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Inflammatory Disease and the Human Microbiome

Amy D. Proal, Paul J. Albert, and Trevor G. Marshall

Abstract: The human body is a superorganism in which thousands of microbial genomes continually interact with the human genome. A range of physical and neurological inflammatory diseases are now associated with shifts in microbiome composition. Seemingly disparate inflammatory conditions may arise from similar disruption of microbiome homeostasis. Intracellular pathogens long associated with inflammatory disease are able to slow the innate immune response by dysregulating activity of the VDR nuclear receptor. This facilitates the ability of other species to gradually accumulate in tissue and blood, where they generate proteins and metabolites that significantly interfere with the body's metabolic processes. The microbes that contribute to this dysfunction are often inherited from family members. Immunosuppressive therapies for inflammatory disease allow pathogens driving these processes to spread with greater ease. In contrast to immunosuppression, treatments that stimulate the immune system seem to allow for reversal of this pathogen-induced genomic dysregulation. [*Discovery Medicine* 17(95):n-n, May 2014]

Introduction

Over just the past decade, molecular tools have revolutionized the field of microbiology. These tools -- which include pyrosequencing, single cell sampling, and shotgun sequencing -- directly extract and clone DNA from communities of microorganisms in the human body. We can subsequently characterize microbes based on their genomic signatures rather than their appearance in a Petri dish.

Indeed, it is now understood that the vast majority of microbes capable of persisting in *Homo sapiens* cannot be cultured in a laboratory. In contrast, studies using these new molecular tools have revealed the presence of thousands of previously unknown microbes in human tissue and blood (Pagani *et al.*, 2012). These microbes persist both in and on the human body, and are collectively referred to as the human microbiome.

Many of these newly characterized microbes have the capacity to contribute to human disease processes. For example, the amniotic fluid is widely regarded as being sterile. However, in 2010, DiGiulio and Relman and their colleagues at Stanford University published a seminal study that used molecular methods to identify eighteen different bacterial taxa in the amniotic fluid of women during preterm labor (DiGiulio *et al.*, 2008). A number of bacterial species identified were previously uncultivated and uncharacterized. The positive predictive value of PCR (polymerase chain reaction) for preterm delivery was 100%.

Discoveries like Relman's allow us to study chronic inflammatory disease from a new perspective. The theory of autoimmunity -- in which the immune system is thought to lose tolerance and generate antibodies against self -- gained traction before molecular-based technologies existed. The human body was logically assumed to be largely sterile. It followed that inflammatory conditions associated with autoantibodies could simply not be tied to infectious processes.

Today however, armed with a growing understanding of the diversity and extent of the human microbiome, we can re-evaluate many of these processes. Much of the inflammation and systemic dysfunction observed among patients with autoimmune and inflammatory disease can now be traced to components of the microbiome, and to the genetic pathways that pathogens within the microbiome dysregulate in order to survive.

The Human Superorganism

Two recent large-scale collaborations spearheaded the use of these metagenomic technologies. One was the Human Microbiome Project (2008-2012), a U.S.-based initiative funded by the U.S. National Institutes of

***Amy D. Proal**, Ph.D., and **Trevor G. Marshall**, Ph.D., are at the Autoimmunity Research Foundation, 3423 Hill Canyon Ave., Thousand Oaks, CA 91360, USA.*
***Paul J. Albert**, M.A., is at the Weill Cornell Medical College, 1300 York Ave., New York, NY 10065, USA.*
Corresponding Author: name (email).

Health (Turnbaugh *et al.*, 2007). The Project generated over 3.5 terabases of metagenomic sequences (Methé *et al.*, 2012). Another initiative was MetaHIT, a Europe-based initiative (2008-2012), which focused primarily on better characterizing the gut microbiome (Ehrlich, 2011).

In conjunction with data generated by other private initiatives, these projects detected and characterized so many novel microbes in *Homo sapiens* that at least 90% of cells in the human body are now understood to be bacterial, fungal, or otherwise non-human in origin. As of March 2014, the Genomes Online database lists 2,723 completed and published bacterial genomes detected in the human body with at least 14,867 in progress (Pagani *et al.*, 2012).

