MICROBIAL AND ENVIRONMENTAL FACTORS IN AUTOIMMUNE AND INFLAMMATORY DISEASES

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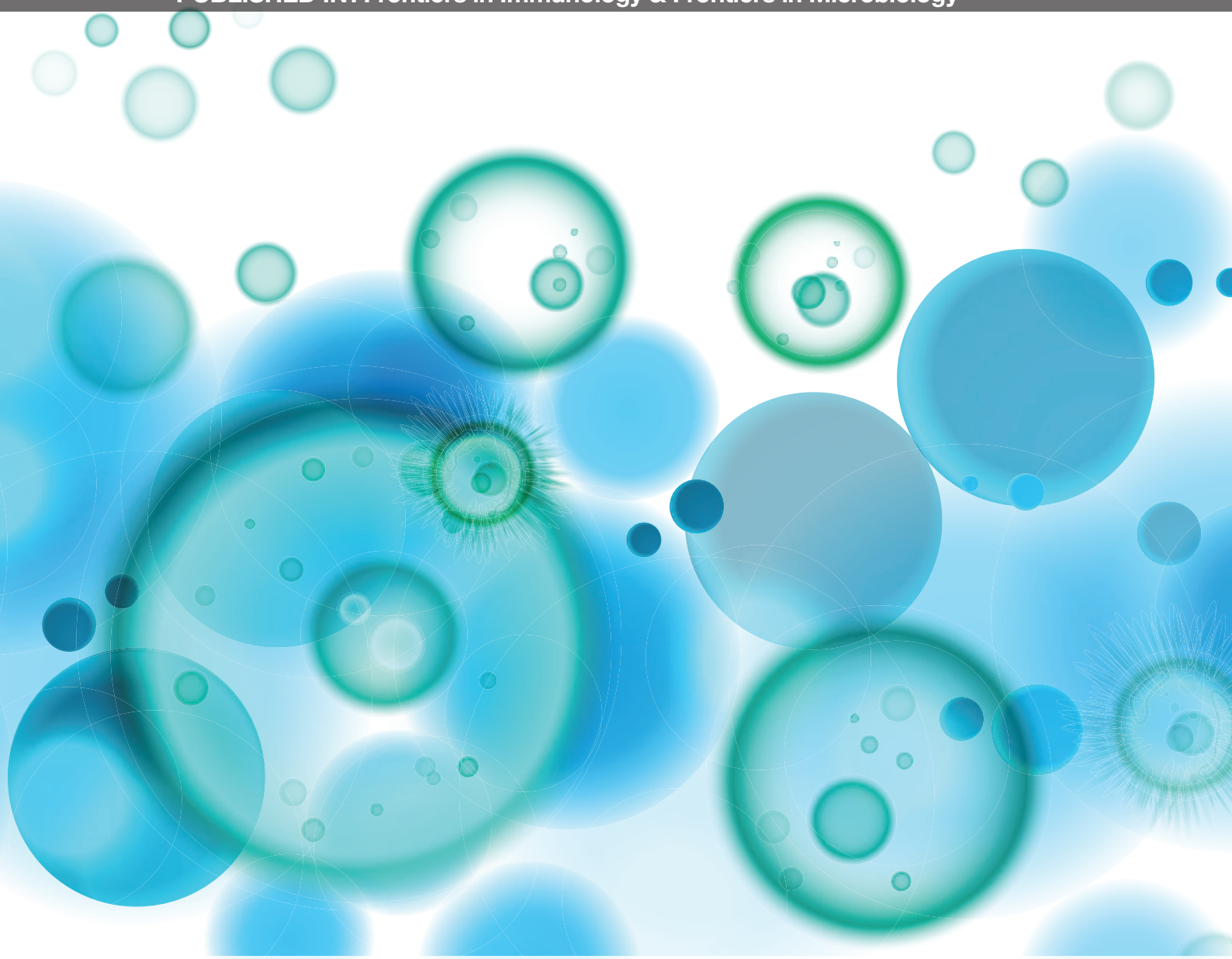

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MICROBIAL AND ENVIRONMENTAL FACTORS IN AUTOIMMUNE AND INFLAMMATORY DISEASES

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In recent years there has been a substantial increase in the number of diseases with the inflammatory component such as such as allergy, asthma, rheumatoid arthritis, inflammatory bowl disease (IBD, which includes ulcerative colitis and Crohn's disease), chronic sinusitis, and many other conditions. The majority of these diseases are multifactorial, with the contribution of genetic and environmental factors. Among the latter, the role of certain microorganisms and viruses in triggering or sustaining the inflammatory process is most controversial. In rheumatoid arthritis, for example, the following bacteria and viruses have been implicated in triggering the disease: *Mycoplasma spp., Proteus mirabilis, Escherichia coli, Staphylococcus spp., Bordetella spp., Acinetobacter spp., the parvoviruses, Epstein-Barr virus*, and *retroviruses*. The list of putative microbial triggers of rheumatoid arthritis is still growing, and it becomes essentially impossible to make a causation link between certain infectious agents and the disease. In the light of these disappointing results there are calls for even larger studies with the use of more advanced and large-scale technologies.

The primary function of the immune system is the maintenance of body homeostasis and protection against any threats to it via several lines of elaborate and complex immune defense. Given even higher complexity that involves the microbiota and the corresponding host-microbe interaction, the conditions for this equilibrium become even more challenging. In the absence of a defined pathogen, for example, the spectrum of microorganisms involved in triggering inappropriate immune responses may include polymicrobial communities or the cumulative effect of several microbial/viral factors. Under the normal circumstances there is a fine-tuned balance between commensal microbiota and the host's immune responses. However, when this balance is compromised, for example in IBD, a massive immune response is launched against commensal microbiota resulting in chronic inflammation.

Besides the microbial/viral factors, the balance of the immune system can be compromised by other causes. Given, for example, the close and inclusive interaction of the immune, nervous and endocrine systems, the list of these provoking factors can expand even more. For instance,

it has been demonstrated that even mild sleep deprivation may increase the production of interleukin-6 and C-reactive protein.

Understanding the complex role of microbial and environmental factors in inflammatory and autoimmune diseases, therefore, is the main subject of this topic.

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EDITORIAL
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Editorial: Microbial and Environmental Factors in Autoimmune and Inflammatory Diseases

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The Editorial on the Research Topic

Microbial and Environmental Factors in Autoimmune and Inflammatory Diseases

Pathogenesis of many autoinflammatory and autoimmune diseases is largely driven by inappropriate or inadequate immune responses toward environmental challenges. Most of them are multifactorial. For example, assessment of disease risk factors in rheumatoid arthritis (RA) brings the share of genetic predisposition to 50–60%, with the rest attributed to environmental factors (1). Thus, the diseases such as RA are well defined by Meda et al. (2) as comprising of both genetic and epigenetic components: "the disease of bad genes and bad luck." While genetic predisposition to RA and other autoimmune and autoinflammatory diseases has been a subject of very extensive research in recent years, the role of environmental factors remains less explored. The research topic articles address the role of various environmental factors that may contribute to the onset of autoinflammatory and autoimmune disorders. They are presented in the following order: purely environmental factors, dysbiotic conditions, and infections of bacterial, fungal, and viral nature.

In their comprehensive review, Anaya et al. discuss what they call the "autoimmune ecology," which includes all levels involved, from the ecosphere to the environmental factors and to the molecular mechanisms of interaction of these factors with the human immune system. The role of microbiota and vaccines, lifestyle habits and socioeconomic status, organic solvents, ultraviolet, and other factors is discussed in the context of how these factors may interact with the innate immunity *via* the toll-like receptor signaling pathway, B-cell activation, the T helper 17, and regulatory T (Treg) cells, posttranslational modifications of self-antigens, and epigenetic modifications. This is a formidable attempt to synthetize the current knowledge of how various environmental factors (collectively called exposome) may interact with the immune system leading to differential outcomes including autoimmune disease.

In the perspective article by Pollard, the silica-induced trigger mechanisms in the pathogenesis of inflammatory and autoimmune diseases are overviewed. Despite the proven epidemiological link between the silica exposure and autoimmune diseases, very little is known about the mechanistic factors leading to this disease. Based on currently available human and animal studies, a putative chain of events is proposed, which begins with activation of the innate immune system and then leads to proinflammatory cytokine production, pulmonary inflammation, activation of adaptive immunity, breaking of tolerance, autoantibody production, and tissue damage. Animal models of the silica exposure, which are used to mimic human autoimmunity, are also discussed.

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In systemic lupus erythematosus (SLE), with the disease onset in adulthood, the preceding prenatal/maternal history could bear important risk factors (Parks et al.). The authors demonstrate specific links between SLE and some early-life factors such as preterm birth, low birth weight, early prenatal/maternal farm exposure, and extended farm residence in childhood.

Several articles highlight a potential link between autoimmune diseases and the gastrointestinal tract disorders such as dysbiosis and bacterial infections. A review article by Ignacio et al. overviews host–microbe interaction and how the innate immune system senses gut microbiota and their metabolites *via* the inflammasomes and toll-like receptors. Disturbances in this fine-tuned cross talk may cause inappropriate modulations of inflammatory pathways thus leading to local and systemic inflammatory and autoimmune diseases.

In their hypothesis and theory article Lerner et al. propose an interesting mechanism, which may explain why a dysbiotic microbiota may have a potential role in autoimmune diseases. Physiologically normal posttranslational modifications of proteins by gut microbiota include cross-linking, de/amination/deamidation, de/phosphorylation, a/deacetylation, de/tyrosination, de/glutamylation, de/glycylation, ubiquitination, palmitoylation, glycosylation, galactosylation, arginylation, methylation, citrullination, sumoylation, carbamylation, and others. The balance of these activities in dysbiosis may be compromised thus generating highly immunogenic or neo-epitopes, which may break the tolerance and induce autoimmunity.

In the opinion article by Saxena, probiotic treatment is proposed as an alternative to the conventional treatment of Guillain–Barré syndrome, an immune-mediated peripheral neuropathy. The main idea is that probiotics may help to normalize the dysbiotic microbiota and replenish Treg cells to promote immune homeostasis.

Regarding gastrointestinal infections, Khaiboullina et al. demonstrate that gastroduodenitis caused by *Helicobacter pylori* is due to the upregulation of serum chemokines CXCL5 and CXCL6. This may lead to the local neutrophil accumulation at the sites of inflammation. *H. pylori* can also be protective against allergy and asthma, and Hussain et al. demonstrate a potential link between the human systemic type 1 T helper and Treg responses to *H. pylori* and allergen-specific IgE levels. The conclusion is that the systemic IL-10^{+} Treg response is likely to play a role in *H. pylori*-mediated protection against allergy in humans.

Chronic infections of the upper gastrointestinal tract can also provoke autoimmune conditions. Fuggle et al. performed extensive meta-analysis of data extracted from relevant publications to elucidate a potential link between RA and periodontitis. There is indeed a significant association between these two conditions. However, when we compare RA and osteoarthritis as a control, there is no significant difference in the prevalence of periodontitis in these two groups.

Some viral infections are the potent inducers of immune response, while others such as hantavirus infection are considered as having a much lesser impact. However, Morzunov et al. demonstrate a differential expression of serum cytokines involved in migration of lymphocytes, natural killer cells, and dendritic cells to the sites of hantavirus infection. There is also a significant upregulation of cytokines regulating leukocyte migration, repair of lung tissue, permeability of endothelial monolayer, and transendothelial leukocyte migration. On the contrary, cytokines associated with the platelet numbers and functions are downregulated. The same research group also reviews the hantaviral proteins and their potential involvement in hantavirus infection (Muyangwa et al.).

Another article from the same group (Boichuk et al.) shows the loss of plasmacytoid dendritic cells (pDCs), which are the major producers of interferon (IFN) type I, in the periphery of subjects with HIV/AIDS. This is presumably due to the decrease in serum cytokines. At the same time, gut-associated pDCs in HIV express the cellular proliferation marker Ki-67 suggesting that gut-associated pDCs are naive and possibly of the bone marrow origin. Gut-associated pDCs display activated phenotypes, with the elevated level of the proapoptotic enzyme, granzyme B. These data support a model of HIV progression where the peripheral pDCs are depleted and replaced by naive bone marrow-derived pDCs with limited IFN-producing capabilities. Ultimately, these pDCs migrate to the gut, with a potential impact on the gut mucosa due to the production of inflammatory cytokines and granzyme B.

Attempts to develop novel treatments against viral diseases such as swine flu, which is caused by the subtype of influenza A virus (H1N1), include the exploration of potentials of traditional medicine (Romero-Pérez et al.). The authors demonstrate some positive effects of stems and roots of *Salacia reticulate* (used in traditional Indian medicine Ayurveda) in a mouse model of H1N1 infection. These effects are presumably due to the increased natural killer cell activity in the host.

A potential role of common microbial and viral infections in the onset of RA is reviewed by Arleevskaya et al. The authors conclude that the optimal immune response toward these infections could be achieved *via* its reasonable adequacy and fine balance of immune system components. In some patients, however, this delicate equilibrium is compromised thus leading to the onset of RA. Contributing factors among these patients could be (i) higher susceptibility to bacterial and viral infections; (ii) greater imbalance of immune system components; (iii) limited capability to control and resolve inflammation; and (iv) compromised interaction at the microorganism–immune system interface. Thus, the disease onset is driven by the combination of genetic and environmental factors.

An important event in launching an appropriate immune response is sensing the invading pathogens by the innate immune system, and one of the important components of it is a protein called pyrin. Manukyan and Aminov give an up-to-date review of structure and function of this protein. They also examined molecular mechanisms of pathogenesis of familial Mediterranean fever, one of the most common hereditary autoinflammatory syndromes caused by mutations in pyrin. The high carrier frequency found in the affected populations from the Mediterranean Basin suggests a selective advantage conferred by the heterozygous state such as protection against presumptive pathogen(s). Identity of the suspected pathogen(s) that had selected for this genetic trait, however, remains elusive.

Another sensing molecule brought up in this topic is pentraxin-3, which serves as a key soluble pattern recognition receptor as well as interacts with the components of complement pathway. Hence, it plays an important role in innate immune responses against several bacterial, fungal, and viral infections. Computational analysis of non-synonymous single nucleotide polymorphisms (nsSNPs) in the *PTX-3* gene suggests that there are 10 high-risk nsSNPs (Thakur and Shankar). Four of them affect the conserved structural and functional residues in the pentraxin-domain thus compromising interaction with the C1q component of complement pathway. This may result in an increased susceptibility to infections among the carriers of these nsSNPs.

SNP-associated risk factors are not necessarily limited to structural/regulatory proteins involved in pathogen sensing, signal transduction, or immune response. An *in silico* analysis of 72 SNPs associated with 43 primary Sjögren's syndrome (SS) genetic risk factors suggests that only 5.6% are associated with the coding sequences (Konsta et al.). Other SNPs include intron sequences (55.6%), regions upstream/downstream of genes (30.5%), and intergenic regions (8.3%). Consequently, a significant enrichment by regulatory motifs (promoter, enhancer, insulator, DNAse peak, and expression quantitative trait loci) characterizes risk variants for SS (94.4%). Analysis of risk variants for SS among histone markers in B cells, monocytes, and epithelial cells shows their close association with promoters and enhancers in B cells and with enhancers in monocytes.

In conclusion, articles in this research topic made a very significant contribution to our understanding of the role played by environmental factors, dysbiotic conditions, and infections in triggering autoimmune and autoinflammatory diseases. Since this is a rapidly expanding area of research, many other factors contributing to the onset of these diseases are not covered here. We are confident, however, that further studies will expand the list as well as bring a better understanding of mechanisms involved in the onset of autoimmune and autoinflammatory diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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The Autoimmune Ecology

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Autoimmune diseases (ADs) represent a heterogeneous group of disorders that affect specific target organs or multiple organ systems. These conditions share common immunopathogenic mechanisms (i.e., the autoimmune tautology), which explain the clinical similarities they have among them as well as their familial clustering (i.e., coaggregation). As part of the autoimmune tautology, the influence of environmental exposure on the risk of developing ADs is paramount (i.e., the autoimmune ecology). In fact, environment, more than genetics, shapes immune system. Autoimmune ecology is akin to exposome, that is all the exposures – internal and external – across the lifespan, interacting with hereditary factors (both genetics and epigenetics) to favor or protect against autoimmunity and its outcomes. Herein, we provide an overview of the autoimmune ecology, focusing on the immune response to environmental agents in general, and microbiota, cigarette smoking, alcohol and coffee consumption, socioeconomic status (SES), gender and sex hormones, vitamin D, organic solvents, and vaccines in particular. Inclusion of the autoimmune ecology in disease etiology and health will improve the way personalized medicine is currently conceived and applied.

Keywords: environment, ecology, autoimmune disease, polyautoimmunity, personalized medicine

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Abbreviations: AA, adjuvant-induced arthritis; aa, amino acids; ACPAs, anticitrullinated protein autoantibody; ADs, autoimmune diseases; AE, autoimmune ecology; AhR, aromatic hydrocarbon receptor; AITD, autoimmune thyroid disease; ANCA, anti-neutrophil cytoplasmic autoantibody; AOR, adjusted odds ratio; aPL-abs, antiphospholipid antibodies; APS, antiphospholipid syndrome; ASIA, autoimmune/inflammatory syndrome induced by adjuvants; BAFF, B-cell activating factor; CD, celiac disease; CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; CMV, cytomegalovirus; CrD, Crohn's disease; CREB, cAMP response element-binding protein; CVD, cardiovascular disease; DAMPs, damage-associated molecular patterns; DAS 28, disease activity score-28; DCs, dendritic cells; DEPAP, 1,2-di-oleyl ester; DMARDs, disease-modifying antirheumatic drugs; dmC, deoxymethylcytosine; DNMT1, DNA methyltransferase 1; dsDNA, double-strain DNA; EAE, experimental autoimmune encephalomyelitis; EBV, Epstein–Barr virus; EPW, expert panel workshop; ERK, extracellular signal-regulated kinase; EULAR, European league against rheumatism; FICZ, 6-formylindolo (3.2-b) carbazole; GC, germinal center; GD, Graves' disease; GO, Graves opthalmopathy; GSE, gluten-sensitive enteropathy; GST, glutathione S-transferase; HMGB1, high mobility group box 1; hnRNP, heterogeneous nuclear ribonucleoprotein; HR, hazard ratio; HRT, hormonal replacement therapy; HT, Hashimoto's thyroiditis; IFN, interferon; Igg, immunoglobulin; IRAK1, interleukin 1 receptor associated kinase 1; IRF, interferon regulatory factor; LN, lupus nephritis; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; MD, mean difference; MDHAQ, multi-dimensional health assessment questionnaire; MHC, major histocompatibility complexes; MS, multiple sclerosis; MTX, methotrexate; MYD88, myeloid differentiation primary response protein 88; NCV, new cluster variables; NF-κB, nuclear factor-κB; NLRs, nod-like receptors; OC, oral contraceptive; OR, odds ratio; OSs, organic solvents; PAD, peptidylarginine deiminase; PAMPs, pathogen-associated molecular patterns; pAPS, primary antiphospholipid syndrome; PBC, primary biliary cirrhosis; PBMC, peripheral blood mononuclear cells; PolyA, polyautoimmunity; PRR, pattern recognition receptors; PSC, primary sclerosing cholangitis; PTMs, post-translational modifications; RA, rheumatoid arthritis; RCTs, randomized controlled trials; RF, rheumatoid factor; RPL, recurrent pregnancy loss; RR, relative risk; SE, shared epitope; SES, socioeconomic status; SHM, somatic hypermutation; SIR, standardized incidence ratios; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index; SLICC/ACR, Systemic Lupus International Collaborating Clinics/American College of Rheumatology; SMD, standardized mean difference; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein; SPD, secondary progressive disease; SS, Sjögren's syndrome; SSc, systemic sclerosis; T1DM, type 1 diabetes mellitus; TAK1, TGFβ-activated kinase 1; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; Th17, T helper 17 cells; TIRAP, TIR domain-containing adaptor protein; TLR, toll-like receptor; TNF, tumor necrosis factor; TRAF6, TNF receptor associated factor 6; TRAM, TRIF-related adaptor molecule; Treg, T regulatory cells; Tresp, T-responder cells; TRIF, TIR domain-containing adaptor protein inducing IFNβ; UC, ulcerative colitis; UV, ultraviolet.

INTRODUCTION

Public health problems are increasingly complex, especially environmental and social threats arising from global changes, driven by rapid industrialization, population growth, over-consumption of natural resources, and inappropriate use of technology (1). Understanding patterns of health and disease requires that public health attention be given to not only personal behaviors, biological traits, and specific risks but also the social and physical environmental characteristics that shape human experience (1).

Ecology originated in natural sciences (botany and zoology) during the late nineteenth century as the study of the interaction between plants and animals with the surrounding environment, involving interdisciplinary research of biology and earth sciences. Early in the twentieth century, social scientists applied ecological principles to study human behavior and community organization (2). The word ecology is derived from the Greek *oikos*, meaning "household," and *logos*, "study." Thus, the environmental house includes all the organisms in it and all the functional processes that make the house habitable. Ecology is literally the study of "life at home" with the emphasis on "the totality or pattern of relations between organisms and their environment" (3). Perhaps the best way to delimit modern ecology is to consider the levels of organization seen as an ecological spectrum (**Figure 1**) and an extended ecological hierarchy (4).

At this basic level, human ecology can be thought of as the study of the environmental conditions in which human beings developed, and humans' relationship with the ecosystems that support them. The same principles apply to the ecology of any species, but in the case of humans, their number on the planet, their presence in almost all terrestrial ecosystems, and their impact are largely the product of the human capacity for culture (5). The term "ecosphere" was sometimes used as an alternative for "biosphere," thus considering the physical and biological aspects of the Earth as a unit, rather than biology alone (6).

Analysis of the environmental influence on chronic diseases has suffered continuous transformations. Concern about the environment's effects on health has caught fire with joint health, urban-planning conferences and strategy sessions, pending legislation, and more new scientific studies (7). A framework for thinking about the organization of health care, medical education, and research has been provided that goes beyond the human ecology definition (8). This has resulted in a definition involving the relationships between people and their health care environment known as the "ecology of medical care" (4, 8, 9).

Over the years, a number of trans-National Institutes of Health (NIH) committees and NIH-supported workshops have examined the impact of the environment on autoimmune disease (AD) (10). In 2012, the National Institute of Environmental Health Sciences Expert Panel Workshop reviewed approaches for defining environmentally associated ADs in three contexts (11): (a) identifying the necessary and sufficient data to define environmental risk factors for ADs meeting current classification criteria; (b) establishing the existence of and criteria for new environmentally associated ADs that do not meet current disease classification criteria; and (c) identifying specific environmental agents that induce AD in individuals, typically in a clinical setting. They emphasized that additional efforts in all these areas are needed to achieve true consensus in this field and to define classification criteria that can distinguish environmentally associated AD cases from others with high sensitivity and specificity. Panel findings on studies of the role of environmental factors and development of ADs are shown in **Table 1** (10).

For many perhaps most traits, the interaction with genes and environmental factors might be genetically programed or may be purely stochastic (12). However, recent evidence pointed out that environment, more than genetics, shapes immune system (13). As a corollary, just as multiple genetic risk factors influence the development of a given AD, multiple environmental factors are also required at different periods in life, or in a specific temporal sequence to affect the immune perturbations that result in AD (4). Thus, the autoimmune ecology (AE) refers to the analysis and study of interactions among individuals and their environment leading to a break of immune tolerance and, therefore, to the development of one or some ADs in such individual. It is also likely that diverse ADs may result by the interaction of multiple environmental factors. This supports the fact that AE is a crucial component of the autoimmune

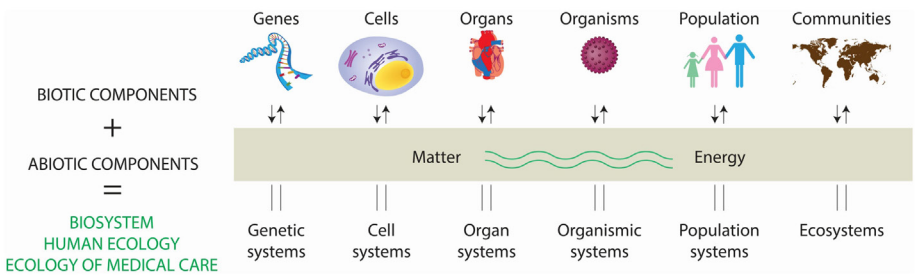

FIGURE 1 | Interaction of living and non-living components in the conformation of the biosystem. A system consist of regularly and interdependent components forming a unified whole. Systems containing living (i.e., biotic) and non-living (i.e., abiotic) components constitute biosystems, and range from genetic systems to ecosystems, all of them influencing their function and homeostasis. This multifactorial biosystem has a major effect in health, resulting into the concept of "the ecology of medical care." Adapted from Ref. (4).

TABLE 1 | Findings on human studies on the role of environmental factors and development of autoimmune diseases.

EPW is confident of the following	EPW consider the following likely, but requiring confirmation	Broad themes to be pursued in future investigations
Chemicals		
Crystalline silica (quartz) contributes to development of several ADs, including RA, SSc, SLE, and ANCA-related vasculitis	Solvents contribute to development of MS	There is insufficient evidence on the role of metals, including those associated with animal models of autoimmunity, e.g., mercury
Solvents contribute to development of SSc	Smoking contributes to development of seronegative RA, MS, SLE, HT, GD, and CD	The identification of single causal agents within groups of exposures is needed (e.g., specific solvents or pesticides contributing to increased risk for the group)
Smoking contributes to development of ACPA-positive and RF-positive RA (with an interaction with the shared epitope genetic susceptibility factor)	Current smoking protects against development of UC	Studies are needed on plasticizers (e.g., phthalates and bisphenol A), some of which may be endocrine or immune disruptors, and have been associated with other immune-mediated diseases There is insufficient evidence on the role of cosmetics in ADs
Physical factors		
An inverse association exists between increased UV radiation exposure and risk of developing MS	Ionizing radiation contributes to development of HT and GD	There is insufficient evidence on a possible protective role of UV radiation on T1D Prospective data are needed on sun exposure as a risk factor for SLE (prior to early clinical symptoms) and dermatomyositis
Biologic agents		
Ingestion of gluten contributes to development of GSE	EBV infection contributes to MS development	Studies are needed on MS and vitamin D in racial/ethnic groups with darker skin (associated with UV-associated vitamin D deficiency), and examining dose-effects Prospective data are needed on vitamin D and other ADs
Ingestion of certain lots of L-tryptophan contributes to development of eosinophilia myalgia syndrome	Early introduction of complex foods contributes to development of T1DM and GSE	Additional studies are needed on associations of food chemicals, dyes, or additives
Dietary intake of DEPAP and oleic anilide-contaminated rapeseed oil contributes to development of toxic oil syndrome	Low dietary vitamin D intake and blood levels contribute to development of MS	Prospective studies are needed on nitrates/nitrosamines and T1DM

Modified from Ref. (10).

EPW, expert panel workshop; ADs, autoimmune diseases; RA, rheumatoid arthritis; SSc, systemic sclerosis; SLE, systemic lupus erythematosus; ANCA, anti-neutrophil cytoplasmic antibody; ACPA, anticitrullinated protein antibody; RF, rheumatoid factor; MS, multiple sclerosis; HT, hashimoto's thyroiditis; GD, graves' disease; CrD, crohn's disease; UC, ulcerative colitis; T1DM, type 1 diabetes mellitus; GSE, gluten-sensitive enteropathy; DEPAP, 1,2-di-oleyl ester; EBV, Epstein–Barr virus; UV, ultraviolet.

tautology and reinforces the theory that ADs share several common mechanisms (14).

The AE is akin to exposome, that is all the exposures – internal (such as the microbiome, described elsewhere in this review) and external – across the lifespan, including physical environment (e.g., occupational hazards, exposure to industrial and household pollutants, water quality, climate, altitude, air pollution, and living conditions and lifestyle and behavior such as diet, physical activity, cultural practices, use of addictive substances, and so on), interacting with hereditary factors (both genetics and epigenetics) to favor or protect against autoimmunity. However, the concept of AE as well as exposome extends beyond these factors to include social factors, such as socioeconomic status (SES), quality of housing, neighborhood, social relationships, access to services, and so on. (15–17).

To further contribute to the evidence about the nurture influence on ADs, herein we provide an overview of the AE. The focus will be on specific environmental factors and associated mechanisms that may be contributing to ADs. First is a review of the immunological and genetic bases of AE, then a review of the epidemiological bases of AE, which are mainly the influence of microbiota, lifestyle habits (i.e., alcohol, cigarette, and coffee), sex hormones, organic solvents (OSs), vaccines, SES on ADs, and environmental influence on polyautoimmunity (PolyA) (**Figure 2**).

IMMUNOLOGICAL AND GENETIC BASES OF AUTOIMMUNE ECOLOGY

Autoimmune disease is characterized by an immune response against self-antigens affecting about 5% of the population (18). Complex diseases have multifactorial origins, which imply that combinations of the many factors involved in autoimmunity produce varying and unique clinical pictures. Gene involvement in AD is inarguable with hundreds of risk loci identified for and shared between different diseases (19). Despite the genetic associations for each distinct AD, much of the heritability remains unaccounted for (20). The heritability of ADs is not fully understood. This establishes that the missing heritability probably reflects the fact that genes do not operate alone, but in the context of the environment leading to the gene–environment interplay (21, 22). That is, the degree of disease risk contributed by one factor will be influenced by the presence or absence of another (23).

Current classification criteria have primarily relied on epidemiological comparisons of disease incidence or prevalence in exposed and unexposed cohorts. However, other approaches have included immunological and animal model studies, which added considerable supporting evidence (24). A challenge in this field has been to clarify how much evidence is required to define a given exposure as a risk factor for ADs (25). Traditional

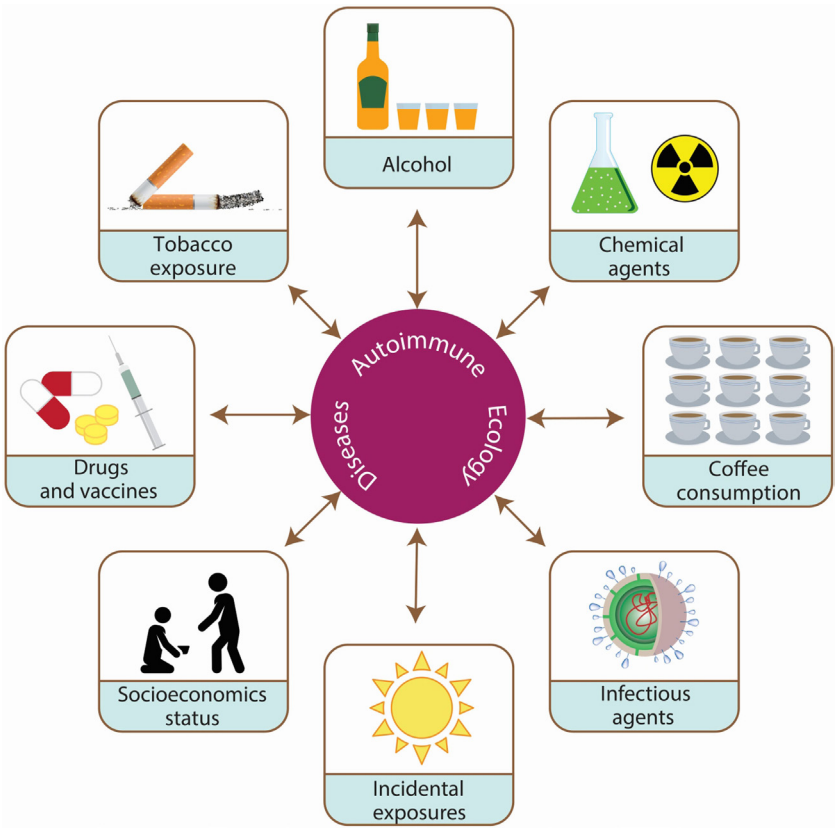

FIGURE 2 | Environmental exposures associated with the development of autoimmune diseases (ADs). ADs are the result of the interaction between hereditary (i.e., genetic and epigenetic) and environmental factors over time. Autoimmune ecology is defined as the environmental exposures (i.e., cigarette smoking, coffee and alcohol intake, socioeconomic status, chemical agents, vaccines, incidental exposures, infectious agents, and microbiota) influencing the development of ADs and their outcome.

epidemiological approaches were established for a number of associations initially outlined in 1965 (26).

The environmental risk factors associated with ADs are varied as are the underlying immune mechanisms that lead to these disorders (**Table 2**). The following sub-topics will be discussed: effects on innate immunity, such as toll-like receptor (TLR) activation by xenobiotics, adjuvant effects, and inflammatory responses; B-cell activation; direct effect impairing the immune function, such as T helper 17 (Th17) and T regulatory cells (Treg); post-translational modifications (PTMs) of self-antigens, and epigenetic modifications, mainly DNA methylation.

TABLE 2 | Environmental factors and mechanisms involved in autoreactivity.

Environmental factor	Main possible mechanisms
Infectious agents (bacteria/viruses)	Innate immune activation via TLRs
Adjuvants	Innate immune activation via TLRs
Sex hormones	Autoreactive B cells
TCDD/Mercury/Silver	Autoreactive B cells
Cigarette smoke	T cell impairment (Th17/Treg cells)
	Modification of self antigens
UV light	Autoreactive B cells
	Epigenetic changes (DNA methylation)

TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TLRs, toll-like receptors; Th, T helper cells; UV, ultraviolet.

