mercury exposure of mHgIA sensitive or resistant strains (Pollard, unpublished results). Thus our findings and those of others [76] suggest that DAF1 may regulate idiopathic and induced models of systemic autoimmunity in a complement-independent fashion.

Table 2: Effect of *Daf1* deletion on mHgIA*.

Serum autoantibodies	
ANA	↑↑↑
AntiChromatin abs	↑↑↑
Serum immunoglobulins	
IgG	↑
IgG1	NC
IgG2a	NC
T-cell activation	
CD4$^+$CD44^{high}	NC
Cytokines	
IFN-γ	↑
IL-4	↑
IL-2	↑
IL-10	↑
IL-17	NC
IL-22	NC
TGF-β	NC

*Data from Toomey et al., 2010 [19].
↑: $P < 0.05$; ↑↑↑: $P < 0.0001$; NC: no change.

5. How Does DAF1 Regulate Immune Tolerance?

Several models of systemic autoimmunity exhibit disease independent of an intact complement system, yet an exacerbated phenotype is observed by DAF1 deletion [19, 21, 76, 104, 105]. This suggests that complement-independent effects of DAF1 are the major contributors to tolerance induction via the interaction of DAF (CD55) with its natural ligand, CD97. CD97 is a member of the epidermal growth factor-like, seven span transmembrane (EGF-TM7) family of proteins, is expressed on macrophages, granulocytes, dendritic cells, and smooth muscle cells, and is rapidly upregulated on activated T- and B-cells [106, 107]. In the mouse alternative RNA splicing produces two isoforms with three or four epidermal growth factor-like (EGF) domains (namely CD97(EGF1,2,4) and CD97(EGF1,2,3,4)) and a third isoform with a protein module inserted between EGF domains 2 and 3 (namely, CD97(EGF1,2,X,3,4)) [108]. DAF (CD55) binds to both CD97(EGF1,2,4) and CD97(EGF1,2,3,4) but not to CD97(EGF1,2,X,3,4) [108].

Structural studies of a model of the CD55-CD97 complex reveal that the sites of interaction of CD97 and complement components occur on opposite faces of CD55 arguing that CD55 can bind to CD97 and complement independently [106]. Activation of human CD4$^+$ T cells in the presence of anti-CD3 and recombinant CD97 (rCD97) results in increased IL-10 production that is IL-2 dependent [109]. Costimulation of naïve human CD4$^+$ T-cells via interaction