Our knowledge of the chronic viruses that persist in *Homo sapiens* is also rapidly evolving. Gordon and colleagues analyzed the fecal virome of monozygotic twins and their mothers (Reyes *et al.*, 2010). Eighty one percent of the reads generated from this virome did not match those of any known viruses. In 2011, Pride *et al.* (2012) found that hundreds of previously uncharacterized bacteriophage species dominate the oral cavity, some of them serving as reservoirs for pathogenic gene function.

The over nine million non-human genes represented by the microbiome (Yang *et al.*, 2009) dwarf the meager 20,500 that comprise the human genome. This knowledge implies a redefinition of the human/microbe relationship. The human body is best understood as a superorganism whose metabolism represents a combination of microbial and human interaction.

While pathogens clearly persist on mucosal surfaces, they are also present in blood and tissue. A microbiome is now known to persist in the lungs (Erb-Downward *et al.*, 2011). Microbial RNA can be easily detected in healthy human blood (McLaughlin *et al.*, 2002), with pathogens such as *Helicobacter pylori* identified by routine testing (AL-Jobori *et al.*, 2011). Polybacterial and chronic pathogens have been detected in atherosclerotic plaque. These include active species such as *Porphyromonas gingivalis* (Kozarov et al., 2005).

The Interactome

The millions of proteins and metabolites expressed by these and other microbes continually interact with those expressed by our own human genomes. The resulting interactions between these foreign and host proteins -- the interactome -- significantly impact all of the body's metabolic pathways. For example, the HIV genome transcribes 19 proteins, yet there are 1,443 direct interactions between just these few proteins and the human genome (Fu *et al.*, 2009).

The expression of our human genes is continually modified by the microbes and their metabolic products. For example, the gene *PTPN22* has been connected to rheumatoid arthritis, lupus, and diabetes mellitus (Goh *et al.*, 2007). However, *PTPN22* expression is also altered by the bacterial metagenome -- it is upregulated as part of the innate immune response to mycobacteria (Lykouras *et al.*, 2008).

Molecular Mimicry Further Complicates the Interactome

These genomic interactions are complicated by the fact that the structures of many nucleic acids and microbial proteins are identical or very similar to those expressed by those of their human hosts. For example, humans and *E. coli* metabolize glucose in nearly the same fashion, so the human superorganism may have difficulty distinguishing proteins and metabolites created by the microbes from those recognized as "self."

Tens of thousands of protein-protein interactions have been documented between just the genomes of *Salmonella*, *Escherichia coli*, *Yersinia*, and the human genome (Krishnadev and Srinivasan, 2011).

Composition of the Body's Microbial Communities Often Changes in Disease

Microbiome composition is often altered in patients with chronic inflammatory conditions. This dysbiosis, or microbial imbalance, has been associated with a growing number of chronic diagnoses including Crohn's disease, ulcerative colitis (Morgan *et al.*, 2012), irritable bowel syndrome (Franceschi *et al.*, 2009), psoriasis, both type 1 (Giongo *et al.*, 2010) and type 2 diabetes (Larsen *et al.*, 2010), and cardiovascular disease (Rajendhran *et al.*, 2013).

Some studies demonstrate direct relationships between microbial composition and disease onset. Amar *et al.* (2011) found that in 3,280 subjects without diabetes or obesity at baseline, pathogens in the microbiome led to 16S rDNA blood serum concentrations significantly elevated in those who went on to develop diabetes.

It is becoming increasingly clear that inflammatory disease processes are not due to acquisition of any single pathogen. Instead, they appear to result from alterations in the complex microbial communities. It follows that Koch's postulates, which dictate that one microbe must

be proven causative of a single disease state, can no longer be supported in the era of the metagenome.

Pathogens Capable of Intracellular Persistence Can Cause Significant Dysregulation

Many of the pathogens that contribute to chronic inflammatory disease persist inside the nucleated cells. When macrophages internalize *Salmonella*, persister cells can form that are resistant to most antibiotic therapies (Helaine *et al.*, 2014). When *E. coli* encounters a macrophage, it can evolve in a manner that allows it to remain alive within the phagosome (Miskinyte *et al.*, 2013).