Innate Immunity

Toll-Like Receptor Activation, Adjuvant Effects, and Inflammatory Responses

Toll-like receptors are membrane bound proteins present in several immune cells, including B-lymphocytes, selective populations of T cells, dendritic cells (DCs), and macrophages as well as non-immune cells, such as epithelial cells and fibroblasts. TLRs recognize invading organisms bearing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (27). PAMPs are conserved molecules derived from microorganisms, such as lipopolysaccharide (LPS), peptidoglycan, flagellin, and microbial nucleic acids, while most DAMPs are endogenous molecules released from dying host cell molecules upon cellular stress or tissue damage, such as oxidative stress and heat shock proteins (28). Activation of TLRs by PAMPs or DAMPs can upregulate inflammatory cytokines and chemokines and engage intracellular signaling pathways to regulate the nature, magnitude, or duration of the host's inflammatory response (29). TLR expression may rapidly change in presence

of cytokines, pathogens, and environmental factors (30). TLRs can be distinguished by their location; TLR1, TLR2, TLR4, TLR5, and TLR6 are present at the cell membrane, while TLR3, TLR7, TLR8, and TLR9 are in cell compartments, such as endosomes (31). There is constant interplay between the innate and adaptive arms of the immune system, and TLRs play a key role in this interaction.

The involvement of microbial PAMPs and endogenous ligands in autoimmunity through the activation of TLR and/or their increased expression acting in synergy with the formation of autoantigen–autoantibody immune complexes has been hypothesized (**Table 3**) (32). High mobility group box 1 (HMGB1) is a protein that acts as a DAMP when is located outside the cell, promoting the pathogenesis of autoimmune diseases. HMGB1 can interact with multiple receptors; in particular, the binding to TLR4 can lead to the activation of NF-κB and production of IL-6 and tumor necrosis factor (TNF)-α by macrophages (33). Furthermore, HMGB1 can bind to PAMPS, such as LPS, double-stranded RNA or DNA, and act through the partner's receptor. This synergism could induce expression of mediators, such as the prostaglandins, to promote pain and inflammation in arthritis (34). The presence of PAMPs in tissues following infection has been associated with autoimmunity. These observations provide indirect evidence that PAMPs may promote autoimmune and chronic inflammatory conditions (35). There is a well-established link between infection and ADs in both clinical settings and animal models. This link has been attributed to either molecular mimicry between pathogen-derived antigens and self-antigens or non-specific activation of innate immunity leading to a breakdown in immunological tolerance and development of self-antigen-specific T cell and antibody responses (35). Several studies in patients with multiple sclerosis (MS) have suggested that the disease may be triggered or exacerbated by infections with pathogens such as *Chlamydia pneumoniae*, *human herpes virus 6*, and *Epstein–Barr virus* (EBV) (36). *Parvovirus B19* is considered one triggering factor for rheumatoid arthritis (RA) (37). The virus was detected by PCR in synovial biopsies from 75% of RA patients compared to 17% of patients with osteoarthritis and other arthritides. Furthermore, EBV DNA and RNA were detected in 34% of RA patients with the shared HLA-DR4 epitope compared with 10% of healthy individuals (38). Infection with *Streptococcus pneumoniae* 7 days after the induction of experimental autoimmune encephalomyelitis (EAE) exacerbates autoimmunity in wild type but not *Tlr2−/−* mice (39). This suggests that pathogens may exacerbate ADs via the activation of TLRs (**Figure 3**). Furthermore, PAMPs are present in the diseased tissues of patients with ADs. For example, peptidoglycans, which can act as ligands for nod-like receptors (NLRs) and TLR2, have been found in various cells and tissues, including in synovial tissue macrophages and DCs isolated from patients with RA (39, 40). Immunization of mice with myelin-derived peptides in complete Freund's adjuvant (CFA) induces active EAE. CFA contains killed *Mycobacterium tuberculosis*, and PAMPs from these bacteria activate innate immune responses, which, in turn, promote pathogenic autoreactive T cell responses. Marta et al. showed that mice deficient in the adaptor protein MYD88 are resistant to EAE (41). This was associated with reduced IL-6 and IL-23 production by DCs and reduced IL-17 and interferon (IFN)-γ production by T cells. This suggests that innate immune responses initiated through TLR or IL-1R signaling are required for the induction of experimental autoimmunity. In the collagen-induced arthritis (CIA) model, which is induced by immunization with collagen and CFA, *M. tuberculosis* in CFA provides a source of PAMPs. Moreover, zymosan, a polysaccharide from the cell wall of *Saccharomyces cerevisiae* that binds TLR2, has been used to induce experimental arthritis in mice. Zymosan-induced arthritis was found to be dependent on TLR2 activation as disease was substantially attenuated in *Tlr2−/−* mice (42). In addition, injection of immunostimulatory DNA sequences into joints of rats promoted development of adjuvant arthritis (43). This suggests that activation of TLR9 may also precipitate the innate immune responses that drive inflammation in joints (35).

Shoenfeld and Agmon-Levin coined the term "Autoimmune/inflammatory Syndrome Induced by Adjuvants" (ASIA) (44). This syndrome is characterized by non-specific and specific manifestations of AD. An adjuvant is any substance that accelerates, prolongs, or enhances antigen-specific immune response. It may stimulate the immune system and increase the response to a vaccine, without having any specific antigenic effect. Activation of the immune system by adjuvants, a desirable effect, could trigger manifestations of autoimmunity. The main substances associated with ASIA are squalene, aluminum hydroxide, silicone, mineral oil, guaiacol, and iodine gadital (45). Alum adjuvants are humoral immune potentiators in vaccine formulations. This property has been attributed to NLRP3 inflammasome activation, an intracellular multiprotein complex that mediates caspase-1 cleavage of the inactive precursor of the proinflammatory cytokine IL-1β, leading to the release of its mature form. Inflammasome-mediated cleavage of pro-IL-1β *in vitro* depends on signals that activate both TLR and nucleotide oligomerization domain-like receptors, such as NLRP3 (46, 47). The adjuvanticity of aluminum compounds is related to their association with uric acid. Alum appears to promote an inflammatory response that results in the release of uric acid from necrotic cells. Uric acid, in turn, is thought to increase the adjuvanticity of alum with an increase in IL-4 levels (45, 48). IL-4 drives the upregulation of monocytic

TABLE 3 | Effects of ligands of TLR2 or TLR4 on different cells in autoimmune diseases.

Disease	Cells	Ligands	Effects
RA	Macrophage	LPS	Enhancing the production of proinflammatory cytokines
SLE	PBMC	LPS	Dysregulation of cytokines and autoantibodies production
SSc	DCs	LPS	Secretion of cytokines
SS	Fibroblasts	LPS	Upregulation of the production of profibrotic and proangiogenic chemokines
	Human salivary cells	Peptidoglycan/LPS	Stimulating CD54 expression and IL-6 production
	PBMC	LPS/Zymosan A	Inducing the production of IL-23/IL-17

RA, rheumatoid arthritis; LPS, lipopolysaccharide; SLE, systemic lupus erythematosus; PBMC, peripheral blood mononuclear cells; SSc, systemic sclerosis; DCs, dendritic cells; SS, Sjögren's syndrome. Adapted from Ref. (29).

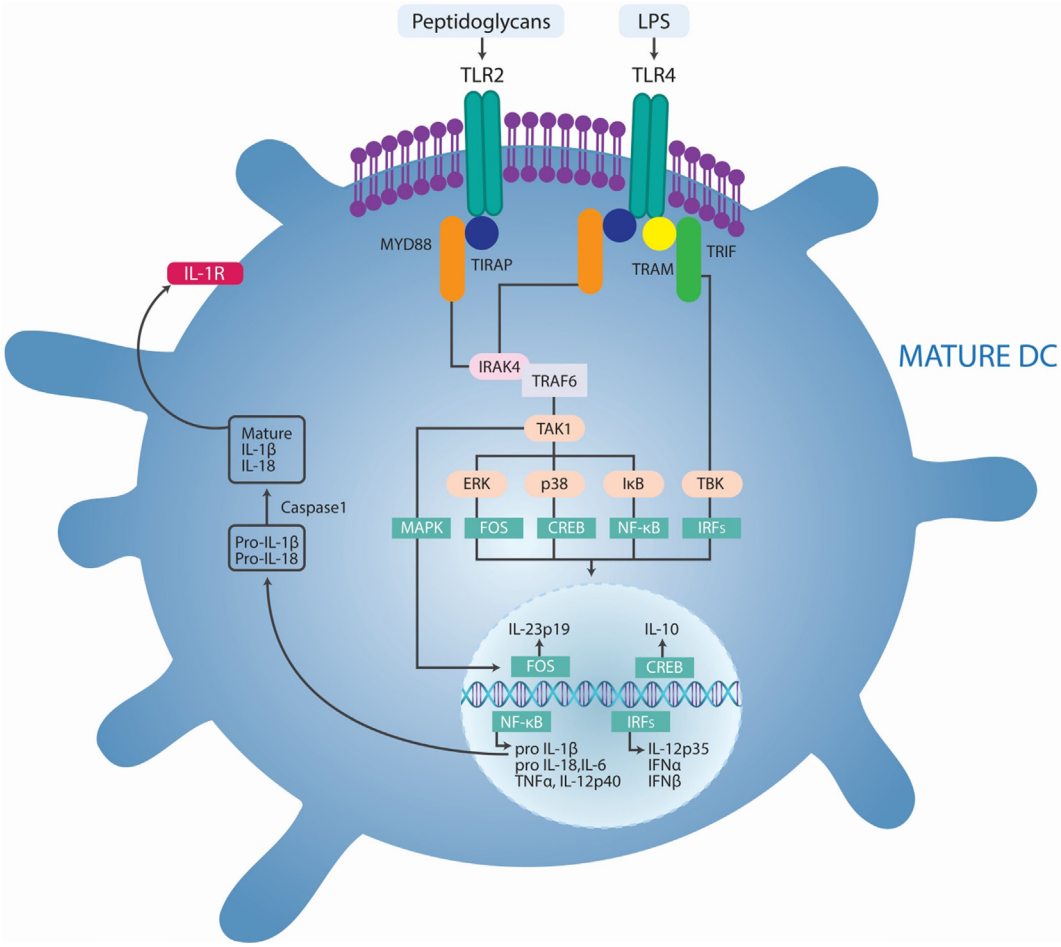

FIGURE 3 | Pathogens-induced TLR signaling cascade in innate immune cells may exacerbate autoimmune diseases. Lipopolysaccharide (LPS) binds to TLR4 and peptidoglycans bind to TLR2, which initiates signaling by recruiting the adaptor proteins myeloid differentiation primary response protein 88 (MYD88), the TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor protein inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM). MYD88 associates with IL-1R-associated kinase 1 (IRAK1) and IRAK4 and recruits TNFR-associated factor 6 (TRAF6). This complex recruits TGFβ-activated kinase 1 (TAK1), leading to phosphorylation of NF-κB inhibitor (IκB), activation of nuclear factor-κB (NF-κB), and consequent transcription of a range of genes coding for proinflammatory cytokines, including tumor necrosis factor (TNF), interleukin-6 (IL-6), pro-IL-1β, and pro-IL-18. In addition, TLR agonists activate the interferon regulatory factor (IRF) pathways, leading to the production of IL-12p35 and type I IFNs. TLR-induced activation of TAK1 also results in phosphorylation of mitogen-activated protein kinases (MAPKs), including p38 and extracellular signal-regulated kinase (ERK). Phosphorylation of ERK promotes the transcription of the gene encoding IL-23p19 and subsequent IL-23 synthesis. Phosphorylation of p38 activates cAMP response element-binding protein (CREB), leading to IL-10 production. IL-1β activates its own receptor, which also signals via the same TLR pathway components to produce IL-1β. Adapted from Ref. (35).

cell surface major histocompatibility complex (MHC) class II, a crucial component in developing innate immunity. Another danger signal hypothesized to enhance the adjuvanticity of alum is host cell DNA that is released from necrotic cells (49, 50). In susceptible individuals, aluminum-based adjuvants can induce AD although this is rare. ADs correlating with alum-based vaccinations encompass conditions, such as RA, type 1 diabetes mellitus (T1DM), MS, and systemic lupus erythematosus (SLE) (**Figure 4**) (45).

Subcutaneous injection of mineral oil has been shown to promote anti-chromatin/DNA autoantibody production even more efficiently than squalene or incomplete Freund adjuvant. This suggests that different types of autoantibodies could be produced in response to different hydrocarbons (51). Moreover, experimental models demonstrated that adjuvant oils induced T-cell-dependent polyarthritis (52).

Silicones are a family of synthetic polymers sharing a silicon oxygen chain with varying organic side groups. The link between silicone and immune-mediated diseases has been reported in the past and is one of the cornerstones of ASIA (53). Silicone is a component of breast implants. Although there is no clear association between silicone and overt ADs (45), patients with severe immune-mediated reactions to implanted silicone devices were found to have increased immunoglobulin (IgG) in the surrounding tissue and higher levels of anti-silicone antibodies compared to asymptomatic implanted patients (54). An adjuvant action linking breast implants with development of autoantibodies has been hypothesized (55). Murine CIA model and the MRL

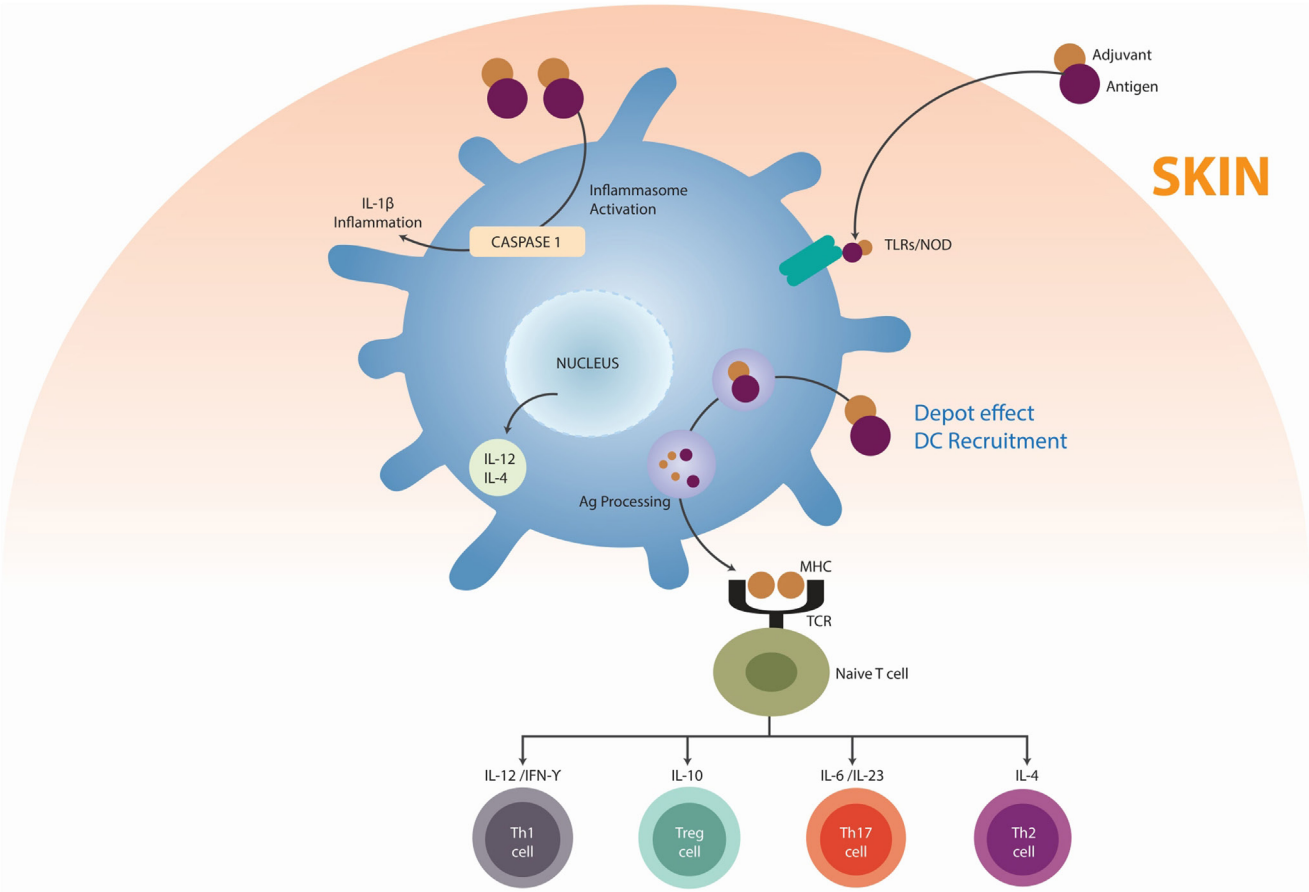

FIGURE 4 | Mechanisms by which adjuvants trigger autoimmunity. Adjuvants (mainly alum) may function as delivery systems by generating depots that trap antigens at the injection site, providing slow release in order to continue the stimulation of the immune system, thus enhancing the antigen persistence and increasing recruitment and activation of dendritic cells (DC) (depot's effect). Other adjuvants, essentially ligands for pattern recognition receptors (PRR), act by inducing the innate immunity by targeting the DC via toll-like receptors (TLRs). Adjuvants can direct support antigen presentation by the major histocompatibility complexes (MHC), inducing the differentiation of a naïve T cell in T helper 1 cells (Th1), T helper 2 cells (Th2), T regulatory cells (Treg), and T helper 17 cells (Th17). Adapted from Ref. (45).

model of murine lupus showed that silicone was responsible for increased circulating levels of IL-2 in both models as well as the production of anti-DNA autoantibodies in the MRL model. Also, in the CIA model, long-term (12 months) silicone implantation resulted in an increased incidence and severity of arthritis (56, 57). Nonetheless, most of these reports indicate that silicone implants may cause a non-specific foreign body reaction, sometimes with autoantibodies.

Damage-associated molecular patterns or alarmins, which are released from dead or dying cells, can also stimulate innate immune responses that lead to autoimmunity. Ultraviolet (UV) radiation promotes necrotic cells that induce danger signals or DAMPs, thus activating TLR4. This activation may be responsible for autoimmune responses (58). Furthermore, TLR4 can bind other endogenous ligands released from damaged cells, including several extracellular matrix components, such as hyaluronic acid oligosaccharides, fibronectin extra domain A, and fibrinogen, which are able to promote the synthesis of chemokines by macrophages. Also the abnormal internalization and transport of double-strain (ds) DNA fragments from necrotic cells into endosomes could induce autoimmune responses after binding to TLR3 (31). A characteristic of SLE is the presence of nuclear debris from the impaired clearance of apoptotic cells that become self-antigens able to bind B-cell receptors, induce TLR9 expression, and activate both TLR9 and B-lymphocytes with the subsequent production of autoantibodies (59). Patients showing increased apoptosis causing the release of DNA and RNA can be sensed by TLR9 and TLR7, respectively (60). Human synovial membrane cultures from patients with RA were found to express TLR2 and TLR4 and to release endogenous TLR ligands that may contribute to destructive inflammation in these patients' joints. Another component with a role in this disease is heterogeneous nuclear ribonucleoprotein (hnRNP), an RNA- and DNA-binding complex that is a target of most nuclear antigen-specific B and T cells in systemic ADs, such as human RA and pristane-induced arthritis in rats. Moreover, hnRNP triggers TLR7- and

TLR9-mediated activation of arthritogenic DCs, which activate pathogenic T cells (**Figure 5**) (61, 62).

B-Cell Activation

A major role of cells in the B-cell lineage is to generate antibody-secreting plasma cells and memory B cells with an enhanced ability to respond to the specific initiating antigen. B cells increasingly emerge as part of a tightly regulated immune activation process with numerous intimate interactions with other immunocompetent cells that have been identified (63). Under normal resting conditions, B cells follow a tightly regulated life cycle with a large number of checkpoints at different stages (64). Dysfunctions at these tolerance checkpoints have been directly correlated with ADs in murine models (65, 66). In the bone marrow, B cells develop from stem cells through a series of precursor stages. During them, they rearrange their variable Ig genes to generate a wide range of unique antigen-binding specificities. B cells are critical for promoting ADs, including SLE (67–69), RA (70), T1DM (71), and MS (72), among others. A unique challenge to the maintenance of self-tolerance in the B-cell compartment is the BCR diversification within B cells that are recruited into T-dependent immune responses and ultimately enter the germinal center (GC) reaction. Somatic hypermutation (SHM) of the IgG variable region genes of GC B cells results in the occasional generation of clones with increased affinity for foreign antigen, these cells being specifically perpetuated and subsequently differentiating into the high-affinity plasma cells and memory B cells that provide long-term immunity. However, the random nature of the SHM process inevitably leads to the generation of self-reactive B cells in the GC that, unless somehow inactivated, have the potential to initiate autoantibody production. The fact that most pathogenic autoantibodies show the hallmarks of SHM and selection strongly suggests that failure to enforce self-tolerance in GCs may contribute to many autoimmune diseases. However, the self-reactive GC B cells are normally kept in check and are only rarely allowed to differentiate into autoantibody-producing plasma cells (73). B cells facilitate autoimmunity by not only secreting autoantibodies but also presenting auto-antigens to T cells and secreting proinflammatory cytokines (74, 75). Consequently, targeting B cells has become one of the most effective treatments for ADs

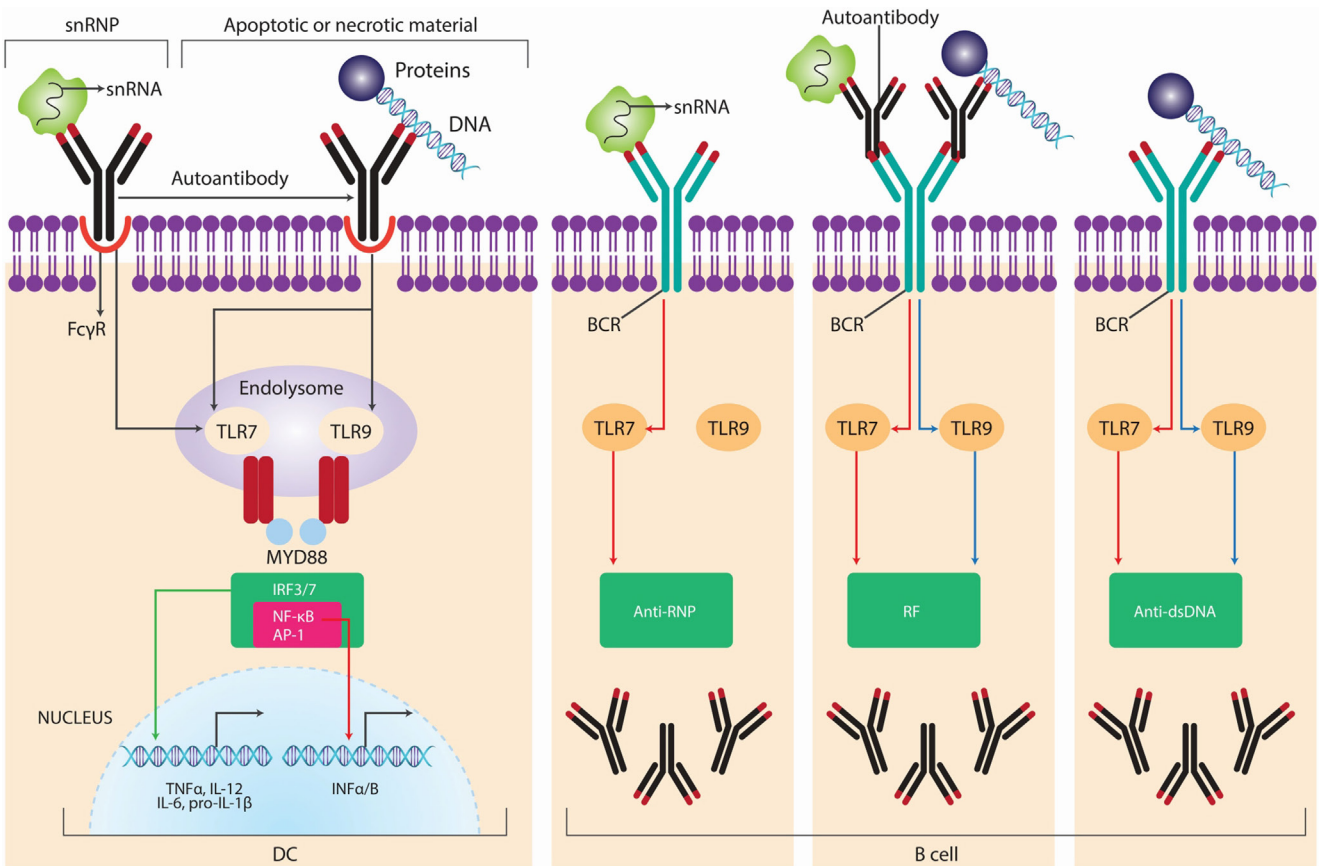

FIGURE 5 | Self nucleic acids recognition by TLRs in autoimmunity. Autoantibodies and autoreactive B-cell receptor BCRs, such as rheumatoid factor (RF) and anti-small nuclear ribonucleoprotein (snRNP) or anti-DNA antibodies mediate the access of self-nucleic acids to endolysosomal toll-like receptors (TLRs), leading to the production of type I interferon (IFN) from dendritic cells (DCs) and autoantibodies of corresponding specificities from B cells. The major snRNP antigen for anti-snRNP autoantibodies contains small nuclear RNA (snRNA), whereas the antigenic targets of anti-DNA autoantibodies are apoptotic or necrotic material containing DNA; accordingly, anti-RNP B cells are activated by TLR7 ligands, whereas anti-DNA or RF-producing B cells are activated by either TLR9 or TLR7 ligands. Adapted from Ref. (62).

(76). Given the biology of B-lymphocytes, certain environmental agents with the following effects are expected to have the potential to enhance B-cell-mediated autoimmunity: (a) agents that induce activation or expansion of autoreactive B cells, such as those that cause enhanced transcription of pro-survival molecules like B-cell activating factor (BAFF) and relaxed negative selection against autoreactive lymphocytes; (b) agents that might induce secretion of pathogenic antibodies, such as those that promote necrosis or apoptosis that may lead to the inappropriate presentation of nuclear components to lymphocytes; and (c) agents that induce secretion of proinflammatory cytokines as endocrine disruptors (66).

Sex hormones, such as estrogen and prolactin, can differentially activate autoreactive B-cell populations from different subsets, e.g., B2 cells, which are responsible for most of the high-affinity pathogenic antibody production and marginal zone autoreactive B cells. Therefore, a possible connection between B-cell-mediated and endocrine disruptors (i.e., environmental estrogens) has been proposed (77). dnMEK + CD2rtTA + (C57 BL/6 × SJL)F1 hybrid animals disclose an impaired extracellular signal-regulated kinase (ERK) resulting in increased IgG anti-dsDNA antibody in female double transgenic mice. Moreover, male mice failed to make anti-dsDNA antibody even when both transgenes were present. Therefore, the failure in males to produce pathogenic antibody must result from sex hormones or other gender-specific differences (77). Sex hormones are thought to be responsible for the female predominance observed in SLE. The role of estrogen, which influences both humoral and cellular immunity, has been widely studied, particularly in the NZB/W F1 mouse model of spontaneous SLE (78). Autoantibody formation is accelerated by estradiol treatment of the females while tamoxifen, an estrogen blocker, ameliorates disease progression. *CD40LG,* an X chromosome gene, encodes a B-cell co-stimulatory molecule transiently expressed on the surface of activated T cells and is overexpressed on T cells from women but not men with SLE (79, 80). However, the CD40–CD40 ligand interaction is crucial to the development of T-dependent immune responses. In mice, CD40L was shown to play an important role in promoting pathogenic IgG autoantibodies and kidney disease. ERK impairment has been found to result in overexpression of CD40L protein on $CD4^+$ T cells in female but not male mice, and the level of CD40L protein was twice the amount in females compared to males (79, 80). Estradiol has a central role in modulating antigen presentation, enhancing B-cell responses by increasing survival of autoreactive B cells and production of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α. RA patients have higher levels of estradiol compared to healthy controls. Animals with CIA show decreased disease activity during pregnancy, and estrogen treatment in non-pregnant CIA mice leads to an increase in IgG1 antibodies, which may explain remission during pregnancy. In mice, estrogen has been shown to inhibit autoreactive B-cell apoptosis thus increasing their survival and contributing to disease severity mainly via engagement of estrogen receptor alpha (81).

Another environmental factor associated with increased risk of autoimmunity is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Its effects on the immune system are primarily mediated through the aromatic hydrocarbon receptor (AhR). TCDD is released through incineration of waste, chlorine bleaching of paper, copper smelting, and wood burning. Nevertheless, nowadays the main source of TCDD is cigarette smoking. During prenatal development, TCDD-exposed mice displayed a clear enhanced autoimmune profile, including increased splenic $CD5^+$ and follicular B cells, increased autoantibody production, and increased autoimmune kidney lesions (82). Similar studies have been done on the effect of mercury and silver on autoimmunity in outbred mice, showing that both were able to induce B-cell activation and anti-nucleolar autoantibody production (83). These results are also supported by human studies showing that 40% of a human population chronically exposed to relatively high levels of mercury (a gold-mining community) exhibit relatively high levels of anti-nucleolar autoantibody in their sera (84).

Chronic infection by B lymphotropic virus can prime B-cell activation, proliferation, and clonal expansion, leading to select more autoreactive B cells (85). Although the mechanisms by which these infection agents can induce autoimmunity are not fully understood, recent studies have shown an accelerated immune senescence due to chronic exposure to latent viruses. Latent cytomegalovirus (CMV) infection in the context of a chronic autoimmune response induces the recently described "chronic infection phenotype" in $CD8^+$ T cells, which retains anti-infectious effector features while exhibiting autoreactive cytolytic potential (86).

T-Cell Impairment

T-lymphocytes are the ones mainly responsible for maintaining self-tolerance, but, additionally, they actively participate in the cell and tissue damage mechanisms in autoimmune-mediated diseases. Th17 are a subset of T helper cells that participate in autoimmune conditions (87). Th17 differentiation is regulated by various cytokines and is closely related to the development of Treg. Th17 differentiation is induced by TGF-β and IL-6 in mice, and by IL-1β and IL-6 in humans (88). AhR activated by its ligand regulates Treg and Th17 cell development (89, 90). Moreover, AhR is essential for Th17 development through the interference of STAT1 activation (91). TCDD is the best-known ligand for AhR. Smokers have a higher risk of autoantibody-positive RA and a more severe disease course (92). The mechanisms by which smoking influences the development and course of RA are not fully understood (See Post-Translational Modification of Self-Antigens, Cigarette Smoking and Cigarette Smoking and PolyA), but the interaction of TCDD with AhR and the subsequently impaired function of Th17 and Treg could certainly be one. Moreover, nicotine, a major component of cigarette smoke, stimulates the α7-nicotine acetylcholine receptors that have immunomodulatory effects. These receptors are expressed on several immune cells, including monocytes, macrophages, T- and B-lymphocytes, DCs, and fibroblasts found in the inflamed synovial tissue of RA patients. In a rat adjuvant-induced arthritis (AA), a model of human RA, nicotine-pretreated arthritic rats displayed upregulation of IFN-γ as well as IL-17. Furthermore, DCs of nicotine-treated arthritic rats showed altered levels of IL-6, IL-12p35, and IL-23, which are critical for differentiating Th1 (IL-12p35) and Th17 (IL-6 and IL-23) cells (92).

Moreover, the IL-27 level was significantly lower in rats with more severe AA. Although the role of IL-27 in ADs is not fully clear, a lower level of IL-27 with the increased severity of AA by nicotine-pretreatment has been reported, assigning a regulatory role to this cytokine in AA (93). Cigarette smoking affects the cell-mediated and humoral immune responses by inducing the release of TNF-α, TNF-α receptors, IL-1, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor. However, smoking has also been associated with decreased IL-6 production through TLR2 and 9, decreased IL-10 production via TLR2 activation, and decreased IL-1β, IL-2, TNF-α, and IFN-γ production by mononuclear cells (94). The release of intracellular antigens via tissue hypoxia or toxin-mediated cellular necrosis may precipitate an immune reaction in susceptible individuals. Smoking may also augment B-cell autoreactivity and stimulate the proliferation of peripheral T-lymphocytes (95).

In addition to IL-27 effects on rats with AA exposed to nicotine, other studies provide evidence of the implication of this cytokine in ADs. *Toxoplasma gondii* triggers the local expression of IL-27, dampening Th17 immunity and inducing chronic inflammation of the CNS (96). *In vitro* experiments showed that IL-27 suppressed the differentiation of Th17 cells triggered by IL-6, TGF-β, and a STAT1-dependent pathway. Although this work clearly shows the direct effects of IL-27 on the Th17 subset *in vitro*, IL-27 might also control the Th17 subset by indirect mechanisms *in vivo*. Indeed, IL-27 combined with TGF-β promotes the generation of suppressive T cells that produce IL-10. Whether these IL-10-secreting T cells can directly control the Th17 subset has not been formally proven as yet, but these observations indicate another possible level of regulation in the activity of IL-27 on the Th17 subset (96). Considering that exaggerated immune responses are also observed in IL-27 receptor-deficient mice infected with *Leishmania donovani*, *Trypanosoma cruzi*, or after helminth challenge, these studies provide a potential mechanism for controlling Th17-driven ADs by parasitic infections (97).