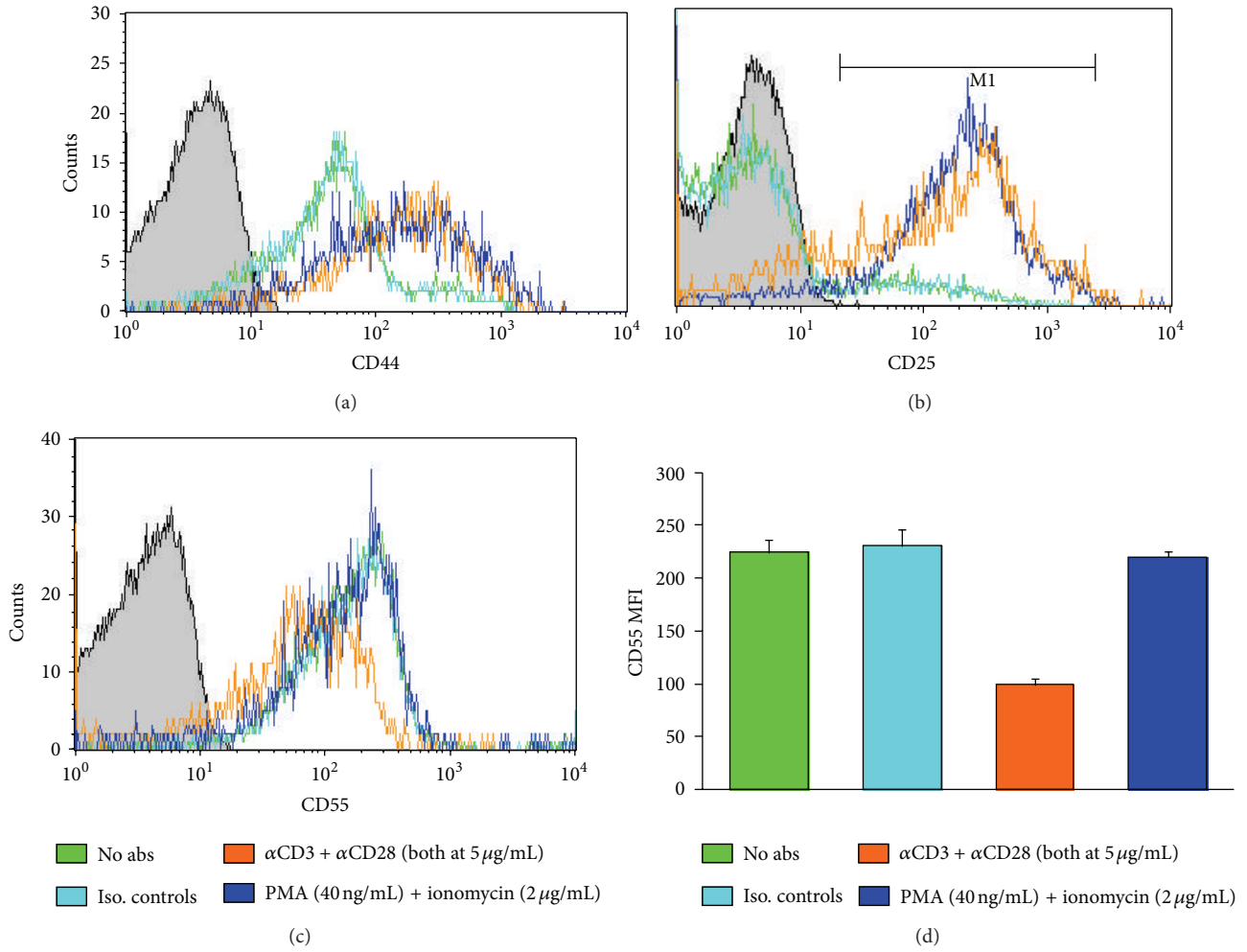

Figure 2: Anti-CD3/anti-CD28 but not phorbol 12-myristate 13-acetate (PMA)/ionomycin lowers CD55 expression of $CD4^+$ T-cells. Lymph node cells from B10.S mice were cultured with no additives (green), Ig isotype controls (light blue), anti-CD3/anti-CD28 (5 μg/mL each) (orange), or phorbol 12-myristate 13-acetate (PMA) (40 ng/mL) and ionomycin (2 μg/mL) (dark blue). Cells were incubated at 37°C and 5% CO_2 for 72 hr and then analyzed by flow cytometry to determine activation status ($CD44^{hi}$, $CD25^{hi}$) and then CD55 expression of activated cells. $n = 4$/group.

of CD97/CD55 leads to T regulatory type 1 (Tr1) activation, expansion, and function [110]. Antibody mediated blockade of CD97-CD55 interaction inhibits proliferation and IFN-γ production [106]. These properties of CD97-CD55 costimulation, particularly IL-2 dependent IL-10, are consistent with stimulation of human $CD4^+$ T cells by another complement regulatory protein, CD46. Activation via CD3/CD46 induces a T_h1 phenotype with significant IFN-γ production [111]. However in the presence of IL-2, immunoregulatory IL-10 is produced resulting in a T regulatory phenotype [111, 112] capable of suppressing antigen-specific T cells [113]. We hypothesize that the immunosuppressive potential of CD55 [24, 75] lies in its interaction with CD97 leading to a T regulatory cell phenotype under appropriate conditions of increasing IL-2, as would happen in an inflammatory response [114]. Thus, like CD46 activation, CD97 mediated activation of CD55 acts to switch T_h1 effector $CD4^+$ T cells toward IL-10 producing immunosuppressive cells [112]. We have shown that activation of murine $CD4^+$ T cells with anti-CD3 and rCD97 leads to a cytokine profile with increased IL-10 and reduced IL-17 and IL-21 compared to a more proinflammatory profile of elevated IFN-γ, IL-2, IL-4, IL-10, IL-17, and IL-21 elicited by conventional costimulation by anti-CD3/anti-CD28 [19]. Increased IL-10 and reduced IFN-γ and IL-17 suggest that rCD97 may be driving T cells to differentiate into T regulatory cells rather than autoimmune promoting T_h1 and T_h17 cells, but it remains to be determined whether rCD97 activated $CD4^+$ T cells have immunoregulatory activity.

Our previous studies had established that activated $CD4^+$ T cells have reduced CD55 expression [103, 115]. However it was unclear if conventional activation of $CD4^+$ T cells with anti-CD3/anti-CD28 could mimic the *in vivo* reduction of CD55 in idiopathic SLE or mHgIA [103].