These intracellular pathogens directly interfere with transcription, translation, and DNA repair at the cellular level. For example, Xu *et al*. (2003) demonstrated that upon infecting a cell, *Mycobacterium tuberculosis* alters the expression of 463 human genes. This kind of interference results in severe dysregulation of the interactome.

Dysregulation of the VDR Nuclear Receptor Leads to Dysbiosis and Chronic Disease

The ability of several prominent intracellular pathogens to dysregulate the Vitamin D Nuclear Receptor (VDR) points to a pathway in the molecular biology by which they can drive the systemic dysregulation associated with inflammatory disease.

The VDR expresses at least 913 genes, many connected to autoimmune and inflammatory processes (Wang *et al.*, 2005). In addition, it lies at the heart of the innate immune response. The receptor expresses TLR2, which recognizes bacterial polysaccharides. It also regulates expression of the cathelicidin and beta-defensin antimicrobial peptides, which play vital roles in targeting intracellular pathogens (Auvynet and Rosenstein, 2009). For example, vitamin D-mediated human antimicrobial activity against *M. tuberculosis* is dependent on the induction of cathelicidin (Liu *et al.*, 2007).

Any microbe capable of dysregulating VDR activity significantly impairs the innate immune response, allowing the pathogen to persist with greater ease. Infection of human B lymphocytes with Epstein-Barr virus downregulates VDR activity by a factor of at least fifteen -- particularly in younger, longer-lasting lymphoblastoid cells (Yenamandra *et al.*, 2009). Persistent *M. tuberculosis* slows VDR activity (Xu *et al.*, 2003). *Borrelia burgdorferi* (Salazar *et al.*, 2009), Cytomegalovirus (Chan *et al.*, 2008), and *Mycobacterium leprae* (Liu *et al.*, 2012) also slow VDR activity to varying degrees. The fungus *Aspergillus fumigatus* secretes a gliotoxin which significantly downregulates VDR expression (Coughlan *et al.*, 2012). Disabling the innate immune system via the VDR pathway is an extremely logical pathogen survival mechanism. Thus, other undetected or uncharacterized microbes likely survive in the same or similar fashion.

Flow on Effects of VDR Dysregulation Further Slow Immune Activity

VDR dysregulation is characterized by rising levels of the active vitamin D metabolite 1,25-dihydroxyvitamin D (1,25-D) and lower levels of the inactive 25-hydroxyvitamin D (Blaney *et al.*, 2009). Our *in silico* data suggests that, when elevated, 1,25-D can interfere with expression of other nuclear receptors, including the androgen receptor, the glucocorticoid receptor, and the thyroid receptor (Proal *et al.*, 2009).

Each of these other nuclear receptors express additional families of antimicrobial peptides (Brahmachary *et al.*, 2006), which can also be compromised by this same VDR dysregulation. This leads to profound immunosuppression. Indeed, decreased expression of cathelicidin over time has been demonstrated in both Crohn's disease (Nuding *et al.*, 2007) and sarcoidosis (Barna *et al.*, 2012).

Successive Infection

Under these conditions, an individual's microbiome may shift towards a composition that promotes disease. We refer to this process, in which the host microbiome shifts gradually away from a homeostatic state, as successive infection (Proal *et al.*, 2010). Infected cells struggle to correctly produce human metabolites in the presence of the pathogenic proteins, enzymes, and metabolites. Any pathogen that decreases antimicrobial peptide expression facilitates the ability of yet other pathogens to persist and cumulatively slow innate immune activity. This creates a snowball effect, where it becomes progressively easier for the host to acquire pathogens as the strength of the innate immune response decreases. Since the immune system strives to target the persistent microbes but never fully succeeds, a stalemate results, and low-grade inflammation accumulates.

Eventually, a person undergoing successive infection may present with symptoms sufficient for an inflammatory diagnosis. The unique symptoms any one person develops vary depending on the location, species, and virulence of the pathogens they have acquired over

time, along with the myriad ways in which the proteins and metabolites created by these microbes cause dysfunction by interacting with those of the host.