6-Formylindolo (3.2-b) carbazole (FICZ), a tryptophan photoproduct, is also a high-affinity ligand of AhR. Two different high-affinity ligands for AhR activation, TCDD and FICZ, can play different roles in the differentiation of Th-cells and the pathogenesis of EAE. On the one hand, FICZ, a high-affinity "natural" AhR ligand, appears to increase only the Th17 population, and therefore, worsen the severity of EAE (89). On the other hand and paradoxically, TCDD appears to expand only the Treg population and prevent EAE (90). Thus, depending on the ligand, AhR is capable of regulating both Treg and Th17 cell differentiation and either protect or worse ADs (98). A study in NFS/*sld* mice, a model of Sjögren's syndrome (SS), showed that neonatal exposure to TCDD causes disruption of thymus selection. This increases the production of Th1 cytokines, such as IL-2 and IFN-γ, from splenic $CD4^+$ T cells and the autoantibodies relevant for SS in the sera of these mice (99). Though not directly demonstrated, changes in the thymic microenvironment in these TCDD-exposed animals could compromise Treg development facilitating autoantibody production (66).

Ultraviolet photoproducts of tryptophan, such as the high-affinity ligand FICZ, could be synthesized *in vivo* through exposure of human skin to UV light (89). UV light, in particular UV-A and UV-B, can induce disease flares in patients with SLE and trigger disease onset (100). Cellular damage in dermal cells from excessive exposure to UV-B ranges from proinflammatory apoptotic to necrotic cell death. The exposure of auto-antigens and release of proinflammatory cytokines by phagocytic cells could ignite the autoimmune response by directly stimulating autoreactive B cells or by providing lupus antigens to preexisting autoantibodies (101). Overall, necrotic cell death might be an ideal adjuvant for self-immunization in a genetically predisposed individual. Likewise, it is well established that lupus immune response is mostly antigen driven. Therefore, a constant supply of self-antigen(s) is needed, which it is likely provided by cell death. Additionally, apoptotic blebs and bodies are potential sources of lupus auto-antigens and targets for autoantibodies (100).

Silica exposure is well known to cause not only pulmonary fibrosis known as silicosis but also influence ADs, such as RA (i.e., Caplan's syndrome), systemic sclerosis (SSc), SLE, and anti-neutrophil cytoplasmic autoantibody (ANCA)-related vasculitis/nephritis (102, 103). Chronic exposure to silica particles activates T-responder cells (Tresp) and Treg. The activated Tresp enter the peripheral $CD4^+$ $CD25^+$ fraction and activated Treg are lost earlier from this fraction due to apoptosis resulting in a reduced inhibitory function. Chronically activated Tresp, in turn, may express Fas-mediated apoptosis inhibitory molecules, such as soluble Fas and so survive longer (104). These fractions may contain autoreactive clones and progress to subclinical manifestation of ADs. These cellular events also increase sIL-2R, reported in ADs as a marker of chronic activation of autoreactive T cells (103).

Post-Translational Modification of Self-Antigens

Most proteins consist of only 20 amino acids (aa), but when considering PTMs, a protein may consist of more than 140 aa. PTMs are defined as covalent modifications occurring in a specific aa in a protein, and include acetylation, lipidation, citrullination, phosphorylation, methylation, and glycosylation. A variety of chemical modifications may be present in a protein, and these modifications are time- and signal dependent. PTMs may arise either by enzymatic modification or spontaneously. Under physiological conditions, proteins are post-translationally modified to carry out a large number of cellular events, from cell signaling to DNA replication. However, an absence, deficiency, or excess of these PTMs can lead these "new" proteins to trigger autoimmunity (105).

The strongest association of an external environmental factor with RA observed so far is smoking, which contributes to local inflammation in the lungs (106). Inflammation, in turn, leads to apoptosis or necrosis of lung cells, calcium release, and activation of peptidylarginine deiminase (PAD) enzymes. PAD enzymes cause a specific PTM called citrullination. This occurs as part of normal intracellular homeostasis, but extracellular citrullination of proteins in tissues is characteristic of many if not all inflammatory conditions (107). Citrullination changes an arginine for a citrulline by deamination of the former. The basic charge conferred by arginine changes into a neutral site in the peptide, an event that may alter the tridimensional conformation of the protein (108).

These citrullinated proteins may subsequently trigger an immune response by binding to HLA-DRB1 shared epitope (SE) molecules (i.e., a 5-aa sequence motif in the third allelic hypervariable region of the HLA-DRβ chain associated with RA) on DCs leading to activation of pathogenic T and B cells, and ultimately promoting anticitrullinated protein autoantibody (ACPAs) formation. Several citrullinated proteins from fibrinogen, vimentin, α-enolase, and collagen type II constitute ACPA targets. Moreover, ACPA molecules contain additional N-linked glycans, and there is strong evidence that the introduction of N-linked glycosylation sites is a result of SHM and that the presence of these unusual sugars can modulate the binding to citrullinated antigens. This thought is sustained by the high rate of non-synonymous SHM detected in ACPA sequences (109). In particular, of the mammalian PADs characterized, PAD-4 may be associated with RA as they express in affected joints (110, 111). Polymorphisms and haplotypes at *PADI4* gene influence the risk of developing RA (112). Although the association is slight, the functional effect is strong.

Klareskog et al. showed that smoking was strongly associated with increased risk of RA in those patients expressing ACPAs, a highly specific serologic RA marker (113). To further elucidate the relevance of this gene–environment interaction circle, the odds ratio (OR) of RA risk was 21 in those individuals bearing a double copy of the HLA-DRB1 SE allele compared to 6 in patients bearing only one copy (114). The presence of HLA-DRB1 SE is the strongest known genetic factor for susceptibility to RA. The possibility that other non-HLA genes may also interact with smoking to increase susceptibility to RA is supported by Mattey et al. study showing the association between smoking and disease severity in those patients bearing a polymorphism at the glutathione S-transferase (GST) M1 locus (115). GST is important in detoxifying the xenobiotic atherogen generated by smoking. Smoking showed interaction with another major susceptibility gene in conferring risk of RA, the *PTPN22* R620W allele (108).

Using lipstick and hair dye has been associated with SLE (101). Lipstick contains several chemical compounds, such as eosin and phthalates, which have been shown to induce photosensitivity and lupus flares, the production of anti-dsDNA antibodies, and progression of renal disease in NZB/W F1 mice, respectively. This is partly and potentially due to the breach of immunological tolerance by molecular mimicry (116). While the risk of inducing lupus by hair dye containing aromatic amines is theoretically present, it is largely refuted by large observational studies in human lupus (25). Mechanistically, the potential lupus-inducing effect of aromatic amines is related to the fact that the amines are metabolized by acetylation, a process that shares a pathway similar to that of hydralazine (101). Thus, among slow acetylators, the accumulation of aromatic amines might induce SLE in genetically susceptible individuals.

Epigenetic Modifications

Epigenetics is the study of changes in gene expression due to environmental influence without alterations in the underlying genetic sequence. Epigenetic modifications are generally classified into three main groups: cytosine genomic DNA methylation, modification of various side-chain positions of histone proteins, and non-coding RNA feedback (4).

Systemic lupus erythematosus is perhaps the best-studied AD with regard to epigenetic modification. Discordance noted in early monozygotic twin studies as well as racial and geographic variations in prevalence suggest a strong interaction between the environment and underlying genomic code in SLE (117). Environmental agents, such as cigarette smoke, mercury, silica, UV light, viral infections, and medications, have been demonstrated to induce oxidative stress. This, in turn, has been shown to inhibit and/or decrease the DNA methyltransferase 1 (DNMT1) level, thus reducing DNA methylation in $CD4^+$ T cells and enhancing autoimmunity (118). In fact, increasing evidence supports reactive oxygen species being not only proinflammatory byproducts of cellular responses to infectious or inflammatory stimuli but also fine-tuning regulators of autoimmune responses varying in time, location, and extent of reactive oxygen species production (119). DNA methylation refers to the methylation of cytosine bases occurring in CpG pairs to form deoxymethylcytosine (dmC) and is a repressive modification. DNA methylation serves to not only help stabilize chromatin but also silence genes inappropriate to the function of any given cell, but for which the cell expresses transcription factors that might otherwise drive gene expression (118).

T cells from patients with active lupus have decreased total dmC content and decreased DNMT1 transcripts relative to patients with inactive disease and normal controls (120). During mitosis, the level of DNMT1 is regulated partly by the activated ERK signaling pathway. Patients with active lupus have impaired T cell ERK1/2 phosphorylation, and the degree of impairment is proportional to disease activity (121). The deficient ERK pathway contribution to lupus has been further supported in mouse models by the finding that inhibiting T cell ERK pathway with a MEK inhibitor could induce autoreactivity *in vitro* and lupus-like autoimmunity in mice by promoting DNA hypomethylation and overexpression of methylation-sensitive genes (122). Decreases in ERK pathway signaling are concomitant with a diminished PKCδ phosphorylation. This direct link between PKCδ and ERK suggests that abnormalities in the PKC–ERK pathway from impaired PKCδ activation may contribute to human lupus through effects on DNA methylation in T and perhaps other cells (101, 123) (**Figure 6**).

Demethylated, autoreactive $CD4^+$ T cells overstimulate antibody production by B cells and kill macrophages, thus releasing apoptotic nuclear material that stimulates lupus-like autoantibodies (124). Moreover, in $CD4^+$ T cells, ITGAL (CD11a) is an integrin involved in both costimulation and cellular adhesion that, upon dimerization with CD18, forms leukocyte function-associated antigen 1. CD11a is overexpressed in SLE patients' $CD4^+$ T cells and correlates with disease activity (125). The upstream ITGAL promoter is demethylated in active lupus patients compared to controls, the degree of demethylation correlating with disease activity. $CD4^+$ T cells from patients with active lupus were also found to overexpress perforin, CD70, CD40L, and KIR due to demethylation in the respective promoters (118). CD40L is encoded on the X chromosome, so T cells from women have one methylated and one unmethylated CD40L gene, while T cells from men have only one unmethylated gene (79). Furthermore, injecting experimentally demethylated $CD4^+$

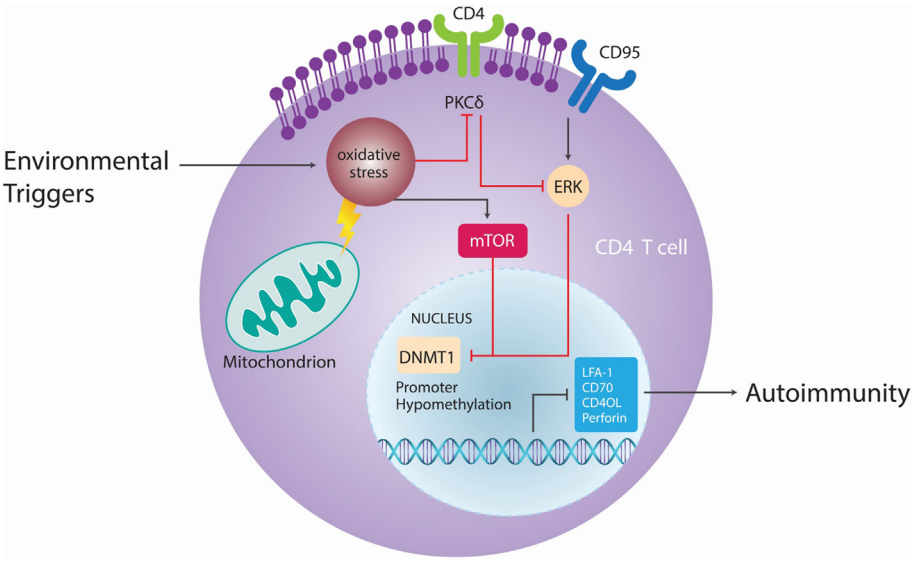

FIGURE 6 | Epigenetic autoimmune ecology pathways. Different environmental factors, such as cigarette smoke, chemicals, UV light, and viral infections cause oxidative stress, which lead to activation of the mammalian target of rapamycin (mTOR) pathway that can directly inhibit DNA methyltransferase 1 (DNMT1). Oxidative stress contributes in $CD4^+$ T cells to site-specific decrease in phosphorylation of protein kinase C (PKC)δ T^{505}, leading to functional loss of PKC and parallel reduction of extracellular signal-regulated kinase (ERK) phosphorylation. As a result of ERK activity, expression of DNMT1 is reduced, DNA hypomethylation occurs and $CD4^+$ T cells become autoreactive. Adapted from Ref. (101).

TABLE 4 | DNA methylation process in autoimmune diseases.

Disease	DNA methylation
SLE	T cells hypomethylation, IFN signature, hypomethylation of CD70, CD11a, CD40L, perforin
RA	T cells and CD40 hypomethylation, synoviocytes hypermethylation
SSc	T cells and fibroblasts hypomethylation, Wnt pathway genes hypermethylation

RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; IFN, interferon.

T cells into syngeneic mice causes anti-DNA antibodies and a lupus-like disease (118).

After exposure to UV-B irradiation, the growth arrest and DNA damage-induced 45a GADD45a levels are increased and accompanied by high levels of CD11a and CD70. The levels of GADD45a are inversely proportional to DNA methylation levels in human lupus $CD4^+$ T cells. The transfection of GADD45a *in vitro* is sufficient to increase expression of these methylation sensitive genes and induce autoreactivity (126). Furthermore, increases in oxidative stress lead to activation of the mammalian target of rapamycin (mTOR) pathway, which can directly inhibit DNMT1 (127).

The importance of epigenetics in initiating and perpetuating autoimmunity is summarized in **Table 4** (117). However, there is no clear evidence of the environmental agent involved in each of the epigenetic mechanisms known for these ADs. Despite great efforts and many studies, several environmental agents have been associated with a variety of ADs, but little is known regarding the specific cellular and molecular mechanisms involved for each causal environmental agent.

EPIDEMIOLOGICAL BASES OF AUTOIMMUNE ECOLOGY

Microbiota

There are different microorganisms that populate the gut known as intestinal microbiota. This process starts at the time of delivery and breastfeeding, and it plays an important role in the homeostasis of the human body. Indeed, there are microorganisms in the microbiota that produce enzymes and molecules, which are not generated by human beings. Therefore, microbiota is important to normal metabolism because it is capable of degrading the different components in food (128, 129). However, microbiota may also influence other systems that do not seem to be related to the gut, such as the immune system. Moreover, it actively participates in the education of the immune system. For example, the development of Th17 and Treg lymphocytes is highly dependent on the interactions of commensal bacteria with host cells in the intestine. That is why it is possible to establish a connection between microbiota and autoimmunity or inflammatory disorders (128–131).

Studies of germ-free (GF) animal models have demonstrated deficiencies in their immune system. It is noteworthy that microbiota is the first barrier against pathogenic microorganisms. They may produce molecules against the pathogens during infection because they occupy the same niche, thus competing for the same place. GF mice show deficiencies in T-lymphocyte differentiation within the lamina propria in the presence of IgA in mucosal layers and alterations in the homeostasis of Th populations (Th1, Th17, and Treg). Furthermore, GF mice spleens show few GCs that indicates abnormal development and maturation of cells in the lymphoid follicles (128–130). As a result, the cytokine production

and the normal maturation process of immune cells are greatly affected (129).

Microbiota varies from one individual to another and even in the same individual over the course of his life. However, there are many factors that influence the composition of microbiota. Initially, newborns are sterile, and their microbiota depends on maternal transfer at the time of delivery, breastfeeding, and skin contact with the mother. For example, there are differences between children that were born by natural delivery and cesarean section, and also between children that were breastfed and those fed with formula. The latter group in both cases is colonized by potential pathogens and, in contrast with first group, they present a lack of beneficial commensal microorganisms (129, 132). Nevertheless, their own genetic background plays an important role in determining the microbiota before contact with the mother's microbiota. For example, animal models with gene alterations related to the innate immune system have problems in the signaling pathways associated with pattern recognition receptor (PRR) recognition (e.g., TLRs and NODs). These models also have important differences in the pattern of microbiota microorganisms compared to wild-type animals (128–130).

In addition, the evolution of medical treatment, stress, and quality of life influence microbiota development. For example, antibiotics are the treatment for infectious diseases, but their use is linked with the loss of beneficial microorganisms. Moreover, they alter the microbiota ecology because they affect the equilibrium between different bacterial species in the gut, which allows opportunistic pathogens to attack the body (129). Finally, diet plays a central role in the homeostasis of the microbiota because it defines which microorganisms can survive in the gut due to differences in the preferences of microorganisms for energy sources. Thus, diet composition is extremely important in microbiota maintenance. Components from plants are the energy source for beneficial microbes and promote their growth over other microbes. It has been suggested that differences in the modern western diet could be causing the rapid increase in diseases, such as asthma (129, 133). For example, one study shows how a switching from a low fat, vegetable-rich diet to a high fat, high sugar diet could alter the microbiota within 1 day (129, 134).

Altogether, there is a lot of variability in microbiota between individuals, and it also varies based on the anatomic area of the human body (135, 136). Fluctuations in microbiota population have been described in patients with ADs (128). In addition, most of the studies have established a relationship between the microbiota inhabiting the gut and its influence on health. Usually, microorganisms that live in the gut are not pathogenic under healthy conditions, and they have a positive effect on the host (130). Nevertheless, some commensal bacteria may drive the preferential development of Treg, while others promote Th17 response and inflammation. These bacteria favor the production of regulatory molecules and cytokines, e.g., Foxp3 and IL-10, which characterize the regulatory cells, Treg in particular. Specifically, species such as *Bacteroides fragilis* and the genus *Lactobacillus* and *Bififobacterium* greatly promote the presence of Treg in the gut. By contrast, a pathogenic phenotype characterized by Th17 response and proinflammatory cytokines is promoted by segmented filamentous bacteria, such as *Firmicutes* (**Figure 7**). Moreover, it has been demonstrated that this kind of bacteria is able to induce the production of IgA in the small intestine. Th17 response certainly has its own positive role in the case of infection control, but it is also critical in the development of inflammatory and autoimmune diseases (128–130, 135).

Any change in microbiota could induce a dysbiosis and a pathological event. At present, the use of antibiotics, such as ampicillin, gentamicin, and vancomycin, may affect the balance toward bacteria that stimulate the Th17 response. At the same time, the consumption of a diet rich in sugar and fat favors a rise in the population of *Firmicutes* and a reduction in the *Bacteroidetes* (128, 129, 135). Finally, the influence of microbiota on the immune balance does not depend exclusively on the presence of the microorganisms, but it also depends on molecules that are produced by microbes and can stimulate specific pathways associated with immune tolerance (129). An example is the anti-inflammatory effect of the short-chain fatty acids, e.g., acetate, propionate, and butyrate. These fatty acids can bind to the G-protein-coupled receptor (GPR43 and GPR120) that is expressed in most of the innate immune system cells and, thus, produce an anti-inflammatory action (129). The anti-inflammatory events induce the maintenance of the epithelial barrier, regulate apoptosis, diminish oxidative DNA damages, and regulate cytokine production, phagocytosis, and neutrophil recruitment. At the same time, the peptidoglycan and the polysaccharide A teach the immune system to recognize potential pathogenic bacteria, and they also help in the correct development of balanced T-cell response in the gut. This balanced response promotes cell–cell interaction by modification of protein expression in immune cells (129).

Different studies have shown the microbiota as a changing ecosystem that has many factors in the model (136). Most of these studies try to determine what these microorganisms are and how they vary over time. However, the study of these microorganisms represents a challenge in terms of methodology because their identification was done by culture, and most of them cannot be grown in laboratory conditions. Therefore, most of them are now being studied with the help of DNA high-throughput analysis. Indeed, analysis of the entire DNA allows the study of all the organisms present in a sample (137, 138).

Due to the current metagenomic results (i.e., the study of the genomes in a specific environment) (139, 140), the analysis of the microbiota is moving forward and new OMICS have been identified. However, the real involvement in host systems does not depend exclusively on the presence of a specific microbiome. For instance, metagenomics would be just a representation of what these microorganisms are. Moreover, molecules produced by microorganisms can interact with the host at different levels leading to diverse outcomes. In consequence, it is important to study gene expression (metatranscriptomics), proteins (metaproteomics), and metabolites (metametabolomics) from a microbiome. These analyses, including possible interactions within the host system and under certain conditions can settle the real mechanisms by which specific microorganisms (present in a particular microbiome) may influence the host system and, thus, lead to complex disease modulation, such as ADs (138).

There are different strategies for analyzing data. Currently, the sequencing of the 16S rRNA gene has made it possible to describe

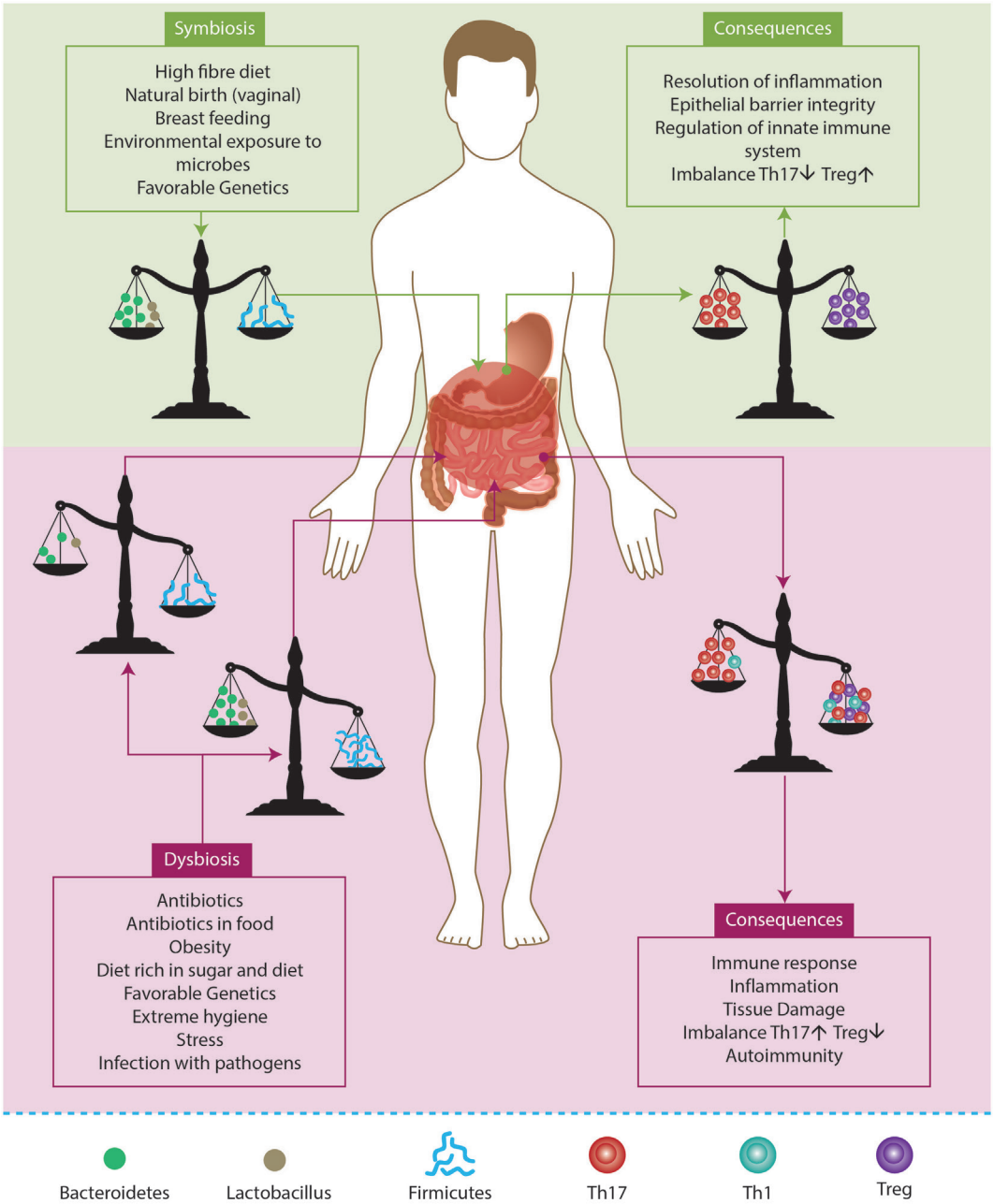

FIGURE 7 | **Factors influencing gut microbiota**. A balancing microbiota in symbiosis influences the equilibrium between T regulatory cells (Treg) and effector T cells (Th1, Th17). As a consequence, the gut can respond to an infection but this also leads to the permanence of microbiota and favors the host. By contrast, if the environment induces the rupture of the equilibrium, it induces a state of dysbiosis. Hence, the percentage of anti- inflammtory microorganisms (e.g., *Bacteroidetes* and *Lactobacillus*) may be lower than proinflammatory microorganisms (e.g., *Firmicutes*). Eventually, this may lead to an inflammatory profile that favors the development of autoimmunity. Adapted from Ref. (129, 130).

prokaryote taxonomic diversity. This gene has been used for this type of analysis for three main reasons. Primarily, it is present in every population member due to its essential role in protein translation in all prokaryotes. Second, 16S rRNA always and only differs between individuals with different genomes. Third, it is considered an evolution marker between species because its function is extremely important, so any change in its sequence can be potentially lethal. Therefore, the 16S rRNA sequence analysis brings us to the construction of operational taxonomic units (OTUs) based on the percentage of similarity between sequences. Then, comparison between multiple databases leads to the identification of species within a sample. Finally, the population becomes a 16S rRNA sequence collection where the number of unique sequences represents the number of microorganisms (136).

Furthermore, high-throughput DNA sequencing technologies have recently become very useful for metagenomic analysis (i.e., pyrosequenciation). Thus, different strategies for different types of genome assembly and annotation within a sample are used to ensure an accurate description (141). In addition, potential proteins encoded in each genome can be analyzed based on DNA sequence. As a result, it is possible to assign a role or function for a specific microorganism within the microbiome based only on their genome capabilities (137).

The relationships between infection and ADs and its main mechanisms have been outlined (i.e., host–guest interaction). Nonetheless, most of the interactions and mechanisms that influence this relationship are still unknown. Microorganisms may alter and deregulate gene transcription, translation, and human metabolic processes. This means that the effect induced on the host by a microorganism is not caused by the presence of the microorganism itself but also by the metabolic and genetic polymorphism of the microorganism. In particular, intracellular pathogens may have a direct influence on gene regulation and protein expression inside host cells (131, 132, 142).

In the last few years, most of the large cohort studies have evaluated genetic factors that may predispose to ADs. In addition, expression and proteomic analysis have also been done, and most of them have tried to find a way to establish predictor genetic factors for the diseases. However, these studies do not take into consideration the DNA, RNA, and proteins from microorganisms that could be considered potential "contamination," and which should be considered a source of information that would help complete the overall picture of molecular interactions between infection and ADs and make it understandable (132, 143, 144).

Last but not least, familial autoimmunity and polyautoimmunity should be incorporated into the study of infection and ADs (145, 146). ADs may be associated with a specific family group exposed to a particular environment. Within this environment, family members will be in contact with the same microorganism, and they will develop the same microbiota. As a new common mechanism for ADs, microbiota can be "inherited" from parent to child, and it can also be shared among siblings (135).

Lifestyle Habits and Autoimmunity

Cigarette Smoking

Cigarette smoking has been widely associated with cardiovascular disease (CVD), pulmonary disease, cancer, and increased overall mortality. The association with ADs has also been extensively studied and confirmed based on the direct tissue damage and inflammatory response to tobacco (92).

For RA, a clear causal relationship between tobacco and RA risk was established in 1987 even though previous studies had already recognized its association with RA comorbidities (147–149). Since then, many case-control and cohort studies have evaluated and confirmed this relationship (150–154). Additionally, cigarette smoking was found to increase the risk of seropositive RA (rheumatoid factor (RF) and/or ACPA) both in Caucasians and Latin Americans (155–157). Tobacco also influences the phenotype or clinical expression of disease, and is related to earlier onset of disease (158, 159). For instance, smokers have a worse outcome with greater disability and disease activity, and have usually taken significantly more disease-modifying antirheumatic drugs (DMARDs) combinations or biologics than non-smokers (155, 158, 160).

A dose–response association between cigarette smoking and RA development was also confirmed by a recently published meta-analysis. Three prospective cohort studies and seven case-control studies, including 181,100 subjects and 4,552 RA cases were considered for analysis. Compared to non-smokers, patients who smoked 1–10 pack-years presented an increased risk for RA [relative risk (RR): 1.26, 95% CI: 1.14–1.39]. Those who smoked more than 20 pack-years had an even higher risk, with a RR of 1.94 (95% CI: 1.65–2.27) and, for those with more than 40 pack-years, the RR was 2.07 (95% CI: 1.15–3.73) (161).

A systematic review and meta-analysis on the influence of cigarette smoking on disease activity and joint erosions in RA showed a negative association between smoking and EULAR response (OR: 0.72, 95% CI: 0.57–0.91; $p = 0.005$) and DAS28 remission (OR: 0.78, 95% CI: 0.63–0.96, $p = 0.023$). DAS28 was significantly higher in current smokers [mean difference (MD): 0.29, 95% CI: 0.12–0.44, $p < 0.001$] as well as erosive score [standardized mean difference (SMD): 0.38, 95% CI: 0.04–0.72, $p = 0.028$]. Data were ambiguous for erosion progression during follow-up (OR: 0.93, 95% CI: 0.72–1.2; $p = 0.59$) (162).

As for RA, cigarette smoking is also associated with increased risk of SLE. A meta-analysis showed that for current smokers, compared to non-smokers, the odds for developing SLE were significantly higher (OR: 1.50, 95% CI: 1.09–2.08) (163). Additionally, current smokers with SLE are more likely to have anti-DNA antibodies than non-smokers (164). This last finding is related to the evidence of increased disease activity (i.e., SLEDAI score) observed in SLE patients that are current smokers compared to non-smokers (165). Furthermore, cigarette smoking (i.e., ever smoking) has been associated with CVD in patients with SLE (166). When smoking and coffee factors were combined, the association with cardiovascular risk in SLE remained statistically significant [adjusted odds ratio (AOR): 1.82, 95% CI: 1.05–3.13, $p = 0{,}03$].

Environmental exposure (i.e., ever smoke, coffee consumption, silicone implants, organic solvents, hair dye, and pesticide exposure) influences the risk of developing lupus nephritis (LN) in a Latin American population when examined by latent variable analysis using the two-parameter logistic item response theory model (167).

Exposure to tobacco smoke has also been associated with a higher incidence of other AD, such as MS. Cigarette smoking increases the risk of progressive disease from the moment of diagnosis. Also, current smokers progress from relapsing-remitting disease to secondary progressive disease faster than non-smokers (168, 169). Regarding autoimmune thyroid disease (AITD), a meta-analysis was done including eight observational studies that showed an association between Graves' disease (GD) and current and ever-smoking status, with an OR of 3.02 (95% CI: 2.09–5.22) and 1.9 (95% CI: 1.42–2.55), respectively. An association between Hashimoto thyroiditis and tobacco was also found (OR: 1.92, 95% CI: 1.25–2.93) (170).

Furthermore, a strong association has been found between primary biliary cirrhosis (PBC) and smoking in three large

case-control studies (171–173). In addition, cigarette smoke accelerates the progression of PBC (174), all of these pieces of evidence raising the issue of the influence of tobacco on Th1 response.

In conclusion, cigarette smoking is one of the best-recognized environmental factors associated with AD. Different outcomes have been related to this association: disease onset, progression, activity, and treatment response. Relevant evidence available is summarized in **Table 5** (175).

Alcohol Consumption

Multiple studies have evaluated the relationship between alcohol consumption and the risk of ADs, especially regarding RA and SLE. Most of the available evidence has come from case-control studies, which show a reduced risk for both RA and SLE with a low-to-moderate alcohol intake (157, 176–180). By contrast, some case-control studies (181, 182) and a prospective cohort analysis (183) have shown no association between alcohol and autoimmunity.

Since the evaluation of lifestyle impact on disease by case-control studies may present a recall bias, two prospective studies have been done on the evaluation of alcohol and RA. The first was subdivided into two large cohorts, the Nurses' Health Study NHS and NHSII, showing 580 incident cases of RA in 1.9 million person-years from 1980 and 2008 and 323 incident cases of RA in 1.78 million person-years from 1989 to 2009. In these cohorts, the pooled adjusted hazard ratio (HR) for moderate alcohol use in seropositive RA was 0.69 (95% CI: 0.50–0.95) indicating a protective effect of alcohol consumption (184). In the second cohort study, 34,141 women were followed from 2003 to 2009 with 197 incident cases of RA identified. Here, there was a statistically significant 37% reduced risk of RA among women who drank more than four glasses of alcohol per week, compared to those who drank less than one glass per week or non-drinkers (RR: 0.63, 95% CI: 0.42–0.96) (185).

Additionally, two meta-analyses for RA and one meta-analysis for SLE have been done to evaluate risk associated with alcohol intake. For RA, one of the meta-analyses included nine studies (six case-control and three cohorts) comparing drinkers vs. non-drinkers and finding an adjusted OR of 0.78 (95% CI: 0.63–0.96). The protective effect was stronger in seropositive RA (0.52, 95% CI: 0.36–0.76) than that in seronegative RA (0.74, 95% CI: 0.53–1.05). Subgroup analysis by study design identified a significant association in case-controls but not in cohort studies (186). The second study considered eight prospective studies that contained 195,029 participants and 1,878 RA cases. Dose–response meta-analysis of the study data revealed that, compared to no-alcohol consumption, the adjusted RR was 0.93 (95% CI: 0.88–0.98) for 3 g/day of alcohol consumption, 0.86 (95% CI: 0.76–0.97) for 9 g/day, 0.88 (95% CI: 0.78–0.99) for 12 g/day, 0.91 (95% CI: 0.81–1.03) for 15 g/day, and 1.28 (95% CI: 0.94–1.73) for 30 g/day, indicating an inverse association between low-to-moderate alcohol intake and RA risk (187). For SLE, six case-control studies and one cohort study were included for analysis, and an overall significantly protective effect was found when considering SLE patients treated for <10 years (OR: 0.72, 95% CI: 0.55–0.95) (188).