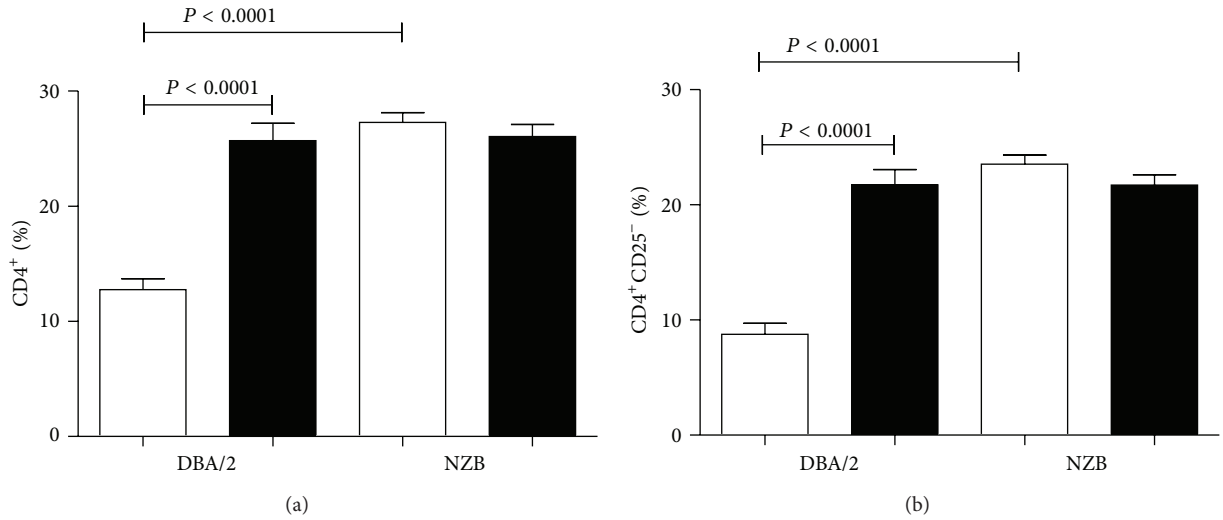

Figure 3: $CD4^+$ T-cell expansion in mHgIA-resistant DBA/2 consists of CD25 negative cells. NZB and DBA/2 mice were exposed to PBS (white bar) or $HgCl_2$ (black bar) for 5 weeks. Splenocytes were then cultured in the presence of PMA/ionomycin and analyzed for percent $CD4^+$ T cells (a) and $CD4^+CD25^-$ T cells (b). Percentages represent cells in total spleen cells. $n = 4$/group.

Experimental comparison between anti-CD3/anti-CD28 and PMA/ionomycin activation (Figure 2) revealed that anti-CD3/anti-CD28 activation does indeed reduce CD55 expression of $CD4^+$ T-cells by about 50% in B10.S mice which is very similar to the reduction found in mercury-exposed B10.S mice and constitutively in autoimmune prone NZB mice [103]. Unexpectedly, PMA/ionomycin activation of $CD4^+$ T cells did not affect CD55 expression (Figure 2) indicating that direct intracellular signaling of protein kinase C (PKC) does not impact CD55 expression and that CD55 reduction is mediated by events occurring at the cell surface. Whether $CD4^+$ T cell activation in the presence of anti-CD3/rCD97 also reduces CD55 remains to be determined.