Microbes are notoriously competitive. Some pathogens successfully outcompete larger populations of less aggressive microbes. Serious dysfunction occurs if keystone species are lost (Cho and Blaser, 2012). Further, the dominance of certain microbial species may increase or decrease depending on the state of the interactome. For example, the guts of infants with atopic eczema are characterized by decreased microbial diversity (Abrahamsson *et al.*, 2012). However, in bacterial vaginosis, vaginal microbiome composition becomes much more diverse and taxon-rich than that of healthy individuals (Oakley *et al.*, 2008).

Comorbidities

Patients with one inflammatory diagnosis frequently develop another. For example, composition of the lung microbiome can predict the onset of rheumatoid arthritis (Demoruelle *et al.*, 2014). Figure 1 demonstrates the overlap in disease presentation among patients with a broad range of inflammatory conditions.

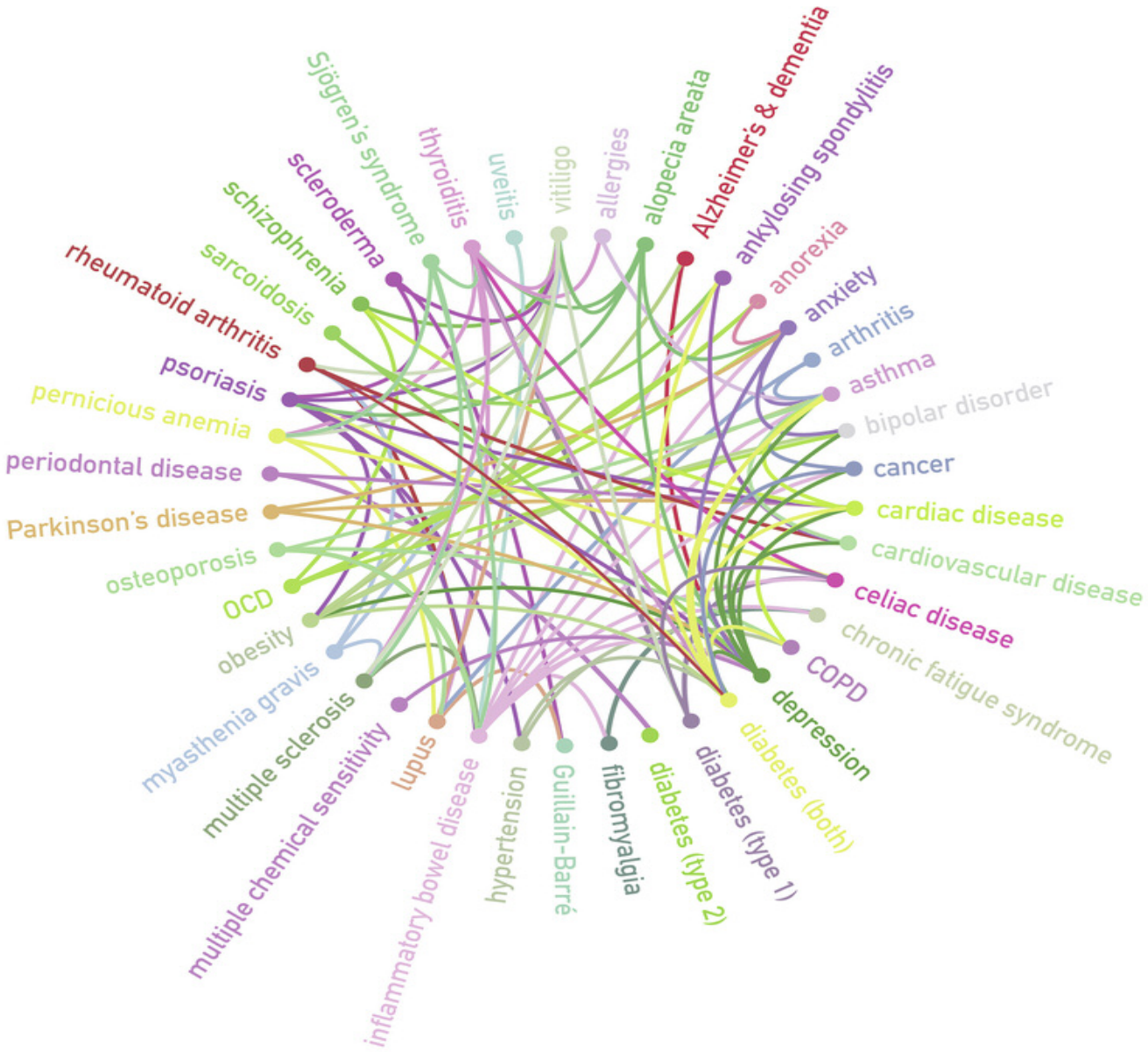

Figure 1. Comorbidities among common inflammatory diseases. Each "spoke" of this wheel represents a published study appearing in MEDLINE, which shows a significant statistical relationship between one disease and another.

Comorbidities directly reflect the successive infectious process, in which different inflammatory conditions may be driven by the same underlying mechanisms. Consequently, seemingly disparate conditions are best studied concurrently. The line between cause and effect is frequently unclear. For example, obesity is often believed to cause diabetes. Yet both conditions have now been tied to changes in microbiome composition (Larsen *et al.*, 2010; Turnbaugh *et al.*, 2006) and obesity itself has been declared a disease.

The microbiome is also involved in neurological conditions including anxiety, depression, and obsessive compulsive disorder (Gonzalez *et al.*, 2011). Thus, successive infection may also contribute to the development of neurological disease. For example, the PRIME study found that in healthy, European, middle-aged men, baseline depressive symptoms were associated with an increased risk of coronary heart disease in the short-term, and with stroke over the long-term (Majed *et al.*, 2012).

Familial Aggregation