Despite the evidence showing the protective effect of alcohol in two common ADs, it should be remembered that the evidence for the harmful effects of alcohol remains stronger than its benefits (181). Thus, no promotion of alcohol intake to prevent ADs is recommended.

Coffee Consumption

A few studies have been done to examine the effect of coffee on the risk of ADs. A meta-analysis evaluated the association between coffee or tea intake and the risk of RA. This analysis included five studies (three cohorts and two case-control) involving 1,279 cases of RA and 133,622 controls. The final effect size showed a significant association between total coffee intake and RA incidence (RR: 2.426, 95% CI: 1.060–5.554, $p = 0.036$). When stratified by seropositivity, a significant association between coffee consumption and seropositive RA incidence was found (RR: 1.329, 95% CI: 1.162–1.522), but no association in seronegative RA (RR: 1.093, 95% CI: 0.884–1.35, $p = 0.411$) (189). Another study evaluated the relationship between coffee intake and methotrexate (MTX)

TABLE 5 | Association between smoking status and autoimmune disease onset and evolution.

Disease	Disease onset			Evolution			Reference
	Ever	Current	Past	Ever	Current	Past	
RA	POR 1.89 (1.56–2.28)[a] POR 1.27 (1.12–1.44)[b]	POR 1.87 (1.49–2.34)[a] POR 1.31 (1.12–1.54)[b]	POR 1.76 (1.33–2.31)[a] POR 1.22 (1.06–1.40)[b]	NA	EULAR response POR 0.72 (0.57–0.91)	DAS28 remission POR 0.78 (0.63–0.96)	(161, 175)
SLE	NA	POR 1.5 (1.09–2.08)	POR 0.98 (0.75–1.27)	Mean SLEDAI 15.63 (12.96–18.3)	Anti-DNA seropositivity OR 4.0 (1.6–10.4)	Mean SLEDAI 9.64 (7.61–11.67)	(163, 164)
MS	AOR 1.32 (1.10–1.60)	NA	NA	NA	Progression to SPD HR 2.5 (1.42–4.41)	NA	(168, 169)
AITD	POR 1.90 (1.42–2.55)	POR 3.30 (2.09–5.22)	POR 1.41 (0.77– 2.58)	Development of GO POR 2.53 (1.70–3.77)	Development of GO POR 2.18 (1.51–3.14)	NA	(170)

RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; MS, multiple sclerosis; AITD, autoimmune thyroid disease; POR, pooled odds ratio; AOR, adjusted ODDs ratio; HR, Hazard ratio; NA, not available; EULAR, European League Against Rheumatism; DAS28, disease activity score-28; SLEDAI, systemic lupus erythematosus disease activity index; SPD, secondary progressive disease; GO, Graves opthalmopathy. All results in parentheses correspond to 95%CI.

[a]*Results in men.*

[b]*Results in women.*

Adapted with author's permission from Ref. (4).

efficacy in RA, measured by DAS28 score, Multi-Dimensional Health Assessment Questionnaire (MDHAQ) score, and duration of morning stiffness with no statistical difference between groups (190).

Concerning SLE, a cross-sectional study was done on 310 Colombian patients to evaluate the risk factors associated with the appearance of CVD as the leading cause of mortality. The estimated AOR for coffee consumption was 1.75 (95% CI: 1.01–3.04, $p = 0.043$). Additionally, to isolate the interaction of smoking and coffee consumption, two regression models were made, showing an independent effect of coffee and tobacco on CVD. Their interaction remained significant (AOR: 1.82, 95% CI: 1.05–3.13, $p = 0.03$), thus demonstrating their synergism (166).

The association between coffee consumption and ADs has also been evaluated in latent autoimmune diabetes in adults with no statistically significant association (191). However, other studies have demonstrated the opposite relationship between maternal coffee consumption and developing advanced β-cell autoimmunity in the offspring (highest quarter vs. lowest HR: 0.62, 95% CI: 0.40–0.97, $p = 0.04$) (192).

Two studies have evaluated coffee consumption, primary sclerosing cholangitis (PSC), and PBC. The first study included data from 480 patients with PSC, 606 with PBC, and 564 healthy volunteers. Interestingly, 24% of patients with PSC had never drunk coffee compared to 16% of controls ($p < 0.05$), and only 67% were current drinkers compared to 77% of controls ($p < 0.05$). Furthermore, patients with PSC consumed fewer lifetime cups per month (45 vs. 47 for controls, $p < 0.05$) and spent a smaller percentage of their lifetime drinking coffee (46.6 vs. 66.7% for controls, $p < 0.05$). Moreover, among PSC patients with concurrent ulcerative colitis, coffee protected against proctocolectomy (HR: 0.34, $p < 0.001$). By contrast, patients with PBC and controls did not differ in coffee consumption parameters (193). The second study included 240 patients with PSC and 245 contro l subjects. The results suggested a protective effect of both cigarette smoking and coffee consumption at the age of 18 with the development of PSC ($p < 0.05$ and $p = 0.048$, respectively) (194).

Socioeconomic Status and Autoimmunity

The social environment includes social, political, economic, and cultural norms and values that result in an unequal distribution of resources that can negatively impact health. Many studies have evaluated the association between SES and chronic diseases, showing greater morbidity and mortality in people with lower socioeconomic position (195).

SES contributes to the observed difference in the prevalence of immunological disorders based on time and geographical distribution (**Figure 8**). As better public health measures have

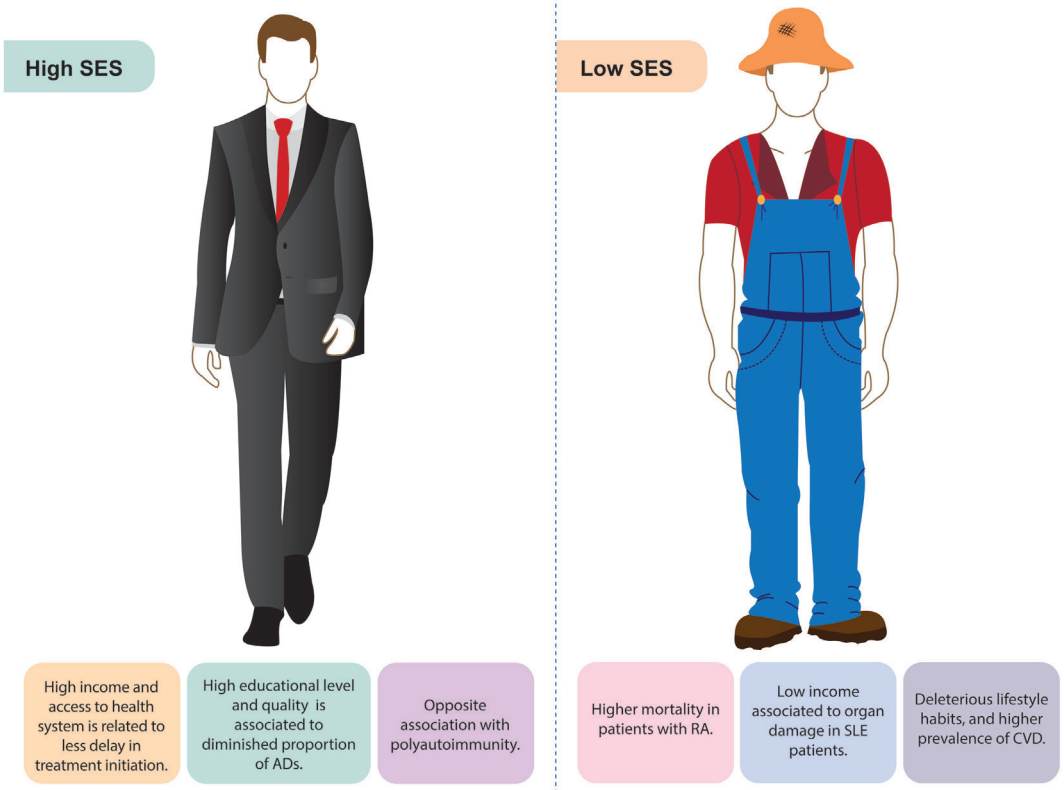

FIGURE 8 | Socioeconomic status (SES) and autoimmune diseases (ADs). Some of the main findings associated with autoimmune diseases related to high or low socioeconomic position are shown.

been taken since the industrial revolution, there has been a major decline in infectious diseases with a simultaneous increase of ADs. This has been cataloged as the hygiene hypothesis (196). According to this theory, social and environmental exposures have a direct impact on immune tolerance and response, supporting the association between SES and AD development.

In RA, different studies have reported a reduced risk as the educational and income level rise (197–199). Furthermore, a correlation was found between 11 years of education or less with a two-fold increase in poor prognosis (200). For instance, the lower the patient's educational level, the greater the chance of mortality and the lower their functional capacity (201). In terms of self-assessment, health quality is usually low in patients with RA who are older, female, less educated, obese, unemployed, and less affluent than other groups (202, 203). Many RA patients tend to be unable to work due to their low functional capacity. Thus, they become dependent on the state for health services and social welfare support (204).

A recently published study was done on 1,209 RA patients to examine the association between SES and DMARDs treatment delays, disease activity, joint damage, and disability. Disease activity was determined by DAS28, joint damage was determined from hand radiographs by Sharp score, and physical disability was determined by MDHAQ. On average, patients with lower SES waited 8.5 ± 10.2 years after onset of RA symptoms to begin DMARD treatment compared to those in the middle and upper SES who waited 6.1 ± 7.9 years ($p = 0.002$) and 6.1 ± 8.6 years ($p = 0.009$), respectively. Moreover, patients with the greatest DMARD lag showed greater DAS28, Sharp, and MDHAQ scores ($p < 0.001$) (205).

In terms of mortality, a retrospective cohort study was done on the Taiwanese population to evaluate the association of individual SES/neighborhood SES, and mortality rates in 23,900 RA patients from 2004 to 2008. Analysis revealed that 5-year mortality rates were worse among RA patients with a low individual SES compared to those with a high SES ($p < 0.001$). Additionally, patients with low SES in disadvantaged neighborhoods had the highest risk of mortality (HR: 1.64, 95% CI: 1.26–2.13, $p < 0.001$) (206).

For SLE, there is a tendency to earlier onset and worse outcomes in people of Hispanic, Asian, or African ancestry compared to Caucasians. This may be partly related to overall poor SES, including less structured families, fewer years of formal education, occupational status, household income, higher poverty, and inadequate health insurance (199). The costs of SLE can be different depending on the health care system. The impact of indirect costs is influenced by poor physical and mental health, low social support, low educational level, unemployment, and high disease activity (199, 207). Moreover, patients with elevated mortality and incidence of end-stage renal disease reported greater levels of poverty (199).

A cross-sectional study done on Mexican SLE patients evaluated the relationship between SES and organ damage. Lupus activity and organ damage were measured using the SLEDAI and Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR) scale. The analysis included 143 SLE female patients, of which 42.7% presented organ damage. Patients with organ damage had lower monthly household incomes and were more frequently unemployed. Low monthly income was not associated with lupus activity or self-reported altered health status (208).

The relationship between low SES and cardiovascular risk factors in SLE has also been evaluated. A longitudinal cohort study included 1,752 SLE patients. Regression analyses stratified by ethnicity and low income were strongly associated with most traditional cardiovascular risk factors in Caucasians, but only with smoking and diabetes in African Americans. In Caucasians, low income increased the risk of both myocardial infarction (OR: 3.24, 95% CI: 1.41–7.45, $p = 0.006$) and stroke (OR: 2.85, 95% CI: 1.56–5.21, $p = 0.001$); in African Americans, these relationships were not seen. Moreover, low education was associated with smoking in both ethnic groups (209).

For MS, African Americans have been reported to start at a younger age, in comparison to Caucasians or Hispanics. Access to neurology consultation and rehabilitation services was determined by health insurance, poverty, and living in rural areas. Additionally, important improvements in quality of life related to educational level and employment status in MS patients were identified (210, 211).

In conclusion, SES has a major impact on health as it affects biological functions. In general, low SES has been associated with worse outcomes and prognosis in ADs. The biological relevance of SES relies on ancestry and environmental exposure (199).

Sex Hormones, Gender, and Autoimmunity

Differences in AD manifestations by gender involve immunomodulation by sex hormones, non-hormonal factors encoded by genes on the X and Y chromosomes, and immunological phenomena unique to pregnancy (212). Over 75% of patients with ADs are estimated to be women, and hormones are important in regulating the onset, severity, and progression of the disease (213). In fact, AD is considered the fourth leading cause of disability for women (214).

Many studies have postulated that oral contraceptive (OC) exposure exacerbates SLE activity, but the evidence has been controversial. Two randomized controlled trials (RCTs) addressed this issue. The first RCT was single-blinded, and included 162 women with SLE who were randomly assigned to a combined OC (30 μg ethinyl estradiol), a non-eluting intrauterine device, or a progestin-only pill for 12 months. There was no statistical difference in the flare rate between the groups (215). The second RCT was double-blinded, and evaluated a combined OC (35 μg ethinyl estradiol) versus placebo for 12 months. It included 183 women with SLE with inactive disease, and analysis showed no statistical difference in flare rate (216).

A systematic review and meta-analysis evaluating the association between exposure to exogenous sex hormones (i.e., estrogens) and SLE (217), showed a significant association between hormonal replacement therapy (HRT) exposure and increased risk of SLE (RR: 1.96, 95% CI: 1.51–2.56, $p < 0.001$) (**Figure 9**). There was no association between HRT exposure and specific outcomes of disease. Six meta-analyses were run evaluating different outcomes: death, all flares, major flares, thrombosis, and coronary disease. None were significant (217). Moreover, analyses seeking an association between OC exposure

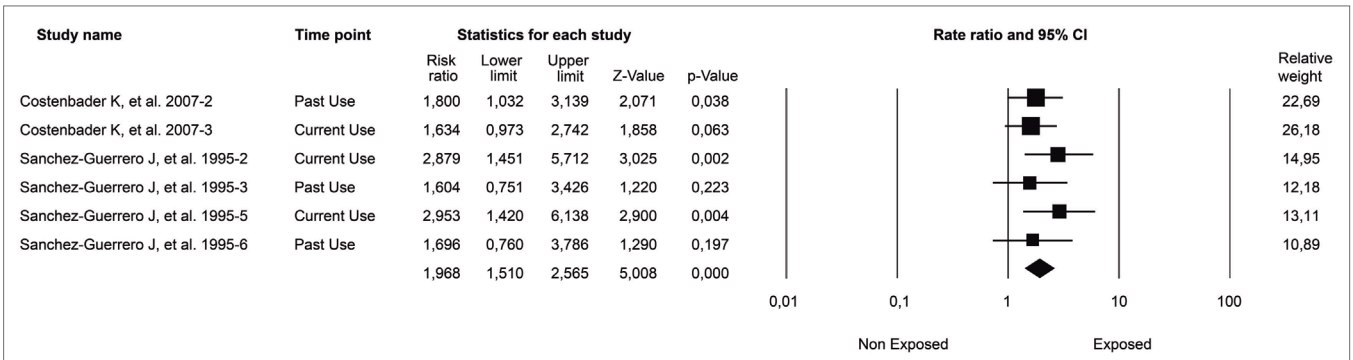

FIGURE 9 | **Association between hormonal replacement therapy (HRT) exposure and risk of developing SLE**. Forest plot of studies meta-analyzed. Final common effect size based on a random model. CI: confidence interval; SLE: systemic lupus erythematosus; HRT: hormonal replacement therapy. Adapted with authors' permission from Ref. (217).

and different SLE outcomes were not significant with the exception of a marginal result in a meta-analysis, including the SLE outcome for patients with "ever-use" status and stratified by age. In conclusion, HRT exposure increases the risk of SLE in healthy women. Considering the selection bias of the studies included in the meta-analysis, which in general, excluded women with high disease, or antiphospholipid antibodies (aPL-abs), or history of thrombosis, generalization of results in the present study was limited and the recommendations for using these on women with known SLE must be followed cautiously (217).

Another meta-analysis found no statistically significant association between OC exposure and RA risk (RR: 0.88, 95% CI: 0.75–1.03) (218).

Like SLE, SS and AITD are also highly prevalent among women (219). Several studies have recognized gender-associated characteristics of disease and severity. For instance, women with SS have more anti-Ro antibodies and Raynaud's phenomenon than men (220). Additionally, men with SS are at higher risk for lymphoma and neurological involvement than women. Accordingly, male gender is considered a risk factor for lymphoma in patients with SS (213, 221, 222). Additionally, AITD represents the main cause of hypothyroidism during pregnancy ranging between 5 and 20% in prevalence with an average of 7.8% (223).

Larger trials are required to assess the long-term effects of hormone therapy (e.g., contraception or postmenopausal) on the course of ADs and to identify patient characteristics associated with an increased risk of flares in hormone therapy exposure.

PolyA in the Context of Autoimmune Ecology

As previously mentioned, the fact that ADs share several clinical signs and symptoms (i.e., subphenotypes), physiopathological mechanisms, and genetic factors has been called autoimmune tautology (12). The clinical evidence of autoimmune tautology highlights the co-occurrence of distinct ADs within an individual and becomes known as PolyA. Although there are several studies evaluating AE through the various ADs, there are few clinical studies of specific shared environmental factors favoring PolyA (224). Some examples of specific associated factors are shown below.

Vitamin D Levels and PolyA

T1DM and MS, two organ-specific ADs, frequently occur together (i.e., PolyA) in the Sardinian population (225). Patients with MS in Sardinia have a sixfold higher risk of T1DM than healthy controls. Although the frequency of the risk HLA group (HLA-DRB1*03) associated with T1DM and MS is highly increased in Sardinia, this genetic group does not seem to account for all T1DM–MS PolyA. Rather, the environment may be the most relevant element determining this risk, perhaps in a setting where susceptibility haplotypes do not decrease the chance of co-occurrence of the two (226). Vitamin D is a shared environmental factor evaluated for these conditions. There is evidence showing a preventive role of vitamin D in MS and T1DM. A latitudinal gradient characterizes both diseases. T1DM and MS become increasingly common as distance from the equator increases and implicates common factors, such as Vitamin D deficiency (226). Although this environmental factor is shared, it has not been systematically evaluated in individuals who have this PolyA.

Recently, a study in celiac disease (CD) was done on 145 patients searching for PolyA prevalence and related risk factors. The study participants were divided into two groups. Group 1 was the CD-alone group consisting of patients without any other ADs, while group 2 was the PolyA group consisting of patients with one or more accompanying ADs. When the groups were compared in terms of demographic features and laboratory data, vitamin D deficiency was the environmental factor associated with PolyA along with the following risk factors: female gender, family history for ADs, antigliadin IgG positivity, antinuclear antibody positivity ≥1/80 titer, and any musculoskeletal disease (227).

A retrospective cross-sectional study enrolled 133 women with recurrent pregnancy loss (RPL) for a 2-year period together with laboratory experiments (228). Sixty-three of them (47.4%) had low vitamin D levels (<30 ng/ml). The prevalence of aPL-abs (anticardiolipin IgG and IgM) was significantly higher in the low vitamin D group (VDlow) (39.7%) than in the normal group (VDnl) (22.9%) (OR: 0.22, 95% CI: 1–4.7, $p = 0.05$). Furthermore, anti-ssDNA (19.0 versus 5.7%, OR: 3.76, 95% CI: 1.1–12.4, $p = 0.05$) and anti-thyroperoxidase antibody (33.3 versus 15.7%; OR: 2.68, 95% CI: 1.2–6.1, $p = 0.05$) were significantly higher in VDlow than in VDnl. Although authors did not study

the clinical confirmation of AITD, antiphospholipid syndrome (APS), and SLE through validated clinical criteria in the whole group, a serologic PolyA of AITD and SLE is displayed in a highly APS-potential group of patients (RPL with aPL-abs positivity). Since autoantibodies are predictors of disease, it is important to vigilantly following the clinical course of patients with RPL and VDlow for prediction of PolyA (APS plus AITD and SLE).

For APS and SLE PolyA, a relationship between vitamin D levels and the co-ocurrence of these diseases has also been evaluated. Serum vitamin D levels were measured in 179 European APS patients and 141 controls (229). Of these, 113 APS patients were diagnosed with primary APS (pAPS) and 66 with the previously so-called APS secondary to SLE (i.e., PolyA). The mean levels differed between patients with APS alone (18 ± 9 ng/ml) and PolyA with SLE who disclosed lower levels of vitamin D [14 ± 8 ng/ml ($p = 0.004$)]. However, upon individual analysis, both levels were significantly lower than that for the controls.

Schoindre et al. (230), evaluated serum 25(OH) Vitamin D levels in 170 patients with SLE who were prospectively followed up for 6 months. In a multivariate analysis contrasting with the previously mentioned results, absence of defined APS ($p = 0.002$) was one of the predictive factors of lower 25(OH)D levels, which showed an opposite relationship between low Vitamin D levels and PolyA with APS. These contrasting results should be analyzed carefully because there are few studies evaluating the influence of vitamin D levels in APS–SLE PolyA.

Similarly, Schneider et al. (231) evaluated 25(OH)D levels and cytokine profiles in 172 patients with SLE. There were 11 patients (6.4%) having PolyA with SS and the same number with APS. Although the main focus of the study was not the 25(OH)D levels in those PolyA patients, no associations were found between the presence of the previously so-called secondary APS nor secondary SS and vitamin D concentration.

All of the above results are linked to the fact that Vitamin D interacts with the immune system. It takes part in the regulation and differentiation of immune system cells directly and indirectly. Current data link vitamin D deficiency to many ADs, including T1DM, MS, inflammatory bowel disease, SLE, and RA. It is essential for part in the genetic regulation of cytokine production, VDR expression and affects biological processes by which these cells interact. Overall, vitamin D confers an immunosuppressive effect. Vitamin D has been shown to be clinically beneficial for animal models, and initial observations indicate that vitamin D supplementation may help prevent MS and T1DM (232). Further research is expected regarding the interaction between Vitamin D levels and PolyA.

Cigarette Smoking and PolyA

There are few or no studies designed to evaluate the relationship between cigarette smoking and PolyA. Nevertheless, this association has been evaluated as a secondary end point in studies researching PolyA globally.

A recent publication (233) analyzed the association between smoking and aPL-abs in SLE patients and explored the relationship between smoking, aPL-abs, and vascular events (arterial and venous, VE). Out of a total of 367 SLE patients evaluated, 118 were aPL-abs positive at inclusion. In regression-adjusted models, regular smoking was associated with aCL IgG, aβ2GP1 IgG, LA, and triple aPL-abs positivity. Furthermore, former and current smokers were more likely to have a history of arterial events than never smokers.

A possible explanation for these results is that smoking may contribute to oxidative alterations, which, in genetically predisposed individuals, may give rise to an autoimmune response, epitope spreading, and induction of aPL-abs resulting in the SLE–APS PolyA and its association with cardiovascular events.

There are various studies showing the independent relationship between smoking, APS or aPL-abs, and the specific cardiovascular compromise subphenotype in SLE patients. Recently, Fernández-Nebro et al. (234), identified smoking (OR: 1.48, 95% CI: 1.06–2.07) and aPL-abs (OR: 1.57, 95% CI: 1.13–2.17) among other independent variables as risk factors for CV events through a multivariate analysis. The study involved 374 SLE patients (10.9%) from a total of 3,658 patients, who suffered at least one CV event. In addition, Ahmad et al. (235) evaluated the strength of association between traditional cardiovascular risk factors and carotid plaque development in 200 women with SLE and 100 controls. When combined in a final model, classic and SLE-related factors, they showed pack-years of smoking as well as serologic PolyA (aCL and/or any LAC) among other variables (any azathioprine exposure, age, and previous arterial events) as predictors of subclinical atherosclerosis. Likewise, in a multiethnic SLE cohort (LUMINA) of 570 patients (236), 51 developed at least 1 venous thrombotic event after SLE diagnosis. Through a cox proportional hazard model, an independent association with smoking and positivity for LAC was demonstrated among other variables related to venous thrombosis.

Additionally, an independent association between APS PolyA (OR: 4.71, 95% CI: 1.81–12.2, $p < 0.001$) and smoking/coffee interaction (OR: 1.82, 95% CI: 1.05–3.13, $p = 0.03$) with CVD was previously shown in a cohort of 310 patients with SLE through a logistic regression model (166).

In a Colombian cohort of 376 patients with SLE (237), we evaluated the PolyA with autoimmune hypothyroidism and its related factors. Multivariate analysis and a classification and regression tree model (CART) were used to analyze data. After adjusting for gender and duration of disease, ever smoking (AOR: 6.93, 95% CI: 1.98–28.54, $p = 0.004$), and PolyA with SS were persistently associated with AITD. Ever-smoking exposure as environmental factor was confirmed as a main factor associated with AITD PolyA in SLE patients through the CART model (**Figure 10**).

The association between smoking exposure and PolyA has been seen not only in SLE but also in other ADs. For example, the coexistence of ADs (i.e., PolyA) in SS and related factors was evaluated earlier in a cross-sectional study involving 410 patients. Of these, 134 (32.6%) had PolyA. In the regression analysis, duration of disease, positive smoking status (AOR: 2.86, 95% CI: 1.18–6.94, $p = 0.02$), and a history of spontaneous abortions remained significantly associated with PolyA (238).

Lastly, 955 consecutive RA patients (1987 ACR criteria) and their nuclear families were included in a single-center cohort study (239). The history of 23 ADs, the factors related to PolyA, and familial autoimmunity were investigated. The mixed cluster

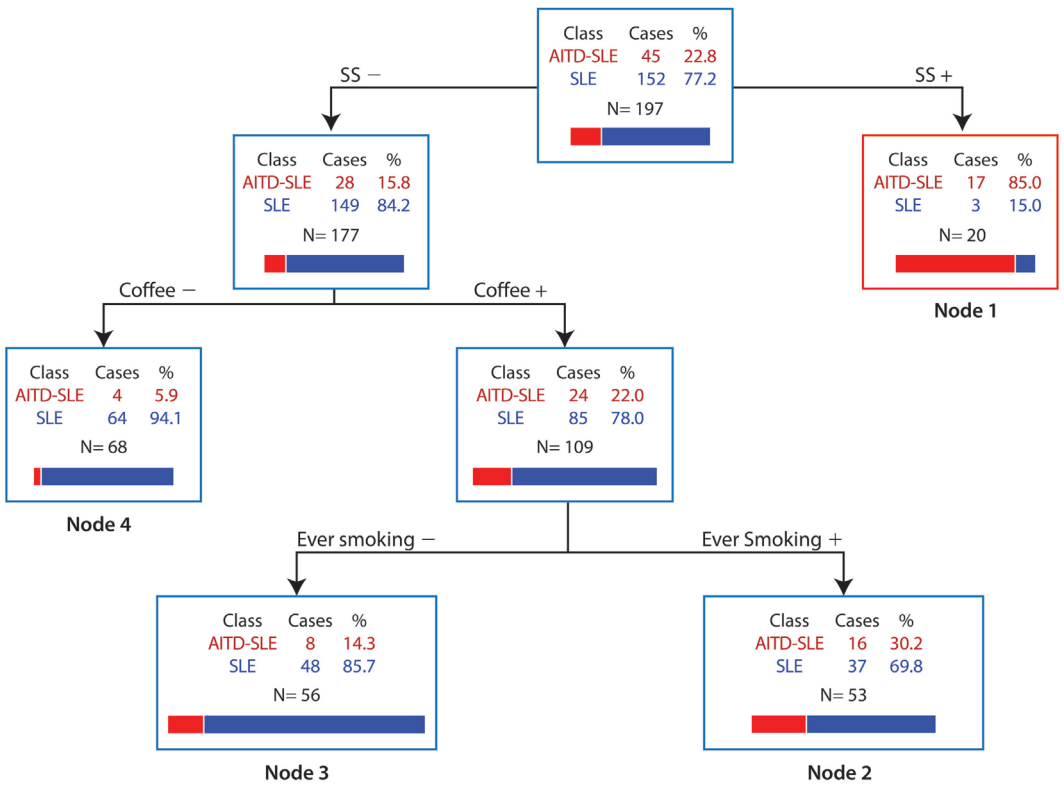

FIGURE 10 | Predictive factors associated with AITD PolyA in SLE. Sjögren syndrome and ever smoking are the main factors associated with the development of AITD in SLE. AITD, autoimmune thyroid disease; PolyA, Polyautoimmunity; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome. Adapted from Ref. (237).

methodology, based on multivariate descriptive methods, was used to summarize sets of related variables with strong associations and common clinical context. Then, for each set of related variables, new cluster variables (NCV) were derived for each patient. This was done on two sets of variables: the first were variables related to toxic substances consumed by patients from which we derived the toxic consumption profile as NCV; and the second was related to severity variables from which we derived the NCV of severity profile. The X^2 and Fisher's exact tests were done to establish differences between categorical variables (original and NCV) and PolyA. There were 130 (13.6%) patients who met the classification criteria for at least one other AD. Main factors influencing PolyA were familial autoimmunity, familial RA, and toxic consumption profile. For toxic consumption profile NCV, the proportion of PolyA in RA patients increases with more severe toxic exposure (no toxic consumption, followed by just coffee consumption exposure, then by coffee plus tobacco exposure, and finally, by coffee consumption plus hair dye).

A summary of the effects of cigarette smoking on the development of ADs is shown in **Figure 11**.

Socioeconomic Status, Infections, and PolyA

As mentioned previously, several factors, such as SES, may explain differences in the prevalence of immunological disorders based on time and geographical distribution. Research on the influence of SES in PolyA is scarce. The influence of SES on the course of ADs in admixed and heterogeneous socio-demographic populations was evaluated in a cross-sectional analytical study in which 1,096 consecutive patients with RA ($n = 678$), SLE ($n = 258$), and SS ($n = 160$) were included. Significant association between low SES and a stronger autoimmune response was observed (240). In addition, low SES influences the presence of arthritis, Raynaud's phenomenon, and discoid lupus in SLE. As expected, an association between low SES and low educational level was observed in SLE and SS; while in RA, a low SES was also associated with housewives. The association between PolyA and low SES in SS patients (OR: 3.692, 95% CI: 1.49–9.18, $p = 0.005$) should be highlighted. These results could be related to the admixture of the evaluated population, environmental factors, or the quality of health insurance and deserve further research. The results of this study contrast with those of others. For instance, PolyA between RA and AITD plus associated factors was evaluated in a cohort of 800 RA patients (241). The prevalence of AITD in RA patients was 9.8%. A lower AITD frequency was found in the lowest educational level compared to the highest one. Therefore, a low educational level (a surrogate of low SES) was positively associated with AITD–RA PolyA (OR: 0.16, 95% CI: 0.03–0.88, $p = 0.036$). These results should be analyzed in the context of the mentioned "hygiene hypothesis" or a high infectious agent exposure (exposure is interpreted as a poor hygiene problem). Epidemiological studies indicate a direct link between the decreasing infectious burden and the rising incidence of immunological disorders

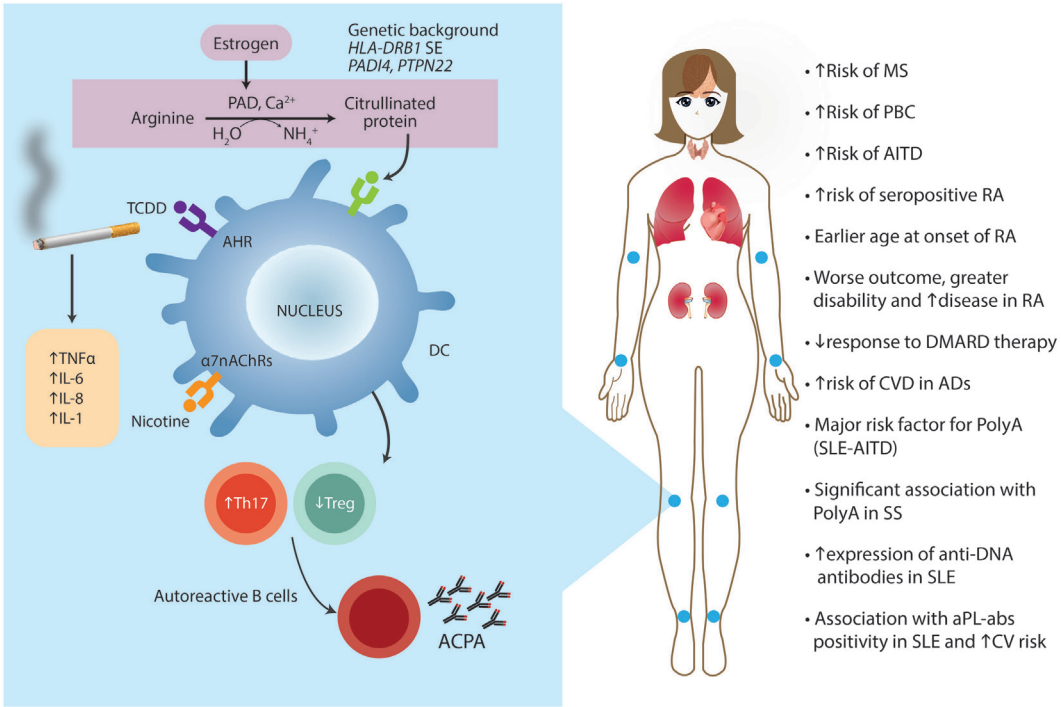

FIGURE 11 | Cigarrete smoking and autoimmunity. In predisposed individuals carrying certain autoimmune alleles at *HLA-DRB1, PTPN22,* and *PADI4* genes, cigarette smoke triggers citrullination by activating PAD enzyme, a mechanism that is estrogen dependent. Citrullinated proteins act as auto-antigens and induce the formation of anticitrullinated protein autoantibodies (ACPAs) by autoreactive B cells. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), released during cigarette smoking, acts as the best-known ligand for aromatic hydrocarbon receptor (AhR). AhR activation induces boosts T helper 17 (Th17) cells. Nicotine stimulates the α7-nicotine acetylcholine receptors (α7nAChRs), and increases TNFα, IL-6, IL-8, and IL-1 synthesis. The effect on T regulatory (Treg) function is controversial. All of these molecular pathways are responsible for the clinical manifestations listed on the right. PAD, peptidylarginine deiminase; SE, shared epitope; DC, dendritic cell; MS, multiple sclerosis; PBC, primary biliary cirrhosis; AITD, autoimmune thyroid disease; RA, rheumatoid arthritis; DMARD, disease-modifying antirheumatic drug; CVD, cardiovascular disease; ADs, autoimmune diseases; PolyA, polyautoimmunity; SLE, systemic lupus erythematosus; SS, Sjögren syndrome; aPL-abs, antiphospholipid antibodies.