The increased expression of IL-10 in human and murine $CD4^+$ T cells stimulated by anti-CD3 and rCD97 [19, 109] suggests a regulatory T-cell phenotype [110, 116, 117]. As CD55 is required for this response, we asked whether a difference in CD55 expression might affect the generation of regulatory T cells. NZB and DBA/2 mice have reduced and elevated CD55, respectively, and exposure to $HgCl_2$ exacerbates autoimmunity in NZB while DBA/2 mice are resistant [103]. Thus regulatory T cells may be more common in DBA/2 than NZB mice. To examine this possibility NZB and DBA/2 mice were exposed to $HgCl_2$ and splenocytes were then cultured *in vitro* in the presence of PMA/ionomycin and analyzed for the presence of markers of T regulatory type 1 (Tr1) ($CD4^+CD25^-IL\text{-}10^+IL\text{-}4^-$) cells [118]. This protocol increased $CD4^+$ T cells in $HgCl_2$ exposed DBA/2 mice but not in NZB mice (Figure 3(a)). This was an unexpected finding given that DBA/2 mice are resistant to mHgIA, however these cells were primarily of the $CD4^+CD25^-$ type (Figure 3(b)) indicating that they were not conventionally activated $CD4^+$ T cells which can express CD25 [103]. $CD4^+CD25^-$ T cells were then examined for expression of both IL-10 and IL-4 to identify Tr1-like cells (Figure 4). In total spleen, Tr1-like cells were dramatically increased following $HgCl_2$ in DBA/2 mice but were reduced in NZB mice (Figure 4(a)). A similar situation was found when only $CD4^+$ cells in the spleen were analyzed (Figure 4(b)). Finally we asked what percentage of $CD4^+CD25^-$ cells were $IL\text{-}10^+IL\text{-}4^-$ and found that the vast majority in the DBA/2 mice possessed the cytokine phenotype of T regulatory cells while such cells were much fewer in number in the NZB mice (Figure 4(c)). The greater percentage of putative Tr1 cells in PBS treated DBA/2 mice compared to NZB mice and the changing percentages of Tr1-like cells following mercury exposure are consistent with the sensitivity of these strains to mercury-induced autoimmunity. We hypothesize that the constitutively reduced CD55 expression in NZB mice [103] reduces CD55-CD97 interaction and the generation of Tr1 cells but favors T_h1 responses. In contrast, DBA/2 mice, with a higher level of CD55 that is not impacted by mercury exposure [103], are able to maintain a higher level of CD55-CD97 interaction which favors regulatory T-cell generation and tolerance to mHgIA.

Our hypothesis is supported by studies comparing CD28 and CD55 mediated T cell activation. CD28 and GPI-anchored proteins, like DAF1, exist in detergent-resistant microdomains or lipid rafts, and their engagement leads to redistribution and clustering at the site of the T cell receptor (TCR) [119]. Moreover, CD28 cross-linking leads to the formation of lipid raft clusters which exclude CD55 and vice versa [120]. It has also been argued that recruitment and crosslinking of GPI-anchored proteins, such as CD55, are less efficient at T cell activation than that elicited by crosslinking of CD28 [119–121]. These observations raise the possibility that the cell surface density of DAF influences T cell activation by competing with other costimulatory molecules. Thus reduced levels of DAF favor more efficient T cell activation via CD28 crosslinking (Figure 5). Conversely increasing the surface density of DAF1 would affect the effectiveness of CD28-mediated T cell activation. This supports observations

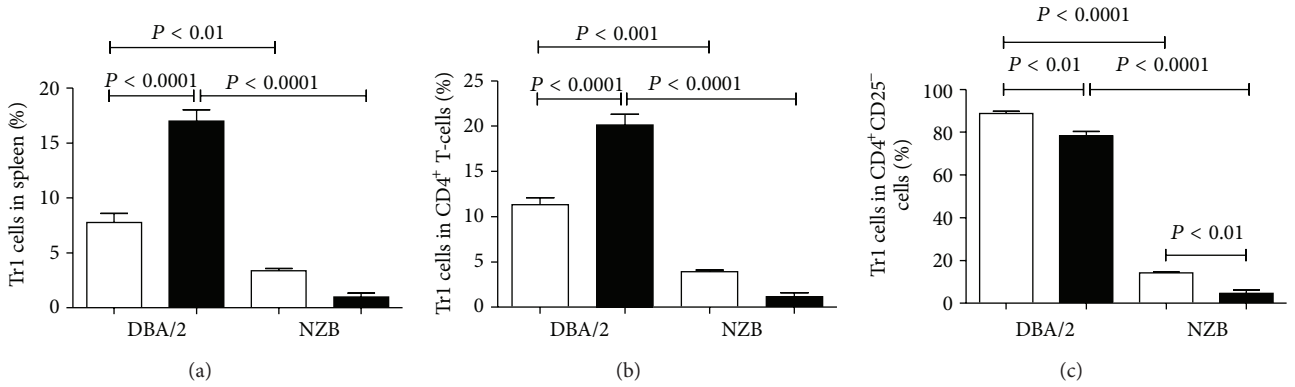

FIGURE 4: $CD4^+CD25^-$ T cells in mHgIA-resistant DBA/2 possess the cytokine phenotype of regulatory T-cells. NZB and DBA/2 mice were exposed to PBS (white bar) or $HgCl_2$ (black bar) for 5 weeks. Splenocytes were then cultured in the presence of PMA/ionomycin and $CD4^+CD25^-$ T-cells analyzed for the cytokine phenotype of $IL\text{-}10^+IL\text{-}4^-$ (Tr1 cells). (a) shows the percent of Tr1 cells in total spleen. (b) shows the percent of Tr1 cells in $CD4^+$ cells. (c) shows the percent of Tr1 cells in $CD4^+CD25^-$ cells. $n = 4$/group.