An understanding of the comorbid conditions makes it easier to recognize familial aggregation. For instance, Benros *et al.* (2014) found that patients with schizophrenia are more likely to develop an autoimmune disease.

Familial aggregation likely results when elements of the microbiome are inherited. For example, infected siblings, mothers, and fathers are all major sources for *H. pylori* acquisition among young children, with the infected mother serving as the main source for childhood (Weyermann *et al.*, 2009). Relman and colleagues have demonstrated that within just weeks of birth, infants develop a microbiome that reflects components of not just their parents' microbiomes but those of their extended families as well (Costello *et al.*, 2013).

Evidence that pathogens are passed down the maternal line is particularly strong. Breast milk delivers a microbiome that varies between women depending on a host of factors (Cabrera-Rubio *et al.*, 2012). The health of the mother also appears to directly impact composition of her milk microbiome. These authors also found that milk from obese mothers tended to contain a different and less diverse bacterial community than that obtained from normal weight mothers.

Autoantibodies

Autoantibodies can be generated in response to microbes. High titers of rheumatoid factor (RF) have been detected not only in patients with rheumatoid arthritis, but also in patients with a number of bacterial, viral, and parasitic infections (Russell *et al.*, 1992).

Autoantibodies are notoriously polyspecific -- antibodies created to target pathogens may additionally target human proteins. This results in "collateral damage" and inflammation. For example, Sutjita *et al.* (1988) found that when normal individuals were injected with tetanus toxoid, at least seven antibodies were produced. One was an autoantibody to cardiolipin. B cells infected with EBV secrete antibodies capable of reacting with dozens of self and non-self antigens including albumin, renin, and thyroglobulin (Seigneurin *et al.*, 1988).

This means that the "autoantibodies" often detected in patients with inflammatory disease may simply be antibodies generated from the accumulation of pathogens into the microbiome. "Autoantibodies" are frequently detected in patients months or years before the onset of clinical disease symptoms. This reflects the gradual increase in microbiome dysbiosis characteristic of successive infection.