(196). Also, social and environmental exposures directly impact immune tolerance and response. Furthermore, several epidemiological studies have investigated the protective effect of infectious agents in allergy and ADs.

Although the relationship between infectious disease and autoimmunity was not intended to be part of this review, some examples regarding the association between infectious disease and PolyA are shown below. EBV and CMV are notorious as they are consistently associated with multiple ADs. A cohort of 1,595 serum samples from 23 different AD groups was screened for evidence of prior infection with EBV and CMV (242). When compared to healthy controls, a significant increase in titers of EBV Viral Capsid Antigen IgG was observed in sera of patients with SLE–APS PolyA as well as in both diseases separately and in patients with other, single ADs (e.g., RA, MS, etc.). Moreover, samples from 82 consecutive SS patients, and 139 healthy controls were analyzed for infectious serology and autoantibodies as well as for relevant genetic mutations (TAP genes) and cytokine levels (243). Vasculitis PolyA among SS patients was significantly related to the presence of *Saccharomyces cerevisiae* antibody IgG (OR: 6.5, 95% CI: 1.14–36.78, $p = 0.05$). No other infectious autoantibodies were related to PolyA.

In contrast to this, when evaluating sera from 260 individuals (120 SLE patients, 140 geographically matched controls) for titers of infectious antibodies (five EBV-Abs), differences in titers from SLE-associated APS were not detected (244).

The results presented here agree with clinical evidence for all of the current theories on how infections could cause AD. Recent evidence suggests that microbe-activating specific innate immune responses are critical while antigenic cross-reactivity may perpetuate immune responses leading to chronic autoinflammatory disease (245). The data regarding cross-talk between PolyA as an expression of specific activated immunological pathways and infectious agents suggest that infections may play both a causative role and a protective role in the pathogenesis of ADs.

Organic Solvents and Autoimmunity

Solvents are liquids that dissolve a solid, liquid, or gas and are broadly classified into two categories: organic and inorganic. OSs are compounds with carbon-containing molecules. They may be broken down further into aliphatic-chain compounds, such as n-hexane, and aromatic compounds with a six-carbon ring, such as benzene or xylene. Common uses of OSs are dry cleaning (e.g., tetrachloroethylene), paint thinner (e.g., toluene, turpentine),

nail polish removers, and glue solvents (acetone, methyl acetate, ethyl acetate), spot removers (e.g., hexane, petrol ether), detergents (citrus turpenes), perfumes (ethanol), nail polish, and chemical synthesis, etc. (246). OSs are capable of altering cellular proliferation, apoptosis, and tissue-specific function. Both the amount and duration of OS exposure are essential in pathology causality. Chronic exposure to OSs might lead to deposits in an organ and consequently to immune infiltration similar to what is observed in ADs. The self-proteins that are modified by OSs may become immunogenic, recognized as foreign and, thus, initiate an inflammatory response and tissue injury (246).

Research in this area began in the 1950s after case reports of patients who developed a scleroderma-like syndrome after exposure to vinyl chloride, epoxy resins, trichloroethylene, perchloroethylene, or mixed solvents (11). The evidence of an association between exposure to OSs and developing AD was recently analyzed through a systematic literature review and meta-analysis. Even though the individual meta-analyses (i.e., each AD considered separately) showed significant association for MS, primary systemic vasculitis, and SSc, the direction and significance of this association did not change when all ADs, considered as a single trait, were analyzed (OR: 1.54, 95% CI: 1.25–1.92, $p = 0.001$). To our knowledge, this is the first evaluation of all ADs as a group with respect to OSs exposure through a meta-analysis. Therefore, the fact that ADs might share several common mechanisms (i.e., the autoimmune tautology) is reinforced (246).

Nevertheless, a recent review of systematic reviews and meta-analyses evaluating environmental risk factors and MS did not show a significant association (247). Likewise, the occurrence of MS among subjects exposed to anesthetic agents was compared to that of those who have never been exposed. This was based on previous evidence that exposure to anesthetic agents, some of which are chemically related to organic solvents, may affect the risk of MS. Therefore, based on two population-based, case-control studies, one with incident cases (1,798 cases, 3,907 controls) and one with prevalent cases (5,216 cases, 4,701 controls), no association was found between occupational exposure to anesthetic agents and risk of MS (248). By contrast, other studies evaluating the relationship between occupational exposure and risk of MS have shown positive associations. In a matched case-control study of 276 first clinical diagnosis of central nervous system demyelination cases and 538 controls done in Australia (2003–2006), Valery et al. examined the association between occupational exposure and risk of central nervous system demyelination. Among women, there was an increase in central nervous system demyelination risk associated with 10 or more years of exposure to livestock (AOR: 2.78, 95% CI: 1.22–6.33) or 6 or more years of farming (AOR: 2.00, 95% CI: 1.23–3.25; adjusted for number of children) (249). Horwitz et al. estimated the occupational risks in relation to MS in an open insurance cohort (all payouts for critical illness insurance from 2002 to 2011 were continuously registered). The MS incidence showed signs of occupation having an overall effect on the risk of MS. The high frequency found within the agricultural segment was attributed to dairy operators, whose incidence of MS was 2.0 times higher than the rest of the study's population (95% CI: 1.2–3.0) (250). Furthermore, Magyari et al., studied whether occupation or physical or social environment influenced the risk of MS differently in women than in men in a cohort consisting of 1,403 patients (939 women, 464 men), identified through Danish MS registry (2000 and 2004), and up to 25 matched controls (251). They found a slight albeit statistically significant excess for six female MS patients who had been employed in agriculture: OR: 3.52; 95% CI: 1.38–9.00, $p = 0.008$ (0.046 when corrected for multiple significance) although the number of cases was small.

Concerning SSc, recent publications have shown an association between OSs exposure and SSc. Marie et al. (252) evaluated the relationship between SSc and occupational exposure between 2005 and 2008 in 100 patients with a definite diagnosis of SSc and 3 matched controls for each patient. Higher ORs for SSc were found for white spirit ($p = 0.0001$), aromatic solvents ($p = 0.0002$), chlorinated solvents ($p = 0.014$), trichlorethylene ($p = 0.044$), ketones ($p = 0.002$), and welding fumes ($p = 0.021$). In addition, in a prospective study involving 142 SSc patients, Marie et al. (253) showed that patients exposed to solvents more frequently developed diffuse cutaneous SSc ($p = 0.001$), digital ulcers ($p = 0.01$), interstitial lung disease ($p = 0.02$), myocardial dysfunction ($p = 0.04$), and cancer ($p = 0.003$). Moreover, these patients were more frequently anti-Scl 70 positive and anticentromere negative. Furthermore, based on those results, some researchers have recently suggested that the association between SSc and occupational exposure be legally recognized as an occupational disorder (254).

Lastly, Li et al. (255) investigated possible associations between occupation and hospitalization for SLE in a nationwide database (Swedish Census to the Hospital Discharge Register 1970–2008). A total of 8,921 male and 42,290 female hospitalizations for SLE were analyzed. Higher risks [standardized incidence ratios (SIR)] among men with the same occupation (SIR > 2.0) were present among workers exposed to OSs in two consecutive censuses: shoe and leather workers (6.93), plumbers (2.21), chimney sweeps (4.54), and military personnel (3.01), etc.

To summarize, the recent expert panel (10) was confident regarding evidence supporting an association between solvent exposure and developing SSc. They agreed that general solvent exposure may also contribute to MS, but more research is needed using improved exposure assessment methods.

In conclusion, an association between OSs exposure and ADs is observed. OS exposure has not yet been sufficiently studied, and to clarify its role in ADs pathogenesis, its relationship with genetics needs to be studied, whether with respect to protection or susceptibility to each AD and the effects on the autoimmune process.

Vaccines

Vaccines represent the most successful and sustainable tactic to prevent and counteract infection. A vaccine improves immunity to a particular disease upon administration by inducing specific protective and efficient immune responses in all of the receiving population. The main known factors influencing the observed heterogeneity for immune responses induced by vaccines are gender, age, ethnicity, co-morbidity, immune system, and genetic background (256).

Autoimmunity is a concern for many vaccines, although AD presentation among immunized individuals is rarely observed. Nevertheless, and as mentioned, ASIA entails autoimmune conditions appearing after the exposure to an external stimuli of an adjuvant, including vaccines (257). In spite of some controversy about the diagnosis and classification criteria of this syndrome (258), we have observed and discussed patients who developed autoimmune conditions after quadrivalent human papillomavirus vaccination (259). However, because of relatively low baseline incidence of many autoimmune conditions, large post-marketing and adequately powered studies are required to evaluate any increased risk of ADs after vaccination. In fact, in most of the clinical trials evaluating vaccines, a systematic screening for ADs is not performed (i.e., testing for autoantibodies and evaluation of familial autoimmunity).

The incidence of narcolepsy, a sleep disorder characterized by loss of hypothalamic hypocretin (orexin) neurons, was increased after the pandemic AS03 adjuvanted H1N1 vaccination in Swedish and Finish but not in Italians (260). The clue for the observed side effects could be related to one type of vaccine, since not all the vaccines against H1N1 were associated with such complication. In fact, the vaccine inducing narcolepsy was suspected to trigger antibodies that can also bind hypocretin receptor 2 (261).

There is no a single mechanism explaining how a vaccine may induce AD. Theoretically, vaccines could trigger autoimmunity by means of cytokine production, anti-idiotypic network, expression of human histocompatibility leukocyte antigens, modification of surface antigens and induction of novel antigens, molecular mimicry, bystander activation, epitope spreading, and polyclonal activation of B cells (262).

There are several case reports of ADs following vaccines (45); however, due to the limited number of cases, the different classifications of symptoms, the long latency period of the diseases, and bias in data interpretation, every attempt for an epidemiological study has so far failed to deliver a connection. Despite this, efforts to unveil the connection between the triggering of the immune system by vaccines and the development of ADs should be undertaken (257).

On the other hand, in patients with AD receiving immunosuppressive therapy, vaccinations are often not offered or provided for a variety of reasons, including the fear of complications or vaccine-related illnesses, a concern for disease flare or reactivation, a perceived lack of effectiveness, or a misunderstanding of current vaccine guidelines (263). Patients with ADs often show decreased immune responsiveness, which in turn would make them vulnerable to infection given their underlying disease and frequent use of immunosuppressive drugs. A recent review highlights that all recommended vaccines may be given to patients before the start of immunosuppressive therapy (264, 265). All inactivated-killed vaccines should be provided to patients with AD, as they provide significant morbidity and mortality benefits. Inactivated-killed vaccines have not been shown to be associated with disease flares in large studies adequately powered to determine this effect. Live vaccines should be used mainly before the start of immunosuppressive therapy and used with caution on patients on active immunosuppressive therapy based on national guideline recommendations (264).

Conclusions and Perspectives: Personalized Medicine and Risk Prediction

Personalized (or precision) medicine, taking account of human individuality, has shown great promise to transform medical care. Since most of genetic factors associated with ADs confer a modest risk of ADs (266) and the immune system is very much shaped by the environment (13), inclusion of the AE in disease etiology and health will improve the way personalized medicine is currently conceived and applied (267).

Better understanding of the complex gene–environment interactions involved in the development of ADs together with the study of epigenetics (which was beyond the scope of this review) will provide insight into personalized interventions for these common and sometimes devastating diseases. Algorithms, constructed based on data gathered at the population level, can shed light on how the parts work together and the causal relationships between them, allowing strategies for prediction and prevention of ADs.

AUTHOR CONTRIBUTIONS

Study conception and design: J-MA, AR-V. Analysis and interpretation of data: J-MA, CR-S, MA, NM-G, AR-V. Drafting of manuscript: J-MA, CR-S, MA, AR-V. Critical revision: J-MA, CR-S, MA, NM-G, AR-V.

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REVIEW
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How Rheumatoid Arthritis Can Result from Provocation of the Immune System by Microorganisms and Viruses

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The pathogenesis of rheumatoid arthritis (RA), similar to development of a majority of inflammatory and autoimmune disorders, is largely due to an inappropriate or inadequate immune response to environmental challenges. Among these challenges, infectious agents are the undisputed leaders. Since the 1870s, an impressive list of microorganisms suspected of provoking RA has formed, and the list is still growing. Although a definite causative link between a specific infectious agent and the disease has not been established, several arguments support such a possibility. First, in the absence of a defined pathogen, the spectrum of triggering agents may include polymicrobial communities or the cumulative effect of several bacterial/viral factors. Second, the range of infectious episodes (i.e., clinical manifestations caused by pathogens) may vary in the process of RA development from preclinical to late-stage disease. Third, infectious agents might not trigger RA in all cases, but trigger it in a certain subset of the cases, or the disease onset may arise from an unfortunate combination of infections along with, for example, psychological stress and/or chronic joint tissue microtrauma. Fourth, genetic differences may have a role in the disease onset. In this review, two aspects of the problem of "microorganisms and RA" are debated. First, is there an acquired immune deficiency and, in turn, susceptibility to infections in RA patients due to the too frequent and too lengthy infections, which at last break the tolerance of self antigens? Or, second, is there a congenital deficiency in tolerance and inflammation control, which may occur even with ordinary infection frequency and duration?

Keywords: rheumatoid arthritis, immune system provocation, Infection, microorganisms, viruses

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INTRODUCTION

Rheumatoid arthritis (RA) pathogenesis, in the most general sense, is largely due to an inadequate immune response in genetically predisposed individuals to environmental challenges, with bacteria and viruses (latent carrier or clinical signs of infection) being the undisputed leaders among these environmental challenges (Brooks et al., 2010; Le Dantec et al., 2015b).

The microbial concept of RA triggering agents has been discussed since the 1870s (Benedek, 2006). But, in spite of this long history, a direct role of microorganisms in the disease is controversial, while the suspected pathogen list is still growing (**Table 1**). The fact is that it is impossible to make a causal link between a specific pathogen and the disease. In light of these disappointing results in understanding RA, there are calls for even larger studies with the use of more advanced technologies (Silman and Pearson, 2002; Carty et al., 2004).

Several Explanations Can Be Proposed for These Conflicting Results

First, in the absence of a defined pathogen, the spectrum of microorganisms involved in triggering RA may include polymicrobial communities or the cumulative effect of several bacterial/viral factors. The simultaneous effects of various pathogens on the immune system in an RA patient may have different direct effects. It has been demonstrated that specific infections (gastrointestinal and urogenital), hypothetically associated with the changes in the gut microbiome, could diminish or increase the risk of RA (Sandberg et al., 2015).

Second, the spectrum of bacteria and viruses, as well as the reaction of the immune system to such pathogens, with or without clinical manifestations of infection, may vary from preclinical to late-stage RA: such that any of numerous pathogens may trigger RA at a preclinical stage, but subsequently lose their influence in the advanced stage (Arleevskaya et al., 2014). Moreover, the delicate balance between pathogens and the anti-infection immune response of an RA patient may be disturbed by several factors including the limited mobility of the patients as well as the drug therapy selected.

Third, infections might not trigger RA in all cases, but only in a subset of the cases. For example, co-occurence with psychological stress and/or chronic joint tissue microtrauma might be needed. Indeed, in Swedish and Finish cohorts only 16–20% of the patients reported infection in the early phase of RA (Leirisalo-Repo, 2005); whereas in a Russian cohort this index was as high as 75% (Arleevskaya et al., 2014). The age at disease onset also seems to be important since younger patients attributed their RA to a previous infection more often than did older patients. (Soderlin et al., 2011).

Fourth, the differences in trigger factors might reflect variations in the pathogenetic mechanisms. Indeed, RA is recognized as being a multigene disorder with a huge number of genetic polymorphisms contributing to the disease pathogenesis. The differences in trigger factors may also be due to geographic, life style, drug or chemical exposures, and ethnic differences (Konsta et al., 2015; Le Dantec et al., 2015a; Lemerle et al., 2016). The diagnostic category of RA includes multiple subtypes of the disease and the different phenotypes of RA might be due to the different genotypes. In fact, the set of clinical signs, which has been known since 1782 (Jonsson and Helgason, 1996), and which we call "RA" may be the outcome of a variety of pathogenic pathways, or in other words, it is a syndrome, i.e., a group of symptoms that arise as a consequence of a number of different diseases (Weyand et al., 1998).

Accordingly, two aspects of the problem of "pathogens and RA" *versus* "RA and infections" are still debated. Is there an acquired immune deficiency in RA patients caused by too frequent and prolonged infections, which break tolerance of self-antigens? Or, is there a congenital deficiency of the tolerance and inflammation control, which may occur even with ordinary infection frequency and duration?

TABLE 1 | Pathogens suspected of triggering rheumatoid arthritis (RA).

Bacteria and viruses	Reference
Acanthamoeba polyphagamimivirus	Shadidi et al., 2002
Acinetobacter spp.	Carty et al., 2004
Alphavirus	Leirisalo-Repo, 2005
Bordetella spp.	Carty et al., 2004
Borrelia burgdorferi	Imai et al., 2013
Campylobacter jejuni	Shadidi et al., 2002
Candida albicans	Hermann et al., 1991
Chlamydophila spp.	Carter et al., 2010
Escherichia coli	Newkirk et al., 2005
Flavivirus	Li et al., 2013
Haemophilus spp.	Carty et al., 2004
Helicobacterpylori	Caselli et al., 1989
Herpesviridae	Shadidi et al., 2002; Silman and Pearson, 2002
Human Immunedeficiency Virus	Carty et al., 2004
Leptospira interrogans pomona	Sutliff et al., 1953
Mycobacterium tuberculosis	Kim H.R. et al., 2006
Mycoplasma arthritidis	Phillips, 1986
Mycoplasma fermentans	Horowitz et al., 2000
Parvovirus	Carty et al., 2004
Porphyromonas gingivalis	Wu et al., 2010
Prevotella intermedia	Martinez-Martinez et al., 2009
Proteus mirabilis	Wilson et al., 2000
Rubivirus	Hart and Marmion, 1977
Staphylococcus aureus	Zahiri Yeganeh et al., 2015
Streptococcus pyogenes	Phillips, 1986
Tannerella forsythensis	Ogrendik, 2009
Yersinia enterocolitica	Maslova et al., 2004

ARGUMENTS FOR THE ACQUIRED VERSUS INNATE HYPOTHESIS

The results of various studies testing the acquired or the genetically determined predisposition to infections in RA are rather contradictory. These differences can be explained by several reasons. First, the conflicting results may be explained in part by features of the patient groups studied, including the particular therapeutic approach used and the specific types of infections tracked by the authors (Vandenbroucke et al., 1987; Widdifield et al., 2013; Sandberg et al., 2015).

Second, evolution of the immune system reaction to pathogens during RA development is usually not taken into

account. Our 10-year follow-up showed that both early stage RA patients and their relatives suffer from more frequent and prolonged minor infections than those individuals without autoimmune diseases in their family history (Arleevskaya et al., 2014). A gradual decrease in the frequency and duration of the infectious episodes was observed in RA patients at a later stage, when they were taken under observation at an early stage and observed for longer than 3 years. It was also observed from this cohort that the frequency and duration of the infectious episodes increased even more in the year before the RA onset, and that all the relatives who developed RA during the observation (i.e., included in the study at the pre-clinical stage) had a pronounced infectious syndrome (Arleevskaya et al., 2014). It is noteworthy that Germano et al. (2014) reported an association between the infection risk and disease activity, while these authors also supported the hypothesis that the infectious syndrome decrease with RA duration. With this in mind, we speculate that there is probably both a congenital and or an acquired deficiency of the anti-infection defense leading to the frequent and prolonged minor infections in early RA patients and their relatives. Attempts to eradicate the infections eventually lead to a certain amount of success, but at the cost of RA onset due to an inappropriate activation/inhibition of various key parts of the immune system. It should be noted, that in late stage RA, microbial colonization (including the increased frequency of heavy *Escherichia coli* and *Staphylococcus aureus* colonization) persists (Arleevskaya et al., 2014). So, in late stage RA, despite the reduction of clinical signs of frequent and prolonged infectious episodes, there are still laboratory signs of dysbiosis. Thus, a delicate balance of microflora and the immune host defense might be disrupted at any time, for example, when there is a change in the therapy. This hypothesis is indirectly confirmed by the data, indicating that a history of previous infections appeared to be among the risk factors for the infectious complications during infliximab and disease-modifying anti-rheumatic drug therapy (Widdifield et al., 2013). We interpret these data in such a way that, in RA patients with a deficiency in their anti-infection defenses, which has been manifested earlier in any infections and compensated for to some extent later, the risk of renewal of infections still remains high.

There are two possible approaches to the problem of "infections and RA". One of them, being of particular importance for practicing rheumatologists, aims to study the susceptibility to infections as a prognostic factor for the infectious complications in RA therapy. The goal is to study all patients without exception, including those with a variety of reasons for the development of infection complications, even in the absence of RA (such as leucopenia, low mobility, diabetes, or other comorbiditiesl; Doran et al., 2002; Soderlin et al., 2011). The results of these studies indicate an increased susceptibility to infections in RA patients. The second approach is to study the role of infections in the provocation and persistence of RA activity. In this case, it seems logical (albeit with obvious damage to the completeness of coverage of the problem) not to extend the study on the patients with the concomitant additional risk factors for the infection development.

THE ACQUIRED HYPOTHESIS

There is a definite list of possible mechanisms by which the pathogen/host interactions could trigger pathological autoimmune responses: molecular mimicry, epitope spreading, polyclonal lymphocyte activation, bystander activation, and viral persistence (Bach, 2005; Kivity et al., 2009; Vojdani, 2014; Floreani et al., 2015).

Molecular Mimicry

Molecular mimicry occurs when foreign antigens bear sufficient structural similarity to self-antigens (i.e., similar epitopes). As a result, an immune response to pathogens could lead to cross reactivity with self-antigens. The similarity of pathogen and self-antigen structures is a widespread phenomenon. By exact peptide matching analysis, it was demonstrated that all human proteins harbor a bacterial penta- or hexa-peptide motif (Trost et al., 2010). Moreover, the study of pathogenic and non-pathogenic proteomes from *Vibrio cholera* and *Mycobacterium tuberculosis* up to *Lactobacillus acidophilus* and *Bifidobacterium adolescentis* showed that the bacterial pathogenicity does not affect the cross-reactivity with human proteins. So, molecular mimicry would seem to be too commonplace to shift on it the blame for the development of autoimmune diseases. In the inflammatory foci a variety of bacterial proteins undergo several different types of post-translational modifications, and most of these proteins have motifs similar to those found in human polypeptides.

Epitope Spreading

Epitope spreading is a phenomenon in which the immune T or B cell response extends beyond the original epitope. In general, epitope spreading is quite a beneficial phenomenon, contributing to the ability of the immune system to attack multiple pathogens (Powell and Black, 2001). Epitope spreading develops in the process of an ongoing immune reaction during an acute or persistent infection and following tissue destruction. Epitope spreading optimizes protection against newly encountered pathogens and assists in clearing inflammatory sites of damaged endogenous proteins of the body's own tissues. Its success is due to the ability to quickly target new epitopes and the ability to adequately destroy pathogens and clear inflammatory sites. It appears evident that, due to RA development, abnormalities occur in the mechanisms that would otherwise constrain epitope spreading only to the diversity of foreign antigens and prevent development of an immune response to self-epitopes. A complete review of these abnormalities and self-epitopes is beyond the scope of the present discussion. We'll briefly examine only some aspects. Epitope spreading can result from a change in a protein structure. One well-known example, protein citrullination, which is a conversion of arginine to citrulline by peptidyl arginine deaminase (PAD), could be due to development of an immune reaction against the original protein or its citrullinated form, but may also arise against other citrullinated proteins.

Currently interest in the well-established link between periodontitis and RA (Helminen-Pakkala, 1968) is going through a renaissance. This reignited interest is due to clarification of the role of citrullinated peptides as epitopes

with pathogenic significance in RA, and identification of the ability of *Porphyromonas gingivalis,* the main pathogen in chronic periodontitis, to citrullinate proteins by using its own PAD (Maresz et al., 2013). Thus, this situation represents a clear example of an autoimmune disease triggered by infection *via* molecular mimicry and epitope spreading mechanisms. When activated, B lymphocytes initially producing antibodies (Ab) against the citrullinated *P. gingivalis* enolase, start producing Ab against citrullinated human enolase, and then against other citrullinated endogenous proteins (reviewed in Lundberg et al., 2010). Quirke et al. (2015) compared the incidence in RA patients of increased levels of serum Ab to arginine containing proteins (enolase, vimentin, fibrinogen), and to the citrullinated modifications according to the occurrence of lung bacterial infections. As compared to RA control patients, lung infections were associated with an elevated level of Ab to citrullinated proteins.

Polyclonal Lymphocyte Cctivation

Microbial molecules can directly induce proliferation and differentiation of T- and B-lymphocytes regardless of their antigen specificity. In the model of murine *Trypanosoma cruzi* infection, it was shown that the number of high rate immunoglobulin secreting cells of both IgM and IgG classes increased up to 100 fold each and a variety of effector T cell activities were revealed, with both Ab and cellular immune reactions being predominantly non-specific (Arala-Chaves et al., 1992). Similar observations have been recorded in various viral, bacterial and fungal infections. The authors provided the results of their experiments, showing that as much as 95% of Ab produced in response to an infectious stimulus fail to bind to the antigens used for the immunization. The polyclonal activation normally produced excessive amounts of usually low-affinity Abs, which are directed against foreign and self-antigens. With regards to expression of the anti-immunoglobulin G Abs (also known as rheumatoid factor, RF) in human RF-positive Tg murine models, results differed according to the timing of the infection. On the one hand and following acute influenza viral infection, an abortive activation of RF-positive B cells and no increase in RF production characterized infected mice as compared to the uninfected ones (Woods et al., 2007); while, on the other hand, the same group also reported that chronic bacterial infection in the RF-positive Tg mice was associated with increased RF production, which resulted from polyclonal lymphocyte activation (Soulas et al., 2005).

In RA, peripheral blood and synovial lymphocyte polyclonal activation is well documented, although its extent is limited since hypergammaglobulinemia does not occur in these patients (Becker et al., 1990; Bucht et al., 1992; Yamamoto et al., 1992; Outschoorn et al., 1993; Brown et al., 1995). When testing the serum levels of Ab free light chains (as a biomarker of B cell activation) in a large cohort of the general population, Deng et al. (2015) reported elevated levels of the polyclonal free light chains 3–5 years before clinical onset of RA, an elevated level that remains during the follow-up of the patients. The authors pointed out that the elevation was moderate, and failed to demonstrate any correlation with mortality in the RA group.

A number of the results demonstrate that the situation with the polyclonal lymphocyte activation in RA is complicated. The data of recent studies, which tested the isotype distribution, antigen specifity and affinity of the serum and synovial fluid RFs and anti-CCP Abs as well as the expression of the inherently autoreactive idiotope 9G4 in preclinical, early and advanced stages of RA, failed to clarify whether such a process is crucial in RA development (Moyes et al., 1996; Verpoort et al., 2006, Ioan-Facsinay et al., 2008; Cambridge et al., 2014, Falkenburg et al., 2015). When analyzing these data, it looks like the anti-citrullinated Abs are produced due to the ongoing immune response to citrullinated proteins, while RFs appear later and persist due to both the polyclonal and antigen-driven activation of B lymphocytes (Renaudineau et al., 2005). Cambridge et al. (2014), speculated that "in RA, autoreactive B cell specificities escape deletion, receptor editing or anergy early in their development, ultimately giving rise to a population of B cells which can also survive entry into the mature B cell compartment in the periphery."

Another important aspect is the imperfection of the polyclonal activation of peripheral blood B-lymphocytes from RA patients. The cell response to bacterial peptidoglycan, pokeweed mitogen, or phorbol myristate acetate stimulation was significantly reduced compared to controls (Abrahamsen et al., 1978; Pardo et al., 1984). Synovial fluid lymphocytes also proliferated poorly when stimulated polyclonally (Abrahamsen et al., 1978; Petersen, 1988). Finally, RA lymphocytes stimulated by the polyclonal B cell activator Epstein-Barr virus (EBV), produced less IgM than controls after 1 week and showed increasing IgM production between 14 and 21 days, whereas, in normal lymphocytes, IgM production decreased during this period (Irving et al., 1985).

Bystander Activation

Autoreactive cells may be expanded and activated by non-specific means or by a combination of non-specific effects with self antigens being released and presented in an inflammatory environment created by infection (Kim B. et al., 2006). Most studies examining non-cognate T cell responses have focused on CD8 T cells, primarily in viral infection models with the stimuli being the products of infected cells, or inflammatory molecules generated by chronically virus-infected tissues or the factors produced by the cells, activated during the viral molecules interaction with toll-like receptors (Miller et al., 1997; Horwitz et al., 1998; Toubi and Shoenfeld, 2004; O'Donnell and McSorley, 2014). However, bacteria, in particular the intra-macrophage bacteria, also may be responsible for the bystander activation (Das et al., 2000; McSorley, 2014; O'Donnell and McSorley, 2014). One of the main factors provoking bystander activation is related to proinflammatory cytokines. At least in experiments studying this phenomenon, the proinflammatory cytokine cocktails or any of these cytokines are typically used for the cell stimulation (Unutmaz et al., 1994; Brennan et al., 2008). Cytokine stimulation of naive and resting memory T cells results in the proliferation of the lymphocytes and display of the effector functions of the memory T cells as measured by lymphokine synthesis and help stimulate immunoglobulin production by B cells (Unutmaz et al., 1994). At the same time,

T cells activated by cytokines in the absence of T cell receptor stimulation also activate monocytes and monocyte cytokine production (Sebbag et al., 1997). Evidently these processes occur in RA pathogenesis (Sebbag et al., 1997; Brennan et al., 2008).

THE INNATE HYPOTHESIS

Human Leukocyte Antigens (HLA)

Recent results from genome wide association studies (GWAS) support a contributory role of genetic and epigenetic factors in the development of RA (Konsta et al., 2015). The most well-studied genetic example is related to the RA associated DRB1*01 and *04 alleles (shared epitopes), which are effective, when expressed, in binding citrullinated peptides, and presenting them to T cell receptors which is the optimal scenario for development of an immune response against citrullinated peptides, as well as for activation of the proinflammatory Th1 cytokine production (Tian et al., 1998; Rosloniec et al., 2002b; Auger and Roudier, 2005; Gourraud et al., 2006; Ohnishi et al., 2006). Moreover in RA patients, an increased expression of the HLA-DR molecules has been reported leading to a significant low-affinity peptide presentation and activation of autoreactive peripheral T cells (Kerlan-Candon et al., 2001; Rosloniec et al., 2002a; Hill et al., 2003; Auger and Roudier, 2005; Gourraud et al., 2006, 2007; Ohnishi et al., 2006).

With regards to associations between infections and HLA-DR peculiarities in RA, HLA-DRB1*0404 was associated with a low frequency of occurence of T cells specific for EBV gp110, a replicative phase glycoprotein which is critical for the EBV infection control (Toussirot et al., 1999). While opposite to this, HLA-DRB1*07, an allele associated with reduced risk of developing RA, was associated with the highest frequencies of the peripheral blood T-lymphocytes specific for gp110. Therefore, it's not surprising that EBV DNA and EBV-encoded RNA I transcripts are present significantly more often in synovial tissue of patients positive for the shared epitope (especially HLA-DRB1*0404–positive; Saal et al., 1999). When considering HLA-DRB1 alleles predisposing to the development of recurrent herpes lymphocytic meningitis and CMV reactivation, results were more contrasted with the characterization of both RA-associated non share epitopes HLA-DRB1*09 and DRB1*15, and RA-associated share epitopes HLA-DRB1*01/*04 (Du et al., 2007; Kekik et al., 2009; Kallio-Laine et al., 2010; Acar et al., 2014).

Non-HLA Genes

Other gene polymorphisms associated with RA development are mainly related to imperfect control of lymphocyte activity (PTPN22, CTLA-4, BTLA, and others) (Plenge, 2009; Oki et al., 2011). Due to these SNPs the immune response becomes difficult to manage (**Figure 1**). The impaired clearance of inflammatory sites caused by an imbalance of the pro-oxidant and anti-oxidant factors and inadequate activity of several enzymes involved in remodeling of the extracellular matrix may also be affected by mutations in the genes of the corresponding factors (Mattey et al., 1999, 2000; Nemec et al., 2006; Ling et al., 2007).

NF-κB and Jak/STAT Pathway

For all the diversity of RA-associated gene SNPs, an enrichment of these RA-associated genes was found in particular in two pathways: the NF-κB and the JAK-STAT signaling cascades (Diogo et al., 2014; Messemaker et al., 2015).