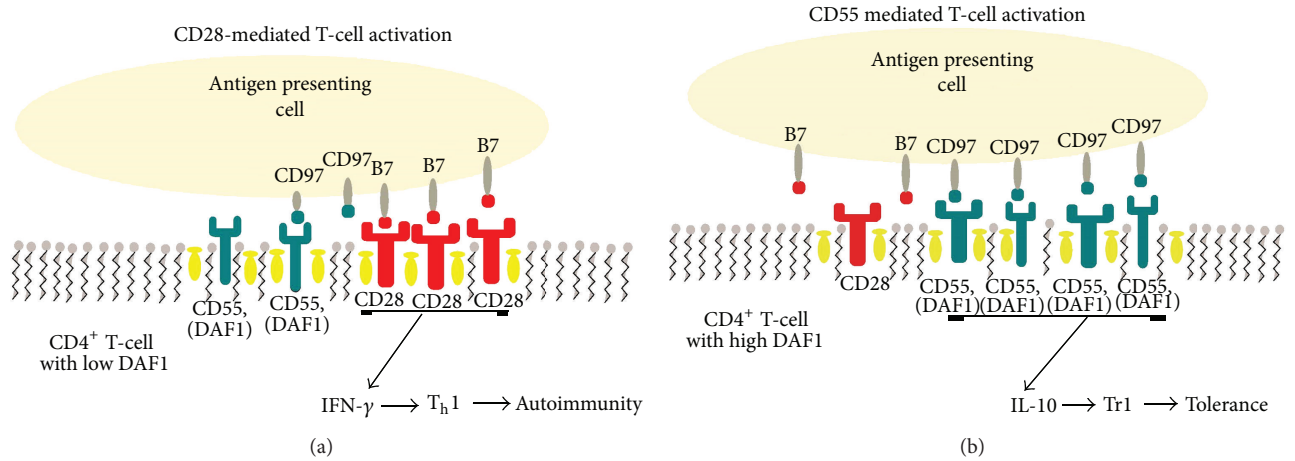

FIGURE 5: Hypothetical DAF function during T-cell costimulation. Conventional T-cell activation via CD28 cross-linking leads to the formation of lipid raft clusters which exclude CD55 (a) resulting in proinflammatory (i.e., IFN-γ) cytokine production which, under suitable conditions, may lead to T_h1-mediated autoimmunity such as mHgIA. Conversely, increasing the surface density of DAF leads to less efficient CD28-mediated T cell activation (b) and in the presence of CD97 potentiates CD55 signaling leading to increased production of IL-10, a T regulatory cell phenotype and immune tolerance.

by us [19] and others [109] that preferential costimulation via CD28 elicits a different cytokine profile than that produced by costimulation via DAF1 (CD55).

6. Conclusion

DAF serves a complex role in the immune system through complement-dependent and -independent functions in the regulation of innate and adaptive immunity. Initial reports on DAF1 in systemic autoimmune disease models suggested that it played a role in regulating adaptive immune responses. In the context of mHgIA our lab has shown that (1) DAF1 is constitutively reduced in mice prone to systemic autoimmune disease, (2) mHgIA is associated with reduced DAF1 expression on T-cells, and (3) interaction of DAF1 with its natural ligand, CD97, can regulate cytokine expression. These results suggest that understanding how mercury exposure reduces DAF1 expression may lead to approaches to regulate DAF in SLE. Evidence in patients shows that decreased levels of DAF on lymphocytes are associated with lymphopenia in SLE as well as in T cells of SS and the endothelial skin lesions of SLE, SSc, and other vasculitic diseases. DAF1 has both complement-dependent and- independent effects by regulating T-cell pro-inflammatory cytokine production *in vivo*. In systemic autoimmune disease models, such as mHgIA, complement-independent effects of DAF1 appear to be the major contributors regulating disease. Based on our mHgIA studies, we hypothesize that CD55 : CD97 interaction at the immunological synapse can regulate CD28 crosslinking and promote a Tr1-like phenotype, either by direct CD55

signal transduction or possibly through blocking of CD28 costimulation, and expression of a Treg cytokine profile.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by NIH Research Grants ES014847, ES020388, and ES021464. This is publication number 25088 from The Scripps Research Institute.

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