Immunosuppression Contributes to Prolongation of Disease by Delaying Resolution

Immunosuppresive therapies represent the standard of care for most inflammatory conditions, particularly those considered to be autoimmune. Corticosteroids, TNF-alpha antagonists, and rituximab are among the many treatments routinely used to slow immune activity. These treatments often provide short-term symptom palliation, but have poor long-term track records when it comes to relapse and stability. No definitive studies have demonstrated that corticosteroids improve long-term prognosis or reduce mortality. Indeed, the opposite is true (Gottlieb *et al.*, 1997).

This pattern is already recognized in the context of acute infection. For example, Earn *et al.* (2014) recently concluded that using antipyretic medications to suppress fever (and subsequently the immune response) in patients with influenza allowed viral particles to spread more easily between people. Thus, while subjects taking the antipyretic medications *felt* fewer symptoms, they were actually more contagious.

As the concept of "autoimmunity" is re-evaluated in light of chronic infection, the utility of immunosuppression must be reconsidered. Temporary symptom relief among patients taking immunosuppressive medications may result because the immune system cannot adequately respond to pathogenic insult. Cytokine and

chemokine levels may decrease, yet this allows pathogens to proliferate more easily.

The secosteroid vitamin D has potent immunosuppressive properties (Kimball *et al.*, 2011). Benefits observed among patients ingesting the substance may subsequently result from the short-term palliative effect described above. Indeed, a number of recent randomized controlled trials have studied the effect of vitamin D supplementation in patients with chronic inflammatory conditions. Most have failed to support a link between supplementation and improved health. In some cases, harm has been reported (Albert *et al.*, 2009; Autier *et al.*, 2014).

Immunostimulation May Better Target the Inflammatory Disease Process

If slowing the immune response in patients with inflammatory disease allows infectious agents to proliferate more easily, then approaches that seek to stimulate immune defenses should result in the opposite outcome. A reinvigorated immune system could more effectively kill pathogens at the heart of the inflammatory disease process.

Yet if the immune system successfully targets pathogens, some elements of the response -- the cytokine storm, for instance -- may cause the host to experience discomfort or an increase in disease symptoms. This is because as chronic pathogens and the cells they once inhabited are killed, toxins, cytokines, chemokines, and debris are released into the bloodstream. This leads to temporary increases in signs and symptoms of disease, and in many cases, temporary fluctuations in the levels of inflammatory disease markers. This phenomenon is called immunopathology (Figure 2).

Over the past decade, in concert with our clinical collaborators, we have developed an immunostimulative therapy that has been used to treat patients with a wide range of chronic inflammatory conditions. The treatment centers on use of a putative VDR agonist, with the goal of reactivating the antimicrobial peptides and other components of innate immunity under VDR control. Although the treatment is in early stages, physicians have started to publish case histories that seem to demonstrate both objective and subjective improvement amongst their patients (Proal *et al.*, 2011; 2013).

Patients using the therapy report high levels of immunopathology, especially during earlier periods of treatment. Yet over time, this immunopathology tends to wane at the same time that symptom improvement is frequently noted (Proal *et al.*, 2011). This pattern strongly suggests that patients on the therapy are indeed targeting pathogens associated with their disease states.

Discussion

The human body is a superorganism in which the human genome continually interacts with the millions of microbial genes from the microbiome. Pathogens have now been identified in tissue and blood previously considered sterile. Those capable of persisting inside