The NF-κB pathway (**Figure 2**) is considered to be a prototypical proinflammatory signaling pathway controlling both RA pathogenesis and viral infection, due to the expression of proinflammatory genes of chemokines, cytokines, receptors, apoptotic regulators, intracellular signaling molecules, and transcription factors (Hinz et al., 2002; Lawrence, 2009). Indeed, stimulation of the NF-κB signaling pathway and inhibition of TNF-related apoptosis (impaired in RA even without this additional exposure) potentiate RA development. Such a pathway could be amplified in the case of infections with CMV and EBV, since the viruses need live, functional and activated "lymphocytes" which could be achieved by controlling the same NF-κB signaling pathway and, in turn, by blocking TNF-related apoptosis (Goodkin et al., 2003).

STATs are a family of proteins which are latent transcription factors activated by extracellular signaling ligands such as cytokines, growth factors and hormones (Abroun et al., 2015). The principal RA players: IL6, TNF-α as well as IFNs, exert their biological functions through the JAK/STAT signaling pathway (**Figure 2**), proteins which are being overexpressed in the various cells in RA (Rottapel, 2001; Shouda et al., 2001). STAT proteins become activated in the cytoplasm by Janus kinases (JAK), a family of tyrosine kinases. These signaling pathways have diverse biological functions, which include participating in inflammation and cell differentiation, proliferation, development and apoptosis. In particular, TNF-α production and NF-κB pathway activation can occur through JAK/STAT signaling (Ahmad et al., 2015). At the same time NF-κB positively regulates STAT5a expression and signaling pathways, and promotes persistent activation (Prosch et al., 2003).

LESSONS FROM HERPESVIRIDAE INFECTIONS IN RA

Herpes Simplex Virus Type 1 and 2 (HSV1/2) Prevalence and Ability to Escape the Immune System Response

Worldwide rates of either HSV-1 or HSV-2 in adults are found to be 60–95% (Chayavichitsilp et al., 2009), and due to such a high level of exposure, some authors have suggested that these viruses should be considered to be part of the normal microbiotic flora (Grinde, 2013). However, diagnosis of an HSV infection based on the presence of viral DNA in blood cells does not provide comprehensive information since HSVs can take up life-time residency in nerve cells during the latency phase and are transported to the mucosa during reactivation phases (Grinde, 2013). The latency *versus* reactivation strategy of the *Herpesviridae* is defined by the balance between viral proliferation and the ability of the immune system to clear the

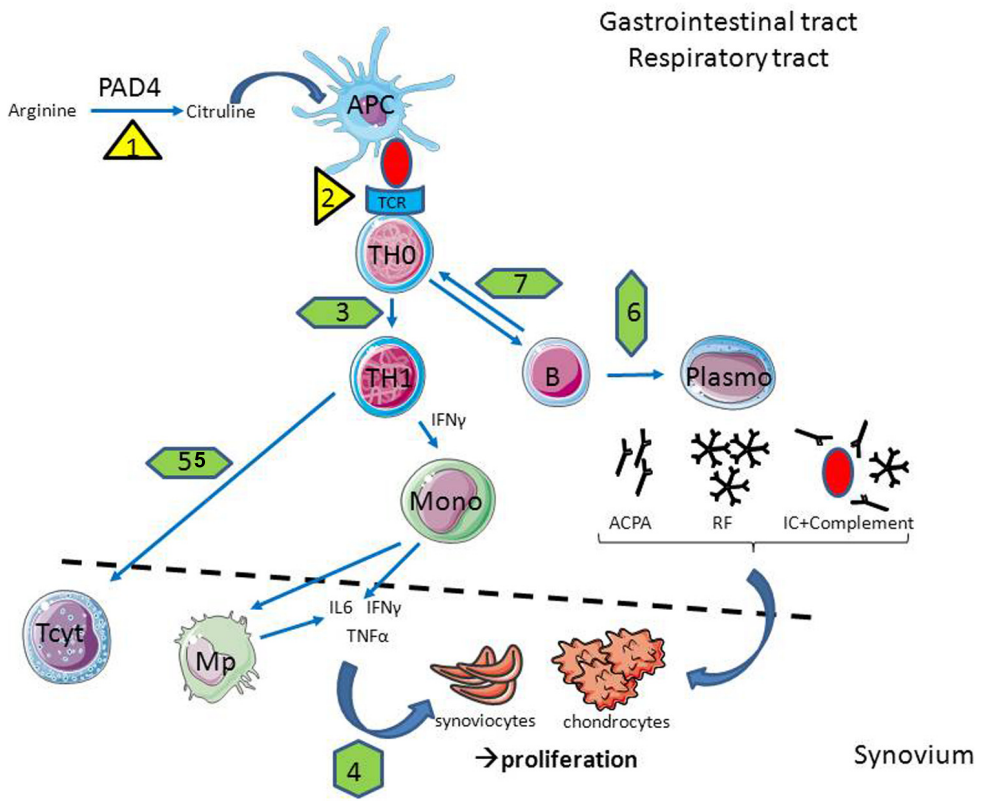

FIGURE 1 | In case of infection, the cellular and molecular players of rheumatoid arthritis (RA) are affected. Infections are able to promote citrullinisation (1) and antigen presentation (2) through HLA-DR overexpression in antigen presenting cells (APC); to interfere with T helper (Th) polarization (3) and cytokine production (4); to reduce lymphocytotoxic activity (5) as well as antibody production (6) and antigen presentation by B cells (7). Abbreviations: Mono, Monocyte; Mp – macrophage; TCR, T cell receptor; PAD4, peptidyl arginine deaminase 4; ACPA, anti-citrullinated protein antibodies; IL, interleukine; IFNγ, interferon gamma; TNFα, tumor necrosis factor alpha; IC, immune complex; RF, rheumatoid factor.

virus (Grinde, 2013). During the latent state, the viral genome is packaged by histones and copied by the host cell's DNA polymerases, along with the chromosomes, primarily when the cell engages in mitosis (Knipe and Cliffe, 2008; Grinde, 2013). This contrasts with lytic replication in which the viral DNA polymerase is engaged, reflecting a viral takeover of the cell. Reactivation may be provoked by various factors, leading to activation of demethylation and histone modification processes, processes that appear to be excessive in RA (Bottini and Firestein, 2013; Wada et al., 2014, de Andres et al., 2015). As a whole, the virus's tactics to avoid immune detection and establish latency works in a significant population – up to 80% in human adults for HSV-1 and about 40% for HSV2 (Akhtar and Shukla, 2009).

According to our data the ratio of people with clinical signs of HSV exacerbation (criteria reviewed in Grinde, 2013) is significantly increased in the early stage of RA as well as among the first degree relatives of these patients (Arleevskaya et al., 2014). However, when considering HSV1/2 DNA detection in blood, joint tissues and synovial fluid cells, an infrequent presence of HSV1/2 infection has been reported in the patients with early diseases (Zhang et al., 1993; Stahl et al., 2000). In late stage RA, the prevalence of the serum IgG anti-HSV antibodies and the viral DNA presence in the blood and synovial fluid cells were similar to that seen in controls (Us et al., 2011). The data on the serum IgM anti-HSV antibody prevalence in RA are contradictory and could not help resolve the discordance observed between the biology and clinical observations (Kurbanov and Mamedov, 2009; Us et al., 2011).

Cellular Sensitivity and Response to Herpes Infection in RA

Since spreading of the virus from cell to cell depends on specific receptors, the presence of these receptors plays a critical role in viral infection and, in turn, in disease exacerbation (Akhtar and Shukla, 2009). Among them, the herpes virus entry mediator (HVEM), a member of the tumor necrosis factor receptor superfamily, serves as one of the entry receptors of HSV (Gavrieli et al., 2006; Cai and Freeman, 2009). In RA, HVEM is overexpressed and contributes to the proliferation and activation of synovial fibroblasts (Ishida et al., 2008). This factor is overexpressed on most cell types found in RA synovial tissues (Kang et al., 2007; Shang et al., 2012). Serum levels of soluble HVEM are increased in RA as well (Jung et al., 2003). So, HVEM overexpression in RA is suspected of contributing to HSV

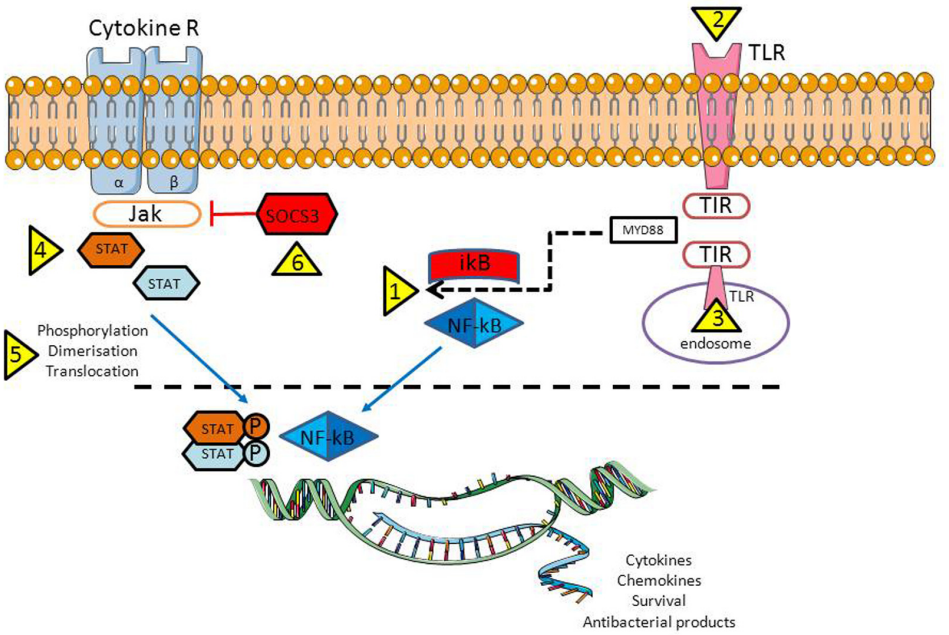

FIGURE 2 | Activation of the NF-κB and JAK-STAT signaling pathways are crucial for proper antiviral response. However, in case of infection, bacteria/viruses have a number of opportunities to inhibit both pathways: trigger NF-κB translocation from cytoplasm into the nuclei (1) through plasma membrane (2, bacteria) or endosomal (3, viruses) TLR activation; by activing various STAT family members that being important for the virus replication (4), by controlling STAT activation/translocation to the nucleus (5); and last but not least by reducing the activity of the suppressor of cytokine signaling-3 (SOCS3), a host negative regulator of the JAK/STAT pathway (6). Abbreviations: TLR, Toll like receptor; TIR, Toll/IL-1R; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor kappa B; IkB – inhibitor of the NF-κB kinase; P – phosphate residue (activated form); Cytokine R, a cytokine receptor with β1 and β2 subunits; JAK, Janus tyrosine kinase familly; STAT, signal transducer and activator of transcription family; SOCS3, suppressor of cytokine signaling 3.

dissemination and to the RA progression directly or indirectly via reception of the viruses.

The epidermal growth factor receptor (EGFR) is another receptor by which HSV enters a cell. EGFR is found to be overexpressed on a variety of synovial tissue cells, and the receptor gene overexpression in bone marrow-derived mononuclear cells is due to an RA-associated SNP (Nakano et al., 2005; Nakamura et al., 2006; Lo et al., 2012; Yuan et al., 2013; Xu et al., 2015).

Immune Surveillance of the Herpesviridae Infection in RA

The capacity of the immune system to effectively control *Herpesviridae* is determined by the adequacy of the virus (i) to directly interact with the immune system through the pattern recognition receptors (PPR), (ii) to modulate cytokine and chemokine production, and (iii) to control invariant natural killers (iNK) T cells, CD8+ cytotoxic T cells (IFN-γ, TNF-α, cytolytic molecules including perforin and granzymes), and the ability of the immune system to establish and maintain a pool of HSV-specific memory/effector CD8(+) T-lymphocytes (**Figure 3**).

Pattern Recognition Receptors

The viruses are recognized by PPR, among which the mannose receptors are considered to be the most important (Summerfield et al., 1995; Seppanen et al., 2009). Some preliminary evidence suggests that mannose receptors may be overexpressed on immune cells of myeloid lineage present in blood and synovial tissue from RA patients (Put et al., 2013).

One member of the secreted soluble PPR family, the mannose-binding lectin (MBL), is a complement component and an opsonic factor binding to HSV. Serum MBL levels vary according to the history of the disease. In early RA, low MBL levels were revealed, which are associated with a higher risk of developing early erosive RA and higher levels of IgM RF and CRP (Jacobsen et al., 2001; Saevarsdottir et al., 2001), and later, the MBL levels are increased (Saevarsdottir et al., 2007). Given the likely increase of MBL expression during RA evolution from early to late stage, the data by Olsen et al are of interest, showing that in the early stage of RA, the MBL gene is down regulated more than threefold compared with late stage RA (Olsen et al., 2004). The importance of MBL in RA physiopathology is reinforced by the observation of a nucleotide polymorphism (SNP) at MBL gene associated RA susceptibility (Ip et al., 2000; Jacobsen et al., 2001), and with HSV infection reccurences, possibly due to impaired recognition of the viruses (Seppanen et al., 2009).

Cytokines and Chemokines

The initial stages of *Herpesviridae* infection are predominantly influenced by the activity of iNK cells to produce type I interferons (IFN), which limit the spread of viruses. The other

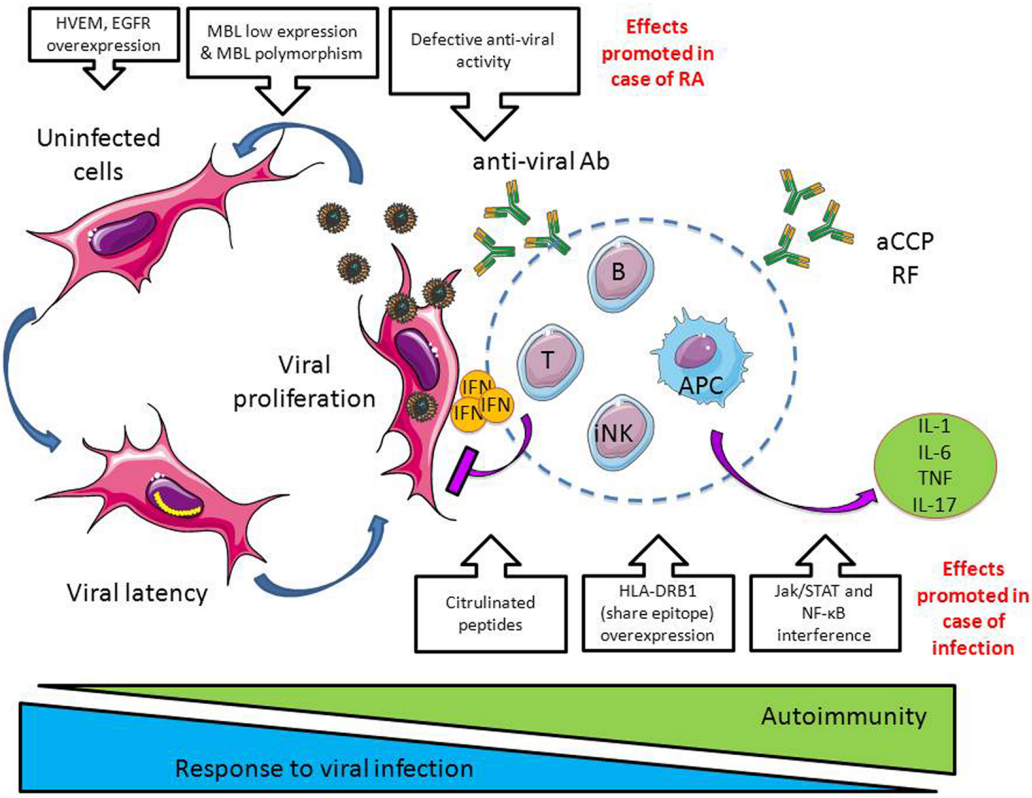

FIGURE 3 | Dangerous liaisons between infections and RA. In case of viral infections such as observed with *Herpesviridae*, the immune system limits viral infection by producing proinflammatory cytokines (e.g., interferon, IFN), anti-viral antibodies and through the activation of cytotoxic cells such as CD8+ T cells and innate natural killer cells (iNK). In case of RA, a defective antiviral activity is observed together with factors promoting *Herpesviridae* propagation (e.g., herpes virus entry mediator, HVEM, and epidermal growth factor receptor, EGFR, overexpression; downregulation of the opsonic factor MBL, mannose binding lectin). At the opposite, infections promote autoimmunity by inducing protein citrulinisation, by promoting HLA-DR overexpression, and by interfering with the pathways involved in the production of cytokines and chemokines, Jak/STAT and NF-κB (see **Figure 2**).

inhibitors of the infection are macrophages (producing IFNs, TNF-α, and IL-6), and lymphocytes that are instrumental in active immune surveillance by producing IFN-γ (Khanna et al., 2003; Khanna et al., 2004; Egan et al., 2013). Activated CD4+ and CD8+ T cells play a pivotal role in clearing the primary infection (Nash, 2000; Egan et al., 2013). After resolution of the primary infection, a small proportion of the primed specific T cell population generates a stable long-term memory cell pool, which activates during reactivation of a latent infection (Crough et al., 2005). Both nonspecific and specific CD8+ T cells infiltrate and persist within virus infected cells, the specific CD8+ T-lymphocytes being the prevailing ones, expressing a late effector memory phenotype and being activated by stimulation from the infected cells (Borysiewicz et al., 1988; Posavad et al., 2000; van Lint et al., 2005; Verjans et al., 2007; Zhu et al., 2007). It appears that the principal role of B cells in the immune response to *Herpesviridae* infection is not to produce neutralizing antibodies but instead to present antigens and secrete cytokines (Deshpande et al., 2000; Youinou et al., 2005, 2009; Gazeau et al., 2015).

While the IFN-γ levels were increased in the synovial fluid of early and late stage RA patients, the serum levels of the cytokines were close to that seen in controls (Bucht et al., 1996). Some authors also pointed out that, although IFN-γ was revealed in the synovial fluid and membrane, IFN-γ T cell producers were absent from peripheral blood and locally present in the joint tissues in small quantities (Ridderstad et al., 1991; De Keyser et al., 1995). T-helper 1 (Th1) cells rather than the antiviral CD8+ cytotoxic T-lymphocyte subset represent the main cellular source of IFN-γ in RA (Schuerwegh et al., 1999). Moreover, experiments using killed influenza virus (Berg et al., 2000), EBV lytic/latent peptide epitopes (Klatt et al., 2005) also support a defective capacity of the immune system to produce IFN-γ in response to viral stimulation. As a consequence, it has been proposed that a low serum level of IFN-γ may be an important factor for recurrence and reactivation of the virus (Minami et al., 2002; Davis et al., 2013; Deng et al., 2015; Motamedifar et al., 2015). A link between cytokine production and HLA alleles associated with an increased risk of RA development, HLA DRB1*0401 and DQ8, has been also established (Taneja, 2015).

At the molecular level, *Herpesviridae* produce proteins that are effective to control various STAT family members and the translocation of NF-κB from the cytoplasm into

the nuclei, which is necessary for (re)activation of the virus (Tsavachidou et al., 2001; Goodkin et al., 2003; Kuchipudi, 2015). As an example, STAT1 regulates the expression of the HSV-1 latency-associated transcript by interacting with its promoter (Kriesel et al., 2004). However, the STAT family proteins also regulate gene expression of a number of antiviral factors. This was elegantly demonstrated in a STAT3-deficient mouse model infected with HSV-1 that produced less IFN-γ and virus-specific CD8+ T cells (Yu et al., 2013). Another consequence of an infection by HSV-1 is its capacity to produce the suppressor of cytokine signaling-3 (SOCS3), a host negative regulator of the JAK/STAT pathway, which, in turn, inhibits the IFN-γ capacity to induce phosphorylation of JAK kinases (Yokota et al., 2004). As a whole, *Herpesviridae* have the opportunity to inhibit proinflammatory cytokine production (Melroe et al., 2004; Cox et al., 2015) through the control of the T cell response (Toussirot et al., 1999; Toussirot et al., 2000; Klatt et al., 2005). Such effect contributes to the reactivation of the *Herpesviridae* infection.

Another gene that is remarkably down regulated in early RA is the CC chemokine receptor 1 (CCR1), which regulates leukocyte migration to infection sites. Inhibition of this factor in herpes-infected mice caused a decreased and shortened recruitment of natural killer cells and led to an impaired antiviral response with a significantly higher viral level, inspite of the markedly enhanced levels of pro-inflammatory cytokines (Sorensen and Paludan, 2004). It is noteworthy that Olsen et al. (2004) concluded that, in total, the early RA signature showed some overlap with that seen in the normal immune response to viral antigens.

Inate Natural Killers and Cytotoxic CD8 T Cells

The iNK subset is a minor population of the innate-like T-lymphocytes. They serve as an early source of cytokines (Opasawatchai and Matangkasombut, 2015). When stimulated by the altered glycolipids of the infected host cells, iNKT rapidly begin to release a variety of cytokines and chemokines, including IFNs and TNF-α, and exert direct cytolysis (Chang et al., 2007; Matsuda et al., 2008; Tessmer et al., 2009; Horst et al., 2012). An adequate response of these cells is required for control of the viral load and protection from the massive tissue damage that can occure in cases of severe viral infection (Grubor-Bauk et al., 2003; Grubor-Bauk et al., 2008). The iNK cells have specific value in keeping the virus in latency.

Several studies have unambiguously reported qualitative and quantitative abnormalities in the iNKT population in early stage RA. Mansour et al. (2015) showed that, in early stage RA, circulating iNKTs were reduced and their frequency was inversely correlated with the disease activity score. The proliferative iNKT response was also defective. Functional iNKT alterations were due to a skewed iNKT-TCR repertoire with a selective reduction of high-affinity clones. Furthermore, the high-affinity iNKTs exhibited an altered functional Th profile with Th1 (in treatment-naive) or Th2-like (in treated patients) phenotype, compared to Th0-like Th profiles exhibited by high-affinity iNKTs in controls. Cell cytotoxicity of iNK in early stage RA is also reduced (Taylor et al., 1993). It is noteworthy that in late stage RA the data, obtained by various authors were controversial: the number of the iNK and their functions were found to be increased, decreased, or didn't differ from that seen in controls (McChesney and Bankhurst, 1986; Taylor et al., 1993; Aggarwal et al., 2014).

The population of the virus specific CD8+ T-lymphocytes and their cytotoxic activity focused on infected cells or the separate *Herpesviridae* peptide epitopes is decreased both in the early and late stage RA in contrast to an abundant and hyperactivated whole population of CD8+ T cells of various specificities (Moss et al., 1983; Gaston et al., 1986; Toussirot et al., 2000; Shimojima et al., 2008; Carvalheiro et al., 2015).

CONCLUSION

Obviously, the ideal immune system response to bacterial/viral aggression should be based on the principles of a reasonable adequacy and a perfect balance between all parts of the immune system. However, for some patients such a delicate equilibrium could be broken, leading to the development of RA. Several factors are suspected for this including:

1. A susceptibility to bacterial and viral infections greater than in the general population;
2. An imbalance of the immune system greater than in the general population;
3. An incapacity to control the inflammatory reactions, and this becomes one of the major factors provoking RA development;
4. Last but not least, disharmony in the relationship of microorganisms and the immune system of an individual predisposed to developing RA, which may be due to both genetic and epigenetic problems.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Gut-associated plasmacytoid dendritic cells display an immature phenotype and upregulated granzyme B in subjects with HIV/AIDS

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Plasmacytoid dendritic cells (pDCs) in the periphery of subjects with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) decrease over time, and the fate of these cells has been the subject of ongoing investigation. Previous studies using animal models as well as studies with humans suggest that these cells may redistribute to the gut. Other studies using animal models propose that the periphery pDCs are depleted and gut is repopulated with naive pDCs from the bone marrow. In the present study, we utilized immunohistochemistry to survey duodenum biopsies of subjects with HIV/AIDS and controls. We observed that subjects with HIV/AIDS had increased infiltration of Ki-67$^+$/CD303$^+$ pDCs, a phenotype consistent with bone marrow-derived pre-pDCs. In contrast, Ki-67$^+$/CD303$^+$ pDCs were not observed in control biopsies. We additionally observed that gut-associated pDCs in HIV/AIDS cases upregulate the proapoptotic enzyme granzyme B; however, no granzyme B was observed in the pDCs of control biopsies. Our data are consistent with reports in animal models that suggest periphery pDCs are depleted by exhaustion and that naive pDCs egress from the bone marrow and ultimately infiltrate the gut mucosa. Additionally, our observation of granzyme B upregulation in naive pDCs may identify a contributing factor to the gut pathology associated with HIV infection.

Keywords: HIV/AIDS, plasmacytoid dendritic cell, pDC, gut, granzyme B

Introduction

Human immunodeficiency virus (HIV) infection is characterized by a rapid loss of peripheral $CD4^+$ T lymphocytes during the acute phase (1, 2). $CD4^+$ T-cell levels partially recover after this phase; however, they gradually decline as the infection progresses, ultimately contributing to the development of acquired immunodeficiency syndrome (AIDS). In order to establish an infection, the viral GP120 envelope glycoprotein must bind to the cell's primary CD4 receptor as well as a coreceptor, either CCR5 or CXCR4 (3, 4). In addition to T cells, macrophages and dendritic cells also express these receptors and are thus susceptible to HIV infection (5, 6). It is therefore not surprising that decreased numbers of dendritic cells are observed in the blood of HIV cases and their presence inversely correlate with plasma viral load. For example, Grassi and coworkers reported that conventional dendritic cells (cDCs) expressing the integrin CD11c showed a significant decline during the course of HIV infection and did not return to normal levels after highly active antiretroviral therapy (HAART) (7). Loss of cDCs as well as plasmacytoid dendritic cells (pDCs) during HIV infection was also reported by Donaghy et al. (8) and similar observations were described by Pacanowski coworkers (9). Consistent with these observations, Azzoni et al. reported that cDC and pDC levels were lower in HIV viremic children when compared with those with undetectable plasma viral load (10). In the same study, they showed that subjects with declining levels of $CD4^+$ T cells are more likely to present with lower DC numbers when compared with those with a stable $CD4^+$ T cell population. These data support a characteristic pathology of HIV infection where declining DC levels inversely correlate with plasma viral load and further suggest that DCs play an important role in HIV pathogenesis.

Plasmacytoid DCs are the primary source of type I IFN and account for over 95% of all IFNα produced by circulating lymphocytes (11). Through the inhibition of viral replication, IFNα would likely play a central role in controlling HIV replication and consistent with this supposition, previous studies have reported that high levels of serum IFNα are often found in asymptomatic HIV carriers (12). In contrast, severe cases of AIDS have been associated with a diminished capacity to produce IFNα and the development of opportunistic infections (13). Furthermore, decreased IFNα levels are reported to correlate with lower circulating pDC numbers (14). Collectively, these data suggest that a decline in circulating pDCs and/or a diminished functional capacity is associated with the lower serum IFNα levels observed in subjects with HIV/AIDS.

A decline in peripheral pDCs during the course of HIV infection is well documented; however, the mechanism responsible for this decline remains a matter of current investigation. *In vitro* studies have shown that pDCs are susceptible to HIV infection and readily support viral replication (15). For this reason, their decline has been suggested to be the result of cytopathic viral replication (16). It has also been proposed that this decline may be the result of redistribution into the tissue, such as the regional lymph nodes (17, 18). Indeed, pDC redistribution into the lymph nodes has been observed in the early stages of simian immune deficiency virus (SIV) infection, the animal model for human HIV infection (17, 19, 20). Nevertheless, depletion of pDCs in lymphoid nodes of subjects infected with HIV has been documented (21), suggesting that the potential site of pDC redistribution remains elsewhere. Recently, Lehmann and colleagues observed pDC accumulation into the gut mucosa of HIV-infected subjects (22). Consistent with observations in SIV-infected macaques, pDCs in HIV cases increased their expression of gut-homing receptors (6, 23), implying that depletion of circulating pDCs is likely a result of their redistribution. However, a recent report by Bruel et al. suggests that the loss of pDCs in the periphery is the result of pDC exhaustion and the apparent redistribution of pDCs to the gut can be explained as pDC precursors migrating from the bone marrow to the gut (24). In a follow-up study to an early report (23), Li et al. observed that, in acute SIV infection of rhesus macaques, gut-homing was imprinted upon pDCs in the bone marrow, which resulted in a decline in pDCs from circulation and secondary lymphoid tissue and subsequent accumulation of hyperfunctional $CD4^+$ pDCs in the mucosae (25).

In the present study, we have investigated the distribution and phenotype of pDCs in human subjects with HIV/AIDS. Consistent with previous reports, our data show a statistically significant decline in circulating pDCs in cases when compared with healthy controls. Using immunohistochemistry, we also observed a significant increase of pDC infiltration into the duodenal mucosal tissue of HIV cases when compared with control biopsies. Additionally and consistent with observations made by Bruel et al. in SIV-infected cynomolgus macaques, we observed that duodenal-associated pDCs in HIV-positive subjects express the cellular proliferation marker Ki-67. Our study supports the previous report of Bruel et al. (24) and Li et al. (25); however, further studies will be required to fully appreciate the contribution of this subpopulation of pDCs to the enteropathy of HIV infection.

Materials and Methods

Subjects

Twenty-three subjects (15 males and 8 females) who were hospitalized at the Republican Center for AIDS Prophylaxis and Prevention, Republic of Tatarstan, were enrolled in this study. Diagnosis of HIV infection was established based on the presence of anti-HIV antibodies using ELISA and confirmed by Western blot. Blood samples were collected from the 23 HIV cases and six of these subjects consented to providing a duodenal biopsy for analysis. Additionally, blood samples were collected from 16 healthy donors. For control biopsies, we utilized duodenal biopsies from eight individuals who underwent routine gastroscopy for gastritis and were otherwise healthy. The Institutional Review Board of the Kazan Federal University approved this study and informed consent was obtained from each study subject according to the guidelines approved under this protocol (article 20, Federal Law "Protection of Health Right of Citizens of Russian Federation" N323-FZ, 11.21.2011) and in accord with the Declaration of Helsinki (2008). Surplus clinical biopsies from subjects with gastritis were acquired under an exemption to IRB as determined by the University of Nevada, Reno Office of Research Integrity (exemption #508962-1).

Clinical Presentation of HIV Cases

Diagnosis of HIV infection was established by detection of anti-HIV antibodies by ELISA and confirmed by Western blot. A total of 23 HIV cases were enrolled in this study (**Table 1**); eight of whom were female (35%) and 15 (65%) were male with an average age of 35.6 years (range, 21–46 years). Mode of transmission included sexual contact (11 cases; 48%) and IV drug users (12 cases; 52%). Enrolled were 12 cases with the severe form (52%), 3 cases with the advanced form (13%), 4 cases with the mild form (17.5%), and 4 cases with non-significant form (17.5%). Sixteen cases (70%) received antiviral treatment while seven cases (30%) remained without virus-specific therapy. Antiviral treatment included nucleoside analogs, non-nucleoside reverse transcriptase inhibitors, and protease inhibitors. Average HIV RNA viral load was 4412.1 ± 342 copies/ml, where 16 cases (70%) had a viral load higher than 250 copies/ml; the remaining seven cases (30%) had an undetectable viral load or less than 250 copies/ml. The mean $CD4^+$ T-cell count was 306.1 ± 56 cells/mm^3. As the disease progressed, some subjects were diagnosed with opportunistic infections, among which were candidiasis, hairy leukoplakia, and tuberculosis. HIV case characteristics and status are summarized in **Table 1** and Table S1 in Supplementary Material.

Antibodies for Flow Cytometry and Immunohistochemistry

PerCP mouse anti-human CD45 was from Beckman Coulter (Brea, CA, USA). PE Anti-Ki-67 (clone B56) and PE mouse IgG1,κ isotype control were from BD Pharmigen (San Jose, CA, USA); PE mouse anti-human GZMB IgG1 (clone GB11) and PE mouse IgG1 isotype control were from Life Technologies (Carlsbad, CA, USA). FITC or APC mouse anti-human CD303 (clone AC144); PE mouse anti-human CD123 (clone AC145); PE mouse anti-human CD80 (clone 2D10); PE mouse anti-human CD56 IgG1 (clone AF12-7H3); APC mouse anti-human CD11c IgG2b (clone MJ4-27G12); APC mouse IgG2b isotype control; and PE mouse anti-human CD4 (clone VIT4) were from MiltenyiBiotec (Auburn, CA, USA).

Flow Cytometry Analysis

Anticoagulated blood was collected in EDTA-containing vacutainer tubes by venipuncture. Whole blood (100 µl) was labeled with anti-human CD45-PerCP, anti-human CD303-APC, and anti-human CD123-PE for 20 min at room temperature, lysed with FACS Lyse (BD Biosciences, San Jose, CA, USA), and analyzed immediately on a FACS Canto II flow cytometer using FACS Diva software (BD Biosciences). A minimum of 300,000 events was collected for each sample and pDCs were identified as being $CD45^+CD303^+CD123^+$.

TABLE 1 | Demographics of HIV cases by World Health Organization (WHO) immunological classification.

WHO HIV-associated immunological classification	CD4 (cells/mm^3)	Sex		Total	
		Male	Female	*n*	%
Severe	<200	8	4	12	52.0
Advanced	200–349	3	0	3	13.0
Mild	350–499	1	3	4	17.5
None/not significant	≥500	3	1	4	17.5
Total		15 (65%)	8 (35%)	23	100

Tissues Preparation and Immunohistochemical Analysis

Limitations on gut tissue samples made flow cytometery analysis impractical; therefore, we characterized duodenum biopsies by immunohistochemistry. Fresh tissues were fixed in 4% paraformaldehyde for 4 h at 4°C and cryoprotected with a 30% sucrose solution in phosphate-buffered saline (PBS) before being paraffin embedded. Immunohistochemical (IHC) staining was performed on 2–3-µm-thick tissue sections. Tissue slides were deparaffinized with xylene and rehydrated through a graded alcohol series. Antigen retrieval was carried out by boiling slides in sodium citrate (0.01M, pH 6.0) at 95°C for 10 min. The slides were next rinsed in PBS and incubated in cold methanol for 20 min at −20°C. Tissue sections were then incubated with human AB serum to block non-specific staining (1 h at 37°C) and then incubated with the labeled antibody overnight at 4°C in a humidified chamber. Slides were then washed (3×; Tween 0.1% PBS) and examined using a Leitz TCS-SP2 RS scanning laser confocal microscope (Wetzlar, Germany) and images were captured with Leitz analysis software.

In order to identify duodenum-infiltrating pDCs, we utilized a panel of fluorescently labeled monoclonal antibodies specific for putative surface cell receptors know to be expressed on mature and naive pDCs. Included in this panel were monoclonal antibodies specific for CD123, which is normally expressed on all mature and BM-derived pre-pDCs; CD303, which is uniquely expressed on circulating pDCs (26) and also on BM-derived pre-pDCs as early as Stage II (27); Ki-67, to distinguish naive pDCs ($Ki\text{-}67^+$) from mature non-dividing ($Ki\text{-}67^-$) pDCs; and the costimulatory marker, CD80, which is not expressed on BM-derived pre-pDCs at any stage, was used as an activation marker. We additionally probed our biopsies for the myloid CD marker, CD11c, which is also expressed on a minor population of BM-derived pDCs, and finally, in order to identify pDCs with cytotoxic potential, we probed our specimens for granzyme B (GZMB), and CD56.

Statistical Analysis

Data are presented as mean/SD. Statistical analysis was performed using Mann–Whitney test for comparisons between individual experimental groups (case and control). Significance was established at a value of $p < 0.05$.

Results

Decreased pDCs in the Periphery of HIV/AIDS Case

Previous studies have shown that subjects with HIV/AIDS have decreased circulating pDCs when compared with healthy controls. To confirm that our study population was consistent with those previously reported, we initially evaluated our subjects for circulating pDCs by flow cytometry. Total lymphocytes were first gated by $CD45^+$/side scatter (data not shown) and then pDCs were identified as a population of $CD303^+/CD123^+$

lymphocytes (**Figure 1A**). The mean pDC count was calculated as a ratio of $CD303^+CD123^+CD45^+$ over total $CD45^+$ lymphocytes. Consistent with the observations of others, we observe that, on average, subjects with HIV/AIDS had significantly lower circulating $CD303^+CD123^+$ lymphocytes when compared with healthy controls, 0.044 ± 0.084 vs. 0.093 ± 0.014 ($p = 0.0031$) (**Figure 1B**).

Increased pDC Infiltration of the Duodenum in Association with HIV Infection

Plasmacytoid dendritic cells exclusively express the surface marker CD303 (BDCA-2) and non-exclusively express CD123, the receptor for the myeloid stimulatory cytokine IL-3 (26, 28, 29). Consistent with ability to become infected by HIV, they also express the HIV receptor CD4 as well as the coreceptors CCR5 and CXCR4. As with other antigen-presenting cells, when activated, they additionally express the B7 costimulatory molecules CD80 and CD86. Therefore, in order to characterize gut-associated pDCs in HIV/AIDS cases and controls, we probed the respective duodenum biopsies with fluorescently labeled anti-CD303, anti-CD123, anti-CD4, and anti-CD80 monoclonal antibodies (30). Consistent with previous reports, we observed a substantial infiltration of $CD303^+$ pDCs in all duodenum biopsies from the HIV-infected subjects; however, we observed significantly fewer infiltrating pDCs in the control biopsies (**Figure 2**). In order to quantify these differences, five

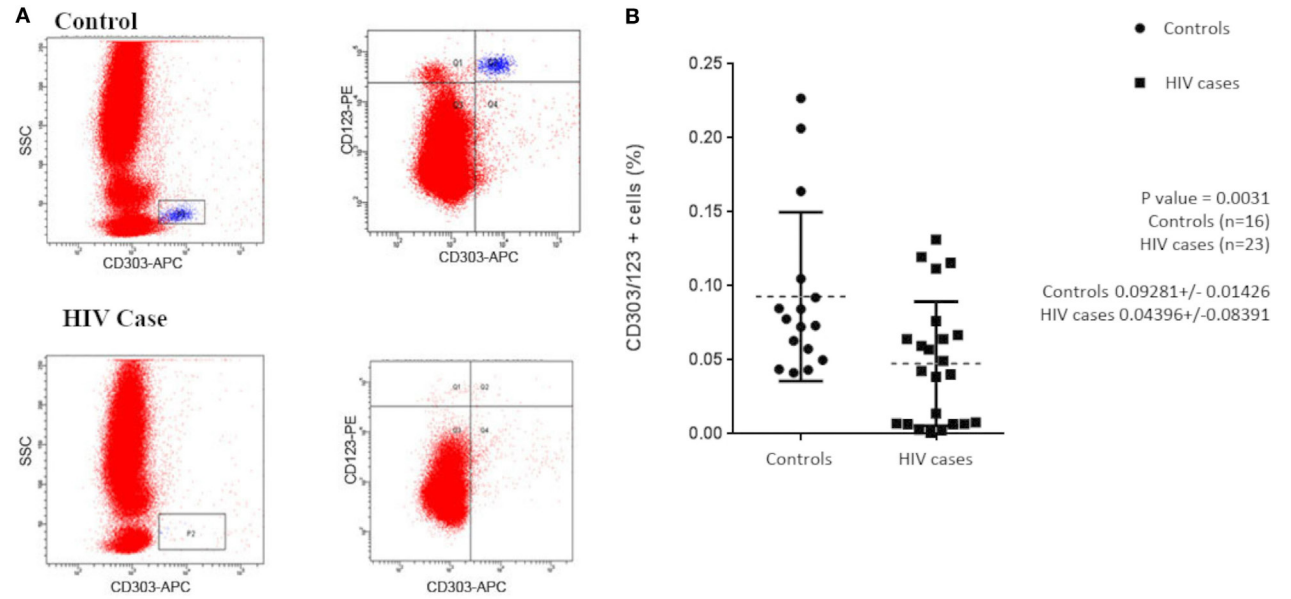

FIGURE 1 | (A) Flow cytometry was used to evaluate circulating plasmacytoid dendritic cells (pDCs) in HIV/AIDS cases and controls. Aliquots of whole blood from 23 cases and 16 controls were labeled with APC-anti-CD303, PE-anti-CD123, and PerCp-anti-CD45. Lymphocytes were initially gated by $CD45^+$/side scatter and pDCs were identified as $CD303^+/CD123^+$ lymphocytes. **(B)** pDCs, as a percentage of total lymphocytes, were used to evaluate differences between cases and controls. Mean pDC counts of HIV cases were 0.044 ± 0.084 and controls were 0.093 ± 0.014 ($p = 0.0031$).

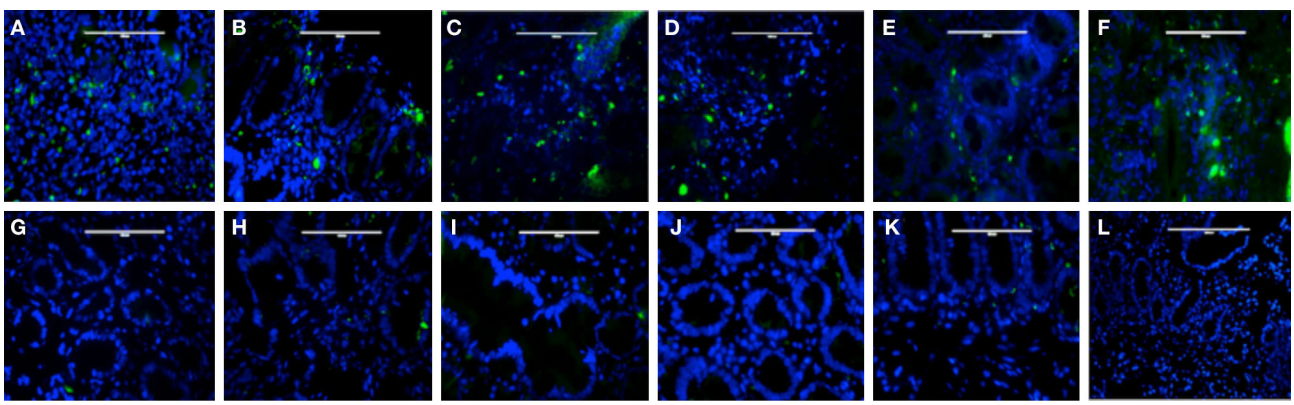

FIGURE 2 | Infiltration of plasmacytoid dendritic cells in duodenum biopsies of six subjects with HIV/AIDS (A–F) and six non-HIV subjects who were evaluated for gastritis (G–L). Biopsies were probed with antibodies FITC-anti-CD303 monoclonal antibody (green) and nucleus localization was determined by TOPO3 staining (blue). Bar represents 20 μm.

microscopic fields were randomly chosen for each subjects in each cohort and used to calculated average pDC infiltration (**Table 2**). We observed a sixfold greater infiltration of pDCs in the gut biopsies of HIV-infected cases over that of the controls ($p < 0.0051$). In order to further characterize the pDC infiltrate, double staining was conducted which confirmed that all CD303$^+$ cells were also all CD4$^+$ consistent with peripheral and pDCs and state II and III BM-derived pDCs (**Figure 3**). Additionally, all CD303$^+$ cells were also CD123$^+$ in the control biopsies (**Figures 4A–C**); however and unexpectedly, the CD123 staining was observably lower or absent for the HIV cohort (**Figures 4D–F**). Finally, double staining for CD303 and CD80 revealed that most of the CD303$^+$ cells in the control biopsies also expressed the activation marker CD80 (**Figures 4G–I**), and all CD303$^+$ cells in biopsies from the HIV cohort were also CD80$^+$ (**Figures 4J–L**).

TABLE 2 | Quantitative analysis of plasmacytoid dendritic cells (pDCs).

Field	Controls						Mean/SD
	1	**2**	**3**	**4**	**5**	**6**	
1	1	1	2	3	1	2	
2	3	2	4	3	0	4	
3	0	5	1	1	3	2	
4	1	1	0	3	2	5	
5	1	1	2	1	2	2	
Sum	6	10	9	11	8	15	9.8/3.1
Field	**HIV cases**						**Mean/SD**
	1	**2**	**3**	**4**	**5**	**6**	
1	10	12	15	9	9	18	
2	12	7	14	15	14	18	
3	16	12	17	10	17	10	
4	10	15	10	13	11	13	
5	11	11	7	17	13	11	
Sum	59	57	63	64	64	70	61.4/4.5

Five microscopic fields were chosen at random and used to count total pDCs in the duodenum of six controls (top) and six cases (bottom). p = 0.0051 by Mann–Whitney.

Gut-Associated pDCs in HIV Cases Display an Immature Phenotype

Using SIV-infected cynomolgus macaques, an animal model of HIV, Bruel and coworkers observed that gut-associated pDCs express the cellular proliferation marker Ki-67 (24). In that pDCs in the periphery do not divide, this observation suggests that the gut-associated pDCs in the SIV-infected macaques are pDCs precursors from bone marrow and not mature pDCs from the periphery. To the best of our knowledge, this observation has not been confirmed in humans. Therefore, we used fluorescently labeled anti-Ki-67 monoclonal antibodies to probe the biopsies of HIV-infected cases and controls. Consistent with the observations of Bruel et al., we observed CD303$^+$/Ki-67$^+$ cells in the biopsies of HIV-infected subjects (**Figures 5A–C**) but not in the control biopsies (**Figures 5D–F**). In order to evaluate the specificity of the primary antibody, we probed the same biopsies with a matched isotype control. An absence of non-specific staining (**Figures 5G–I**) confirmed that the immunoreactivity for CD303 and Ki-67 was indeed specific. A minor population of BM-derived pre-pDCs are also known to express the myeloid DC marker CD11c. Again, consistent with BM-derived pre-pDCs, we observed that most of the duodenum-associated pDCs in the HIV biopsies were also CD11c$^+$ (**Figures 6A–C**); however, no CD303$^+$/CD11c$^+$ cells were observed in the control biopsies (**Figures 6D–F**).

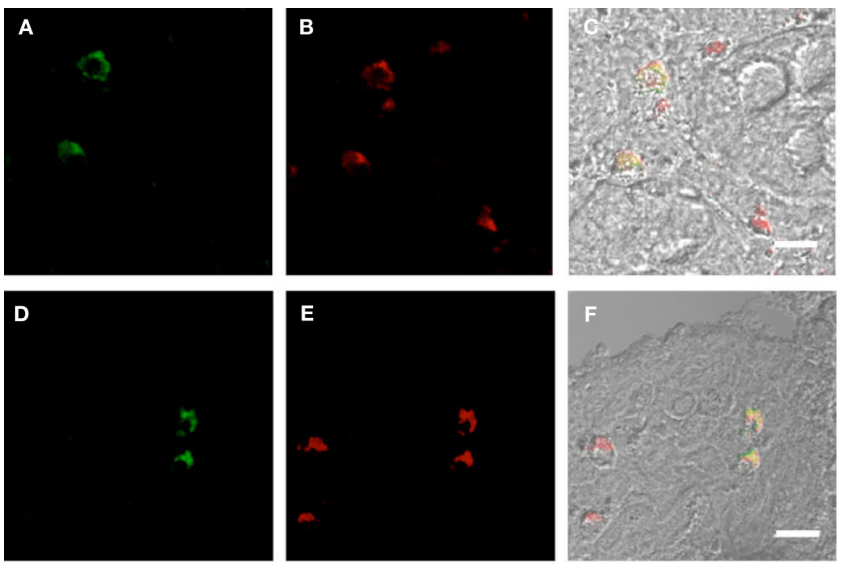

FIGURE 3 | Duodenum biopsies of HIV/AIDS cases and controls evaluated for coexpression of CD303 (green) and CD4 (red). Bar represents 20 μm. **(A)** HIV case duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(B)** HIV case duodenum biopsies probed with a monoclonal PE-anti-CD4 antibody. **(C)** HIV case duodenum biopsies DIC image merged with **(A,B)**. **(D)** Control duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(E)** Control duodenum biopsies probed with a monoclonal PE-anti-CD4 antibody. **(F)** Control duodenum biopsies DIC image merged with **(D,E)**.

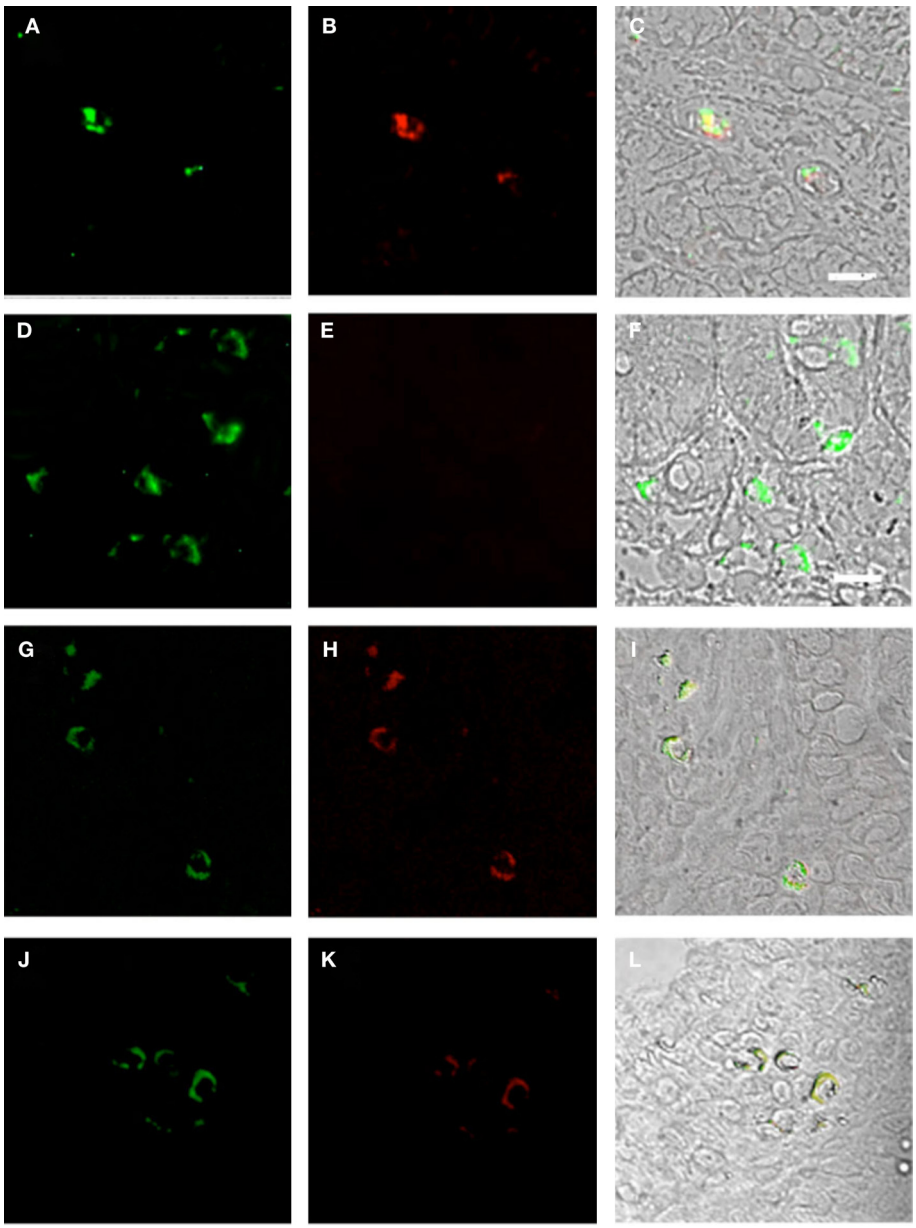

FIGURE 4 | Duodenum biopsies of HIV/AIDS cases and controls evaluated for coexpression of CD303 (green) and CD123 (red) and for CD303 (green) and CD80 (red). Bar represents 20 μm. **(A)** Control duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(B)** Control duodenum biopsies probed with a monoclonal PE-anti-CD123 antibody. **(C)** Control duodenum biopsies DIC image merged with **(A,B)**. **(D)** HIV case duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(E)** HIV case duodenum biopsies probed with a monoclonal PE-anti-CD123 antibody. **(F)** HIV case duodenum biopsies DIC image merged with **(D**,**E)**. **(G)** Control duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(H)** Control duodenum biopsies probed with a monoclonal PE-anti-CD80 antibody. **(I)** Control duodenum biopsies DIC image merged with **(G,H)**. **(J)** HIV case duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(K)** HIV case duodenum biopsies probed with a monoclonal PE-anti-CD80 antibody. **(L)** HIV case duodenum biopsies DIC image merged with **(J,K)**.

Gut-Associated pDCs in HIV-Infected Subjects Display a Killer-pDC Phenotype

Previous studies have reported that the GI tract experiences significant pathology during the course of HIV infection [reviewed by Brenchley and Douek (31)]; however, the mechanism of this pathology is not fully understood. pDCs have the capacity to display a "killer" phenotype, with the ability to lyse target cells in either a GZMB- or TRAIL-dependent manner (32, 33). We therefore speculated that this may contribute to the gut mucosal damage associated with HIV infection. To this end, we probed the duodenum biopsies of HIV subjects and controls for coexpression of CD303 and GZMB and observed significant immunoreactivity

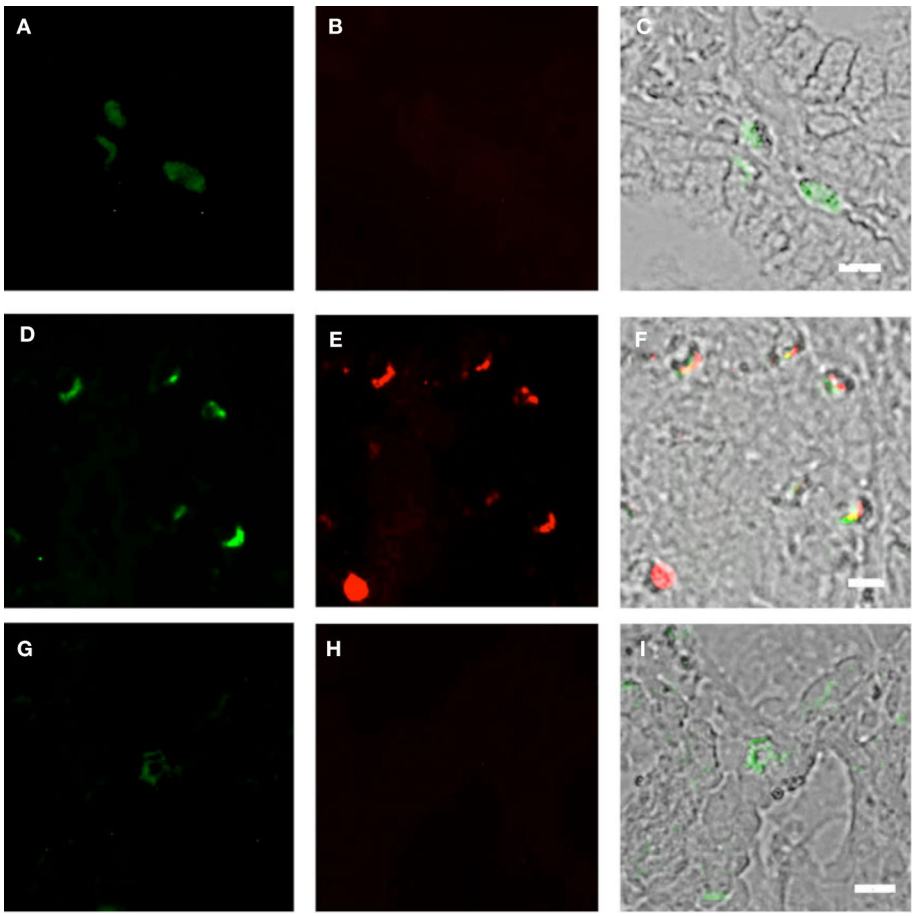

FIGURE 5 | Duodenum biopsies of HIV/AIDS cases and controls evaluated for coexpression of CD303 (green) and Ki-67 (red). Bar represents 20 μm. **(A)** Control duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(B)** Control duodenum biopsies probed with a monoclonal PE-anti-Ki-67 antibody. **(C)** Control duodenum biopsies DIC image merged with **(D,E)**. **(D)** HIV case duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(E)** HIV case duodenum biopsies probed with a monoclonal PE-anti-Ki-67 antibody. **(F)** HIV case duodenum biopsies DIC image merged with **(A,B)**. **(G)** HIV case duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(H)** HIV case duodenum biopsies probed with a PE-Mouse IgG1,κ isotype control antibody. **(I)** HIV case duodenum biopsies DIC image merged with **(G,H)**.

($CD303^+/GZMB^+$) in the HIV cohort (**Figures 7A–C**). In contrast, an absence of immunoreactivity with the anti-GZMB antibody was observed in control biopsies (**Figures 7D–F**). It has also been reported that GZMB-dependent killer pDCs also upregulate the neural adhesion marker CD56; therefore, we additionally probed the same biopsies with anti-CD56 and observed a number of $CD303^+/CDCD56^+$ cells in the biopsies of HIV/AIDS cases (**Figures 8A–C**). In contrast, no $CD303^+/CD56^+$ cells were observed in the controls biopsies (**Figures 8D–F**). Absence of reactivity with matched isotype controls for the anti-GZMB and CD56 antibodies suggested that the binding was specific.

Discussion

As the principal source of type I IFN produced by circulating immune cells, pDCs play a seminal role in the innate antiviral immune response. Under homeostatic conditions, pDCs are primarily found in circulation and mucosal tissues such as the gastrointestinal lymphoid tissue (GALT) and the respiratory tract (34–36). Only small numbers of pDCs are normally present in peripheral tissue; however, their transmigration increases significantly during inflammation (37, 38). Once activated, pDCs have the capacity to release proinflammatory cytokines such as CXCL8 and CXCL10, and therefore, they also have the capacity to contribute to pathology.

Numerous studies have shown that pDCs are qualitatively and quantitatively impacted during HIV infection. For example, Malleret et al. reported a decline in the number of circulating pDCs in animals infected with SIV (17). Additionally, Brown and coworkers observed a rapid decline in circulating pDCs that correlated with their migration to the lymph nodes (19). Other studies have reported a decline in circulating pDC numbers in HIV cases that inversely correlated with viremia (16, 39, 40).

Several hypotheses have been proposed to explain the decrease in circulating pDCs associated with HIV infection, including, virus-associated cytopathic effects and pDC redistribution, from the circulation into tissue (9, 16, 41–44). Kwa and coworkers reported that pDCs infiltrate the colorectal region of the gut in

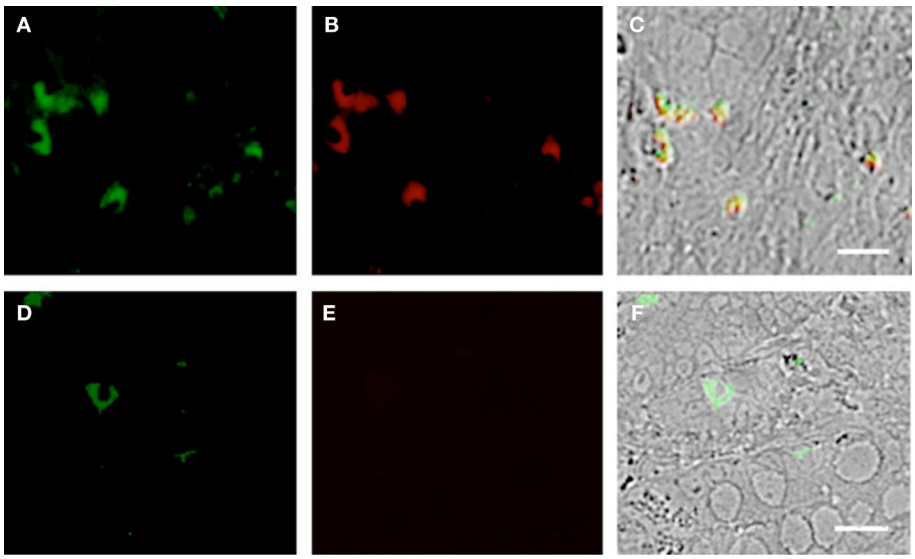

FIGURE 6 | Duodenum biopsies of HIV/AIDS cases and controls evaluated for coexpression of CD303 (green) and CD11c (red). Bar represents 20 μm. **(A)** HIV case duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(B)** HIV case duodenum biopsies probed with monoclonal APC-anti-CD11c antibody. **(C)** HIV case duodenum biopsies DIC image merged with **(A,B)**. **(D)** Control duodenum biopsies probed with monoclonal FITC-anti-CD303 antibody. **(E)** Control duodenum biopsies probed with monoclonal APC-anti-CD11c antibody. **(F)** Control duodenum biopsies DIC image merged with **(D,E)**.

SIV acutely infected rhesus macaques and that the gut infiltrating pDCs were producing high levels of IFNα and other proinflammatory cytokines (45). Additionally, Reeves et al. reported that SIV infection induces the accumulation of pDCs in the gut mucosa (23), and Li et al. observed a fourfold increase in pDC accumulation in jejunum, colon, and gut-draining LNs of SIV-infected rhesus macaques (46). Similar distributions of pDCs have been observed in HIV cases. For instance, Lehmann and coworkers observed increased pDC accumulation in the terminal ileum of HIV-infected subjects (47), and tissue homing was explained by significant upregulation of the gut-homing receptor CD103, when compared with uninfected controls. These data support a model whereby pDCs redistribute from the circulation into the gut during the course of HIV infection. Although the previous data unequivocally supported this supposition, it was recently challenged by the report of Bruel and colleagues whereby they described the depletion of IFN-producing pDCs in the periphery as a result of activation-driven exhaustion, followed by a concomitant increase in gut-associated pDCs (24). They further reported that the gut-associated pDCs are primarily naive Ki-67$^+$ pDCs, suggesting that the BM-derived pDCs egressed from the BM to replace the depleted circulating pDCs and subsequently migrate from the periphery to the gut. Therefore, the current data suggest that the loss of peripheral pDCs during the course of HIV infection is not just a matter of tissue redistribution but a combination of pDC depletion, repopulation, and migration.

During the acute infection stage, pDCs respond with a robust IFN production; however as the acute stage transitions to the chronic stage, they become refractory with respect to their ability to produce IFN (48). BM-derived pDC precursors have little capacity to produce type I IFN and may also have a phenotype different from that of mature circulating pDCs that depends on their stage of development (27). Three subsets of pre-pDCs have been described based on the expression level of CD34 and HLA-DR (Class II) surface markers. All three populations express CD123 (IL-3α chain receptor); however, CD4 expression is absent in the earliest pre-pDCs (Stage I pre-pDCs) but becomes evident in the more developed pre-pDCs (Stages II and III) (27). Additionally, CD184 (CXCR4) is observed in all three stages. These observations suggest that pre-pDCs have the capacity to be infected by HIV at least as early as Stage II.

In the present study, we observed that duodenum-associated CD303$^+$ pDCs expressed Ki-67, but no anti-Ki-67 immunoreactivity was observed in the control biopsies. These observations are consistent with those made in SIV-infected cynomologus macaques by Bruel et al. (24). Ki-67 is nuclear proliferation antigen, which is expressed by cells in a non-G_0 phase of the cell cycle and therefore is indicative of pDCs that were recently mobilized from the bone marrow (19, 49). Additionally, CD303 is not present on Stage I BM-derived pre-pDCs (27); therefore, our data further suggests that duodenum-associated pDCs in HIV-infected subjects are largely consistent with Stage II or Stage III pre-pDCs. We also observed that gut-associated CD303$^+$ pDCs expressed the B7 costimulatory molecule CD80. The B7 costimulatory molecule CD80 is upregulated during *in vitro* HIV infection of pDCs (50); however, circulating pDCs from healthy or HIV-infected subjects show little, if any, CD80 expression. Additionally, characterization of pre-pDCs from healthy BM-donors suggests that these cells do not express CD80 (27). Our observation of CD80 expression by gut-associated pDCs implies that these cells may be activated preferentially in the gut over that of those in the circulation. Also, in contrast to controls, we observed that gut-associated pDCs in HIV-infected subjects express the common myeloid dendritic cell protein CD11c. Although murine pDCs express CD11c at

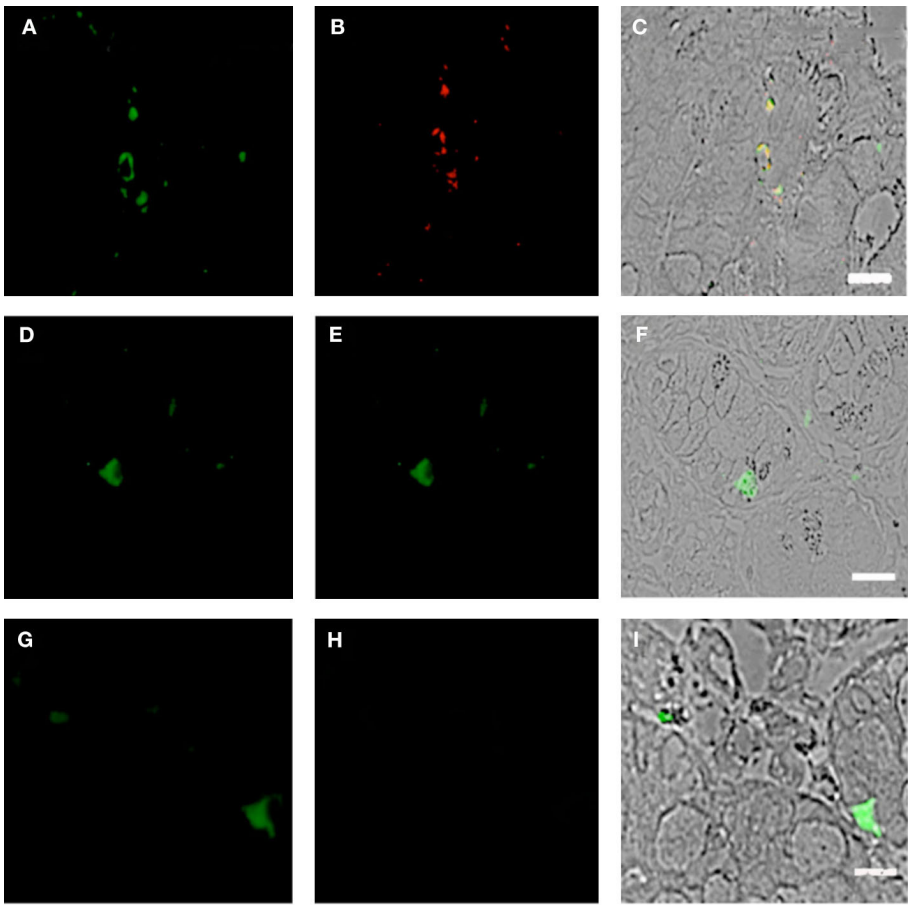

FIGURE 7 | Duodenum biopsies of HIV/AIDS cases and controls evaluated for coexpression of CD303 (green) and granzyme B (GZMB) (red). Bar represents 20 μm. **(A)** HIV case duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(B)** HIV case duodenum biopsies probed with monoclonal APC-anti-GZMB antibody. **(C)** HIV case duodenum biopsies DIC image merged with **(A,B)**. **(D)** Control duodenum biopsies probed with monoclonal FITC-anti-CD303 antibody. **(E)** Control duodenum biopsies probed with monoclonal APC-anti-GZMB antibody. **(F)** Control duodenum biopsies DIC image merged with **(D,E)**. **(G)** HIV case duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(H)** HIV case duodenum biopsies probed with a PE-Mouse IgG1 isotype control antibody. **(I)** HIV case duodenum biopsies DIC image merged with **(G,H)**.

low levels, its expression is typically absent on circulating human pDCs. Notwithstanding, CD11c expressed on a subpopulation of BM-derived pre-pDCs has been described, again supporting the supposition that the gut-associated pDCs in our HIV cohort are naive BM-derived pDCs.