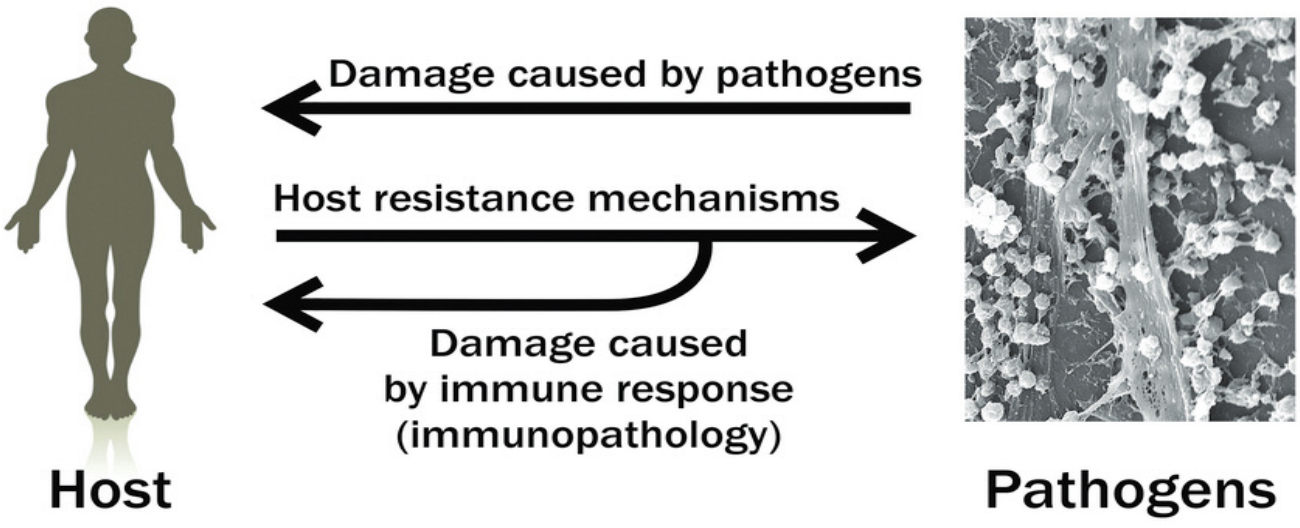

Figure 2. Immunopathology is a cascade of reactions including inflammation, cytokine release, and endotoxin release that occur as part of the immune response against microbes (From Ruslan Medzhitov, La Jolla Immunology Conference, October 2011).

the nucleated cells can directly alter cellular transcription, translation, and DNA repair mechanisms. Dysregulation of VDR nuclear receptor activity by a number of prominent pathogens can slow innate immune activity to the point where microbiome composition shifts away from a state of homeostasis. Under these conditions, a person may develop an inflammatory disease, the nature of which varies depending upon many factors. These include the location and virulence of the pathogens they acquire and the semi-infinite number of ways in which the proteins and metabolites created by these pathogens can cause dysfunction by dysregulating the body's metabolic pathways.

The comorbidities so frequently observed among patients with a wide range of both physical and neurological conditions support the possibility that different inflammatory conditions may develop from common underlying mechanisms. Babies begin to harbor a microbiome just weeks after birth, the composition of which reflects the microbiome of their parents and even those of extended relatives. Thus, the familial aggregation characteristic of inflammatory disease may well result when components of the microbiome are inherited.

The theory of autoimmunity was developed at a time when the human body was believed to be largely sterile. However, as the thousands of species within the microbiome are increasingly characterized, it is more likely that the autoantibodies detected in patients with autoimmune disease are generated in response to pathogens rather than "self."

Immunosuppressive therapies for inflammatory disease may provide short-term relief by slowing the cytokine and chemokine release associated with a healthy immune response towards acquired pathogens. However, pathogens are able to spread with much greater ease over the long term, leading to relapse and instability. Indeed, during the period that immunosuppressive therapies have become the standard of care in the United States, the incidence of nearly every chronic disease has increased. The secosteroid vitamin D has immunosuppressive properties, and should subsequently be evaluated in this context.

It is urgent that we re-evaluate the long-term efficacy of immunosuppressive therapies. In lieu of slowing the innate immune response in patients with inflammatory disease, it seems we should seek to activate it, so that chronic pathogens might be successfully targeted. However, patients on an immunostimulative therapy will inevitably experience immunopathology as toxins and debris generated from microbial death enter the bloodstream. While the resulting symptoms may be difficult to manage, the root cause of the disease is being addressed.

There is a pressing need for researchers to focus on developing tests that might better characterize and measure immunopathology in a clinical setting. Additionally, the current standard of care prioritizes symptom palliation. This means that physicians have few guidelines with which to evaluate the symptom and metabolite fluctuations characteristic of immunopathology. Development of techniques that might help patients better manage the reaction must also become a priority.

Disclosure

The authors report no conflicts of interest.

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