Activated pDCs have the capacity to express the cytotoxic enzyme GZMB; however, its expression is downregulated in the presence of IFNα and upregulated by IL-3 (51). Not only are BM-derived pre-pDCs refractory to the production of type I IFN, we observed gut-associated pDCs in our HIV cohort have low or absent CD123 expression, albeit this observation is only qualitative in that it was not practical to quantify this value by IHC. IL-3 signaling and GZMB expression is intimately connected (51); therefore, a dysregulation in CD123 (the IL-3 receptor) may potentially impact the expression of GZMB. In addition to the cytotoxic and proapoptotic role of GZMB, it also plays a role in inflammation and tissue remodeling (50). Severe tissue damage leading to increased intestinal epithelium permeability is hallmark of HIV infection (52), and this process is not reversed even after long-term HAART therapy (53–55). Although speculative at this point, the presence of GZMB may suggest a potential mechanism that contributes to the decreased regenerative capacity of the gut epithelium and increased mucosal permeability and inflammation associated with HIV infection (56–58). To the best of our knowledge, the expression of GZMB and the downregulation of CD123 in gut-associated pDCs have not been described in HIV; however, future studies will be required to fully elucidate the role of GZMB in HIV-associated gut pathology. Finally, we observed that most $CD303^+$ cells also expressed the neural adhesion molecule CD56. Tel et al. have previously described the coexpression of CD303, CD56, and GZMB by pDCs activated with the preventative vaccine to tick-borne encephalitis virus FSME. These "killer pDCs" possessed the tumoricidal capacity to lyse K562 and Daudi cells in a contact-dependent manner (59). They additionally reported that CD303 and CD56 expression coincided with elevated expression of programmed death-ligand 1 (PD-L1), GZMB, and TNF-related apoptosis-inducing ligand (TRAIL). It is also noteworthy that all pDC neoplasms express the CD56 marker (60), further suggesting

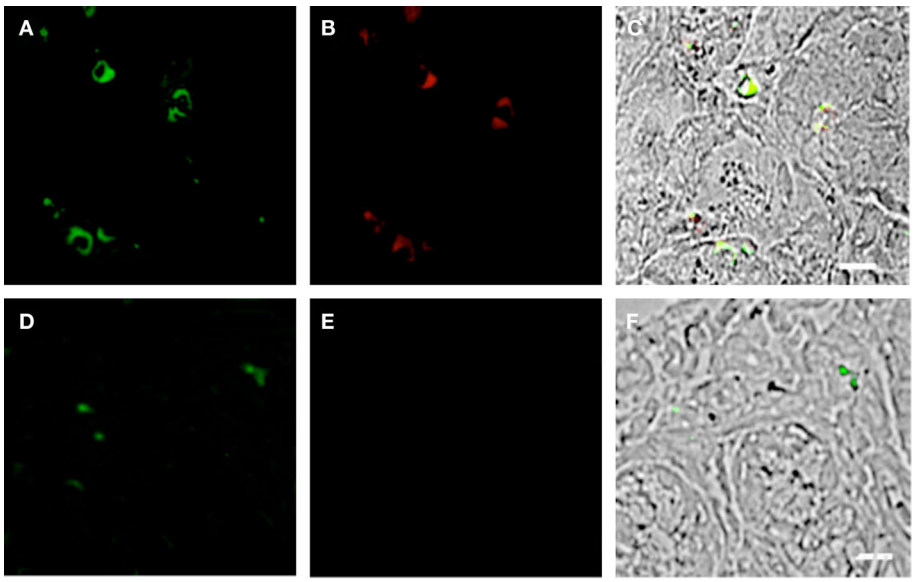

FIGURE 8 | Duodenum biopsies of HIV/AIDS cases and controls evaluated for coexpression of CD303 (green) and CD56 (red). Bar represents 20 μm. **(A)** HIV case duodenum biopsies probed with a monoclonal FITC-anti-CD303 antibody. **(B)** HIV case duodenum biopsies probed with monoclonal APC-anti-CD56 antibody. **(C)** HIV case duodenum biopsies DIC image merged with **(A,B)**. **(D)** Control duodenum biopsies probed with monoclonal FITC-anti-CD303 antibody. **(E)** Control duodenum biopsies probed with monoclonal APC-anti-CD56 antibody. **(F)** Control duodenum biopsies DIC image merged with **(D,E)**.

that CD56 expression by pDCs is not necessarily novel or without precedence. The significance of this marker has yet to be determined on NK cells so the contribution to pDC biology in the context of HIV infection will require further investigations as well.

Conclusion

In summary, our data show that subjects with HIV have, on average, decreased pDCs in the periphery, when compared with healthy controls, consistent with previous reports. Additionally, we show that gut-associated pDCs in HIV cases express the cellular proliferation marker Ki-67, which suggests that the gut-associated pDCs are naive and likely of bone marrow origin. Finally, we observed that gut-associated pDCs have an activated phenotype and also upregulate the proapoptotic enzyme GZMB. When taken together, our data support a model of HIV progression whereby circulating pDCs are depleted and replaced by naive BM-derived pDCs, which have little, if any, IFN-producing capacity. Ultimately, these pDCs migrate to the gut, potentially subjecting the gut mucosa to the inflammatory effects and damage associated with inflammatory cytokine production and GZMB expression. The type I IFN produced by competent pDCs is a critical part of an innate immune response to viral infection. Therefore, a greater understanding of the fate of these cells in HIV infection may lead to strategies that can restore the IFN-producing capacity of the innate immune system.

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Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00485

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Hand to Mouth: A Systematic Review and Meta-Analysis of the Association between Rheumatoid Arthritis and Periodontitis

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Background: Rheumatoid arthritis (RA) and periodontitis are both chronic inflammatory diseases, which demonstrate similarities in terms of mechanism, histopathology, and demography. An association between these conditions has been demonstrated previously but has been called into question more recently.

Methods: The published databases, such as MEDLINE, EMBASE, and PsycINFO, were searched using search terms related to RA and periodontitis. Articles were selected if they included data on the number of people with RA diagnosed with periodontitis (or periodontal disease parameters) compared to a control comparison group. Review articles, case reports, animal model studies, non-English language, and articles with unavailable abstracts were excluded. Data were extracted, critically appraised using the Downs and Black tool, and a random-effect Mantel–Haenszel meta-analysis was performed.

Results: Twenty-one papers met the eligibility criteria and provided data for the meta-analysis; 17 studies (including a total of 153,492 participants) comparing RA to healthy controls and 4 (including a total of 1378 participants) comparing RA to osteoarthritis (OA). There was a significantly increased risk of periodontitis in people with RA compared to healthy controls (relative risk: 1.13; 95% CI: 1.04, 1.23; $p = 0.006$; $N = 153,277$) with a significantly raised mean probing depth, risk of bleeding on probing (BOP), and absolute value of clinical attachment loss in those with RA. When comparing RA and OA, there was no significant difference in the prevalence of periodontitis; however, the risk of BOP was greater in OA than RA.

Conclusion: A significant association between RA and periodontitis is supported by the results of our systematic review and meta-analysis of studies comparing RA to healthy controls. In our meta-analysis, however, this is not replicated when comparing RA to OA controls.

Keywords: rheumatoid arthritis, inflammatory arthritis, periodontitis, periodontal disease, meta-analysis

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INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by joint inflammation and destruction leading to chronic disability, early mortality, systemic complications, and high socioeconomic burden on society as a whole (1). It has a prevalence of 0.5–1.0% in US populations (2). The exact etiology of RA is unknown; however, it is thought to be secondary to an interaction between genetic attributes and environmental exposures, as demonstrated by the established association with smoking (3) and genetic polymorphisms, including HLA-DRB1 (4).

Auto-antibodies to the Fc portion of immunoglobulin are known as rheumatoid factor, and antibodies that form against citrullinated proteins are called anti-citrullinated protein antibody (ACPA) or anti-cyclic citrullinated peptide (anti-CCP). If either of these are present then they confer "seropositivity" that is seen in 70–80% of patients (5) and are associated with more aggressive disease and with the earlier development of erosions. Rheumatoid factor is limited in diagnostic application by low specificity; however, ACPA is 95–98% specific (6).

Anti-citrullinated protein antibody has been shown to be present in RA patient sera up to a decade prior to the development of the disease (7), although the amount of ACPA and inflammatory cytokine levels rises sharply a few months before the synovitis presents (8). It is therefore hypothesized that as well as citrullination of endogenous proteins, a second inflammatory "hit" is required to stimulate the development of RA. Citrullinated proteins are also associated with other environmental factors such as smoking and pathological conditions, including periodontitis.

Peptidyl arginine deiminase (PAD) causes the post-translational modification of arginine to citrulline. It is hypothesized that this citrullination leads to amino acid chains being recognized as auto-antigens, which leads to the development of auto-antibodies and the subsequent autoimmune damage that is the signature for RA. PAD is produced by human cells, for example, in the lung; however, it is also produced by the microbe *Porphyromonas gingivalis* (9).

Peptidyl arginine deiminase production by *P. gingivalis*, an anaerobic prokaryote, has been demonstrated *in vitro*. Due to this organism's role in the development of periodontal disease and the association of RA with periodontitis, it has been hypothesized that *P. gingivalis* provides a causal link among periodontal disease, citrullination, and RA (10).

Periodontitis is a destructive, infectious-inflammatory condition affecting the gums. It is characterized by loss of gingival attachment between the tooth and the gingivae leading to the formation of a periodontal pocket (11). Initially, a biofilm structure develops, which causes localized inflammation in the form of gingivitis. This biofilm is then colonized by anaerobic bacteria that cause further inflammation and neutrophillic activation. Matrix metalloproteinases are spilled during this inflammatory reaction leading to tissue destruction and further exacerbate the attachment loss and deepening the periodontal pocket, resulting in further anaerobic colonization, soft tissue destruction, alveolar bone loss, and, ultimately, tooth loss.

The prevalence of periodontitis varies internationally; however, approximately 10–15% of the global adult population are affected by the condition (12). Known risk factors include smoking, age, diabetes mellitus (13), educational level (14), and immunological diseases (e.g., HIV) (15). Periodontitis, itself is associated with a higher risk of stroke, cardiovascular disease (16), and pneumonia (17).

It is divided into two subtypes; aggressive periodontitis and chronic periodontitis (CP). CP is the more common form affecting an older population with an indolent disease progression; however, aggressive periodontitis is observed in a young population, aggregates in families, and leads to rapid tooth loss and the need for prostheses (18).

Certain bacteria have been implicated in the development of periodontitis, including the "red complex" organisms; *P. gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans*. It is not clear whether there are microbiological differences between the chronic and aggressive subtypes of periodontitis (18).

Epidemiological studies have shown a strong association between periodontitis and RA (19, 20), though these have been hampered by their cross-sectional nature, variability in definition of periodontitis and dental endpoints, the extent of oral examination, and the limited RA information collected.

Shared risk factors, including cigarette smoke, provide possible confounders; however, an increased risk of periodontitis has been demonstrated in a non-smoking RA group (21). In addition, it appears that periodontitis responds to RA treatment (22). There are also pathological similarities between the two conditions in terms of T cell activation, inflammatory cytokine profile and the resultant bone destruction and deformity.

The association between the two diseases has recently been debated with the relevance of *P. gingivalis* being called into question in a large cohort of pre-RA participants (23). Recently, there have been narrative reviews of the literature surrounding the association between periodontitis and RA (24–26). However, there is no recent systematic review or meta-analysis to re-evaluate this association. Our aim was to analyze the association between these two conditions in light of all recent evidence in the form of a meta-analysis and to include all the most recent published studies investigating the association of RA and periodontal disease worldwide to assess the largest dataset possible for meta-analysis.

MATERIALS AND METHODS

Search Strategy

We searched the published databases: MEDLINE *via* OVID, EMBASE *via* OVID, and PsycINFO *via* OVID. No restrictions were placed on date of publication; however, only English language articles were selected. Articles were searched for using the terms "periodontitis," "periodontitis.mp," or "periodontal disease" and "rheumatoid arthritis.mp" or "inflammatory arthritis." All papers which presented data on the number of people with RA diagnosed with periodontitis or periodontal disease measures/assessments, compared to a comparison group [such as healthy controls or those with osteoarthritis (OA)] were included. Review articles, case reports, animal model studies, and those with unavailable abstracts were excluded from the analysis. The titles and abstracts of each citation were independently reviewed by two

authors (Nicholas Rubek Fuggle and Toby O. Smith) and verified by a third (Nidhi Sofat). Full texts of all potentially eligible papers were independently reviewed by three authors (Nicholas Rubek Fuggle, Toby O. Smith, and Nidhi Sofat) with consensus made through discussion on final study eligibility.

Data Extraction

Data were extracted onto a predefined data extraction table. Data extracted included participant number with RA and a comparator (e.g., non-RA healthy controls or OA), age, gender, ethnic origin, marital status, educational status, smoking history, medical history/status (cardiovascular disease, diabetes obesity, osteoporosis, antibiotic usage), RA diagnosis, years of disease (if RA), measures of disease severity (e.g., rheumatoid factor, CRP, ESR, HLA-DRB1), current therapies (e.g., DMARDs, NSAIDS, biologics), and periodontitis measure. These included percentage with periodontitis, probing depth, plaque index, missing teeth, proportion of sites with plaque, bleeding on probing (BOP), and clinical attachment loss (CAL). All data were extracted by two reviewers independently (Nicholas Rubek Fuggle and Toby O. Smith) with any disagreements addressed through discussion.

Critical Appraisal

All included studies were assessed using the Downs and Black critical appraisal tool for non-randomized controlled trials (27). This is a 27-item critical appraisal tool that assessed study quality (10 items), external validity (3 items), study bias (7 items), confounding and selection bias (6 items), and power of the study (1 item). All papers were independently assessed by one reviewer (Nicholas Rubek Fuggle) and verified by a second reviewer (Toby O. Smith).

Data Analysis

Study heterogeneity was assessed through visual assessment of the data extraction table. As there was homogeneity in participant characteristics, periodontal assessment, and study design, a meta-analysis was undertaken. The primary analysis was to estimate the relative risk (RR) of periodontitis for people with RA over people without RA, or another non-RA condition such as OA. The secondary analyses included estimating the RR or mean difference (MD) for measures of periodontal disease between patients with RA and comparative groups (non-RA or OA). Periodontal disease measures included probing depth, plaque index, missing teeth, proportion of sites with plaque, BOP, and CAL. A fixed-effect Mantel–Haenszel meta-analysis was undertaken when the inconsistency value (I^2) was 50% or less and Chi^2 equated $p \geq 0.10$. A random-effect Mantel–Haenszel meta-analysis was undertaken when I^2 was >50% and Chi^2 equated to $p < 0.10$. All analyses were calculated with 95% confidence intervals and forest-plots were constructed, and performed on RevMan Version 5.3 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014).

RESULTS

Search Results

A summary of the search results is presented in **Figure 1**. As this illustrates, from a total of 1182 citations identified from the search strategy, 67 provisionally met the inclusion criteria. In total, 21 papers met the eligibility criteria and provided data for the meta-analysis.

Characteristics of Included Studies

The characteristics of the participants from the included studies are presented in **Table 1**. Seventeen studies analyzed data on periodontitis in RA compared to non-RA healthy control cohorts (21, 28–43). Four studies analyzed data on periodontitis in RA compared to OA cohorts (10, 44–46).

In the RA compared to non-RA analysis, a total of 153,492 participants were analyzed. This consisted of 14,849 people with RA, compared to 138,643 non-RA control participants. Mean age of the RA cohort was 50.9 (SD: 5.7) years compared to 46.4 years (SD: 8.3). In the RA group, duration of RA disease was stated in eight studies (10, 21, 29, 33, 34, 36, 37, 46), ranging from 3.4 months (37) to 16.3 years (34).

In the RA compared to OA analysis, 1378 participants were analyzed. This consisted of 654 people with RA were compared to 724 individuals with OA. Mean age of the RA cohort was 60.2 (SD: 2.6) years compared to 62.5 years (SD: 8.3).

Critical Appraisal

The results of the critical appraisal assessment are presented in **Table 2**.

The evidence-base presented for moderate evidence for both the analysis of RA versus non-RA cohorts and RA versus OA cohort periodontitis measures. Recurrent strengths to the evidence included presenting clear aims and objectives ($N = 21$; 100%), presenting outcome data ($N = 21$; 100%), and participant characteristics data clearly ($N = 21$; 100%), as well as clearly presenting information on periodontitis assessment performed ($N = 21$; 100%), using valid and reliable measures of assessment in all cases ($N = 21$; 100%). The included studies for both assessments also analyzed their data with the appropriate statistical tests ($N = 21$; 100%), provided actual probability values ($N = 21$; 100%), although only nine studies presented estimates on random variability from their data (10, 29, 30, 33, 40–43, 46).

However, recurrent weaknesses in the evidence included poorly blinding assessors to their pathological or non-pathological group, only masked in four studies (10, 36, 44, 46), recruiting cases and controls at the same point in time in only five studies (10, 32, 36, 41, 44), whereas only six studies adequately adjusted their analyses for important confounders such as smoking and alcohol history and antibiotic usage (10, 21, 30, 35, 39, 44). Only 10 papers recruited cohorts that consisted of 50 participants or more per group (10, 21, 30, 35, 36, 39, 40, 42–44). Finally, no studies adjusted their analysis based on different lengths of follow-up or duration between cases and control.

Meta-Analysis: Risk Ratio of Periodontitis in RA versus Non-RA Participants

The results of the meta-analyses are presented in **Table 3**.

The assessment of episodes of periodontitis was assessed in 14 studies. There was a statistically significantly greater risk of periodontitis for people with RA compared to health comparable

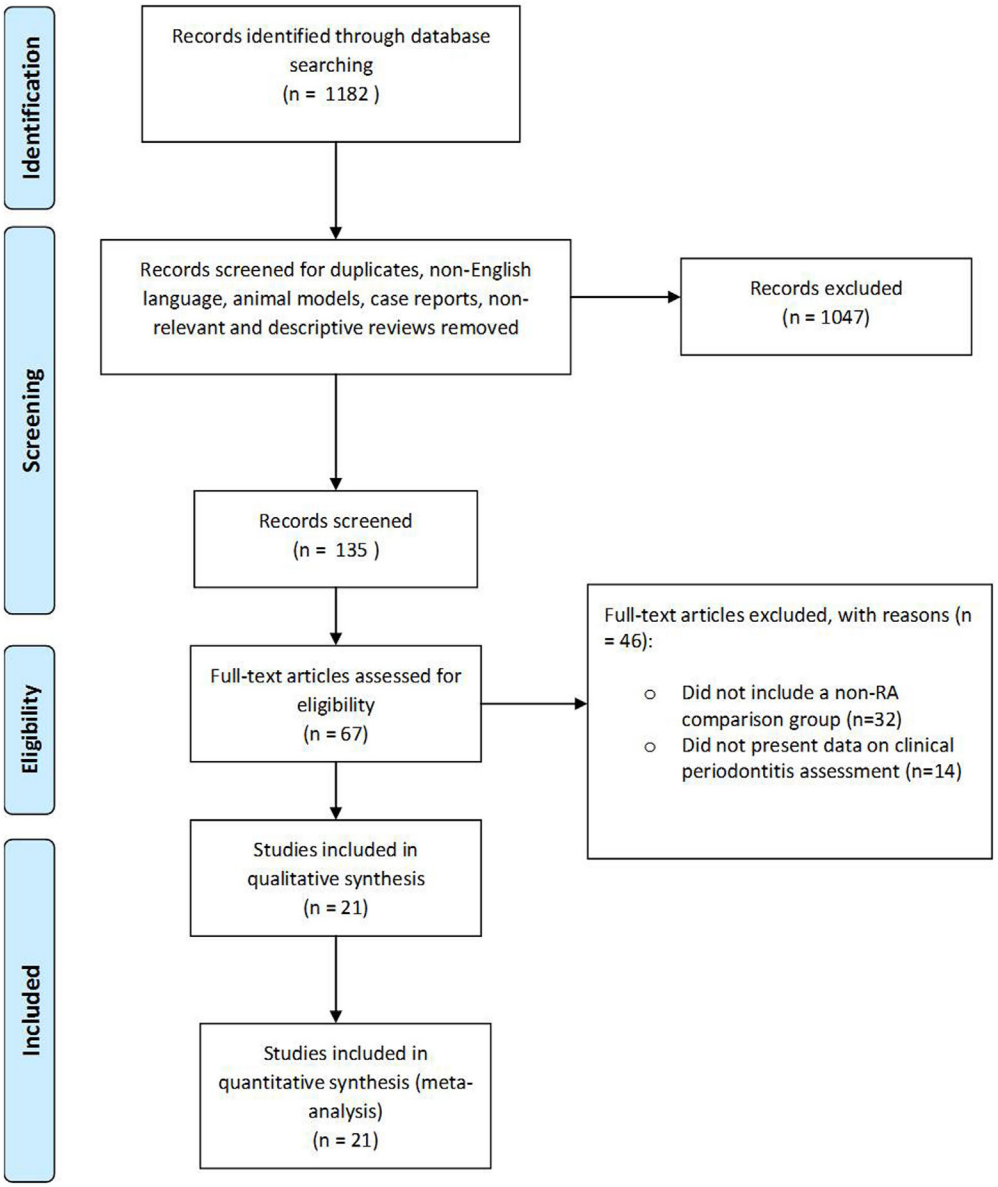

FIGURE 1 | PRISMA flow-chart depicting the results of the search strategy.

cohorts. Those with RA had a 13% greater risk of periodontitis compared to the non-RA cohort, ranging from 4 to 23% (RR: 1.13; 95% CI: 1.04, 1.23; $p = 0.006$; $N = 153{,}277$; **Figure 2**).

The 17 studies were included in the whole RA analysis but not all assessed periodontitis risk (as a whole), with 7 presenting data on specific features of periodontitis, including probing depth, plaque index, BOP, etc., which were reported in **Table 3**. This accounts for the difference in **Figure 2** and the whole dataset that is presented in the characteristics of included studies. On secondary analysis, there was no statistically significant difference in the risk of the probing depth >5 mm (RR: 4.93; 95% CI: 0.84, 28.95) or plaque index as assessed through the frequency of participants >0.6 (RR: 1.23; 95% CI: 0.81, 1.88) or plaque index value (MD: 2.27; 95% CI: −0.16, 4.70) between the RA cohort than non-RA cohort.

There was a significantly greater risk in the RA cohort of the frequency of any BOP in the RA cohort compared to the non-RA cohort (RR: 2.65; 95% CI: 1.00, 7.02; $p = 0.05$), gingivitis index (MD: 0.30; 95% CI: 0.20, 0.41), mean loss of teeth (MD: 2.46; 95% CI: 0.30, 4.63), and periodontal bone loss (RR: 2.05; 95% CI: 1.40, 2.98). There was also a significant difference between the RA and non-RA cohort for probing depth with the RA cohort demonstrating a 0.69-mm greater probing depth than the non-RA cohort (MD: 0.69; 95% CI: 0.26, 1.12).

Although there was no statistically significant difference between the cohorts for the assessment of the frequency of CAL (RR: 3.63;

TABLE 1 | Characteristics of included studies.

Reference	*N* (RA/comparison)	Comparison group	Mean age (RA/comparison)	Gender (% female)	Current smokers (RA/comparison %)	Ex-smokers (RA/comparison %)	Years of RA disease	RA rheumatoid factor + (%)	DAS28
Abou-Raya et al. (36)	50/50	Non-RA control	52.5/52	80/80	N/S	N/S	13.5	86	4.9
Bıyıkoğlu et al. (38)	30/15	Non-RA control	52.7/46.6	N/S	N/S	N/S	N/S	N/S	N/S
Bıyıkoğlu et al. (13)	23/17	Non-RA control	52.7/40.7	78/47	22/35	N/S	16.3	26	N/S
Chen et al. (30)	13,779/137,790	Non-RA control	52.6/52.4	77/77	N/S	N/S	N/S	N/S	N/S
Coburn et al. (44)	287/330	OA	57/58	72/78	15/8	31/23	N/S	N/S	N/S
de Smit et al. (33)	95/44	Non-RA control	56/34	68/57	23/27	40/43	7.4	53	2.4
Dissick et al. (46)	69/35	OA	62/58	17/14	20/17	45/29	14	56	N/S
Esen et al. (41)	20/20	Non-RA control	40.1/44.4	80/95	N/S	10/5	N/S	N/S	3.2
Janssen et al. (31)	86/36	Non-RA control	57/26	56/59	60/42	17/36	N/S	74	2.2
Joseph et al. (39)	100/112	Non-RA control	46.5/45.9	76/86	N/S	N/S	N/S	N/S	N/S
Lee et al. (35)	248/85	Non-RA control	60.1/59.1	88/87	2/5	4/5	N/S	N/S	N/S
Reichert et al. (28)	42/114	Non-RA control	56.1/53.8	22/26	6/3	5/4	N/S	N/S	N/S
Mercado et al. (42)	65/65	Non-RA control	56.4 (total)	75 (total)	7/7	N/S	N/S	N/S	N/S
Mikuls et al. (10)	287/330	OA	59/59	37/40	19/11	43/35	12.6	77	3.2
Mirrielees et al. (32)	35/35	Non-RA control	46.8/43	77/74	11/0	N/S	N/S	N/S	N/S
Pischon et al. (40)	57/52	Non-RA control	52.1/52.1	86/83	21/19	39/21	N/S	N/S	N/S
Potikuri et al. (21)	91/93	Non-RA control	43.9/41.8	84/74	N/S	N/S	2.0	63	7.2
Scher et al. (37)	31/18	Non-RA control	42.2/42.2	68/65	16/6	16/16	3.4 months	92	5.8
Susanto et al. (43)	75/75	Non-RA control	46.5/46.9	80/80	7/7	N/S	N/S	N/S	N/S
Témoin et al. (45)	11/25	OA	62.6/75.0	N/S	N/S	N/S	N/S	N/S	N/S
Wolff et al. (29)	22/22	Non-RA control	51.7/51.9	68/68	3/3	10/10	5.9 months	37	4.6

DAS28, disease activity score 28; N/S, not stated; OA, osteoarthritis; RA, rheumatoid arthritis.

TABLE 2 | Downs and Black critical appraisal results.

Reference	Downs and Black appraisal criteria																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Abou-Raya et al. (36)	✓	✓	✓	✓	X	✓	X	N	N	✓	X	X	X	N	✓	N	X	✓	N	✓	✓	✓	N	N	X	N	✓
Bıyıkoğlu et al. (38)	✓	✓	✓	✓	✓	✓	X	N	N	✓	✓	✓	✓	N	X	N	X	✓	N	✓	X	X	N	N	X	N	X
Bıyıkoğlu et al. (34)	✓	✓	✓	✓	✓	✓	X	N	N	✓	✓	✓	✓	N	X	N	X	✓	N	✓	X	X	N	N	X	N	X
Chen et al. (30)	✓	✓	✓	✓	✓	✓	✓	N	N	✓	✓	✓	✓	N	X	N	X	✓	N	✓	X	X	N	N	✓	N	✓
Coburn et al. (44)	✓	✓	✓	✓	✓	✓	X	N	N	✓	✓	✓	✓	N	✓	N	X	✓	N	✓	✓	✓	N	N	✓	N	✓
de Smit et al. (33)	✓	✓	✓	✓	✓	✓	✓	N	N	✓	✓	✓	✓	N	X	N	X	✓	N	✓	X	X	N	N	X	✓	X
Dissick et al. (46)	✓	✓	✓	✓	✓	✓	✓	N	N	✓	✓	✓	✓	N	✓	N	X	✓	N	✓	✓	X	N	N	X	N	X
Esen et al. (41)	✓	✓	✓	✓	✓	✓	✓	N	N	✓	✓	✓	✓	N	X	N	X	✓	N	✓	✓	✓	N	N	X	N	X
Janssen et al. (31)	✓	✓	✓	✓	✓	✓	X	N	N	✓	✓	X	X	N	X	N	X	✓	N	✓	X	X	N	N	X	N	X
Joseph et al. (39)	✓	✓	✓	✓	✓	✓	X	N	N	✓	✓	X	✓	N	X	N	X	✓	N	✓	X	X	N	N	✓	N	✓
Lee et al. (35)	✓	✓	✓	✓	✓	✓	X	N	N	✓	X	X	X	N	X	N	X	✓	N	✓	X	X	N	N	✓	N	✓
Mercado et al. (42)	✓	✓	✓	✓	✓	✓	✓	N	N	✓	✓	✓	✓	N	X	N	X	✓	N	✓	✓	X	N	N	X	N	X
Mikuls et al. (10)	✓	✓	✓	✓	✓	✓	✓	N	N	✓	✓	✓	✓	N	✓	N	X	✓	N	✓	✓	✓	N	N	✓	N	✓
Mirrielees et al. (32)	✓	✓	✓	✓	✓	✓	X	N	N	✓	✓	✓	✓	N	X	N	X	✓	N	✓	✓	✓	N	N	X	N	X
Reichert et al. (28)	✓	✓	✓	✓	X	✓	X	N	N	✓	X	X	✓	N	X	N	X	✓	N	✓	X	X	N	N	X	N	✓
Pischon et al. (40)	✓	✓	✓	✓	✓	✓	✓	N	N	✓	✓	✓	✓	N	X	N	X	✓	N	✓	X	X	N	N	X	N	✓
Potikuri et al. (21)	✓	✓	✓	✓	✓	✓	X	N	N	✓	X	X	X	N	X	N	X	✓	N	✓	X	X	N	N	✓	N	✓
Scher et al. (37)	✓	✓	✓	✓	X	✓	X	N	N	✓	✓	✓	✓	N	X	N	X	✓	N	✓	X	X	N	N	X	N	X
Susanto et al. (43)	✓	✓	✓	✓	✓	✓	✓	N	N	✓	✓	✓	✓	N	X	N	X	✓	N	✓	✓	X	N	N	X	N	✓
Témoin et al. (45)	✓	✓	✓	✓	X	✓	X	N	N	✓	X	X	X	N	X	N	X	✓	N	✓	X	X	N	N	X	N	X
Wolff et al. (29)	✓	✓	✓	✓	✓	✓	✓	N	N	✓	✓	✓	✓	N	X	N	X	✓	N	✓	X	X	N	N	X	N	X

✓, *satisfied; X, not satisfied; N, not applicable.*

1. Hypotheses/aims/objectives clearly stated.
2. Main outcome measures clearly described.
3. Characteristics of patients/subjects clearly described.
4. Interventions of interest clearly described.
5. Distribution of principal confounders in each group clearly described.
6. Main findings clearly described.
7. Estimates of random variability in the data provided.
8. Important adverse events reported.
9. Characteristics of patients lost to follow-up described.
10. Actual probability values reported.
11. Participants approached representative of entire population.
12. Participants recruited representative of entire population.
13. Staff, places, and facilities representative of majority of population.
14. Blinding of study subjects.
15. Blinding of assessors.
16. Data based on data-dredging clearly stated.
17. Adjustment of different length of follow-up or duration between case and control.
18. Appropriate statistical tests used.
19. Compliance to intervention reliable.
20. Main outcome measure reliable and valid.
21. Intervention groups or case–controls recruited from same population.
22. Intervention groups or case–controls recruited at the same time.
23. Study subjects randomized to the interventions.
24. Was concealed randomization to allocation undertaken.
25. Adequate adjustment made in the analysis of confounders.
26. Patient losses accounted for.
27. Sufficiently powered cohort size.

95% CI: 0.40; 32.88), the RA cohort presented with greater CAL when assessed as an absolute value (MD: 0.99; 95% CI: 0.38, 1.61).

Meta-Analysis: Risk Ratio of Periodontitis in RA versus OA Participants

There was no statistically significant difference in risk of periodontitis between people with RA compared to those with OA (RR: 1.10; 95% CI: 0.81, 1.49; $p = 0.54$; $N = 1344$; **Figure 3**).

On secondary analysis, there was no statistically significant difference in the risk of the probing depth >5 mm in the RA cohort compared to the OA cohort (RR: 1.11; 95% CI: 0.95, 1.31; $p = 0.19$), the frequency of CAL (RR: 1.04; 95% CI: 0.74, 1.48; $p = 0.81$), or in the mean loss of teeth between those with RA compared to OA (MD: 0.10; 95% CI: −0.62, 0.42). There was, however, a significant difference in the risk of BOP, where the OA cohort presented with an 8% greater risk of exhibiting this feature of periodontitis compared to the RA cohort (RR: 0.92; 95% CI: 0.88, 0.97; $p = 0.002$).

DISCUSSION

We performed a meta-analysis to investigate the relationship between periodontitis and RA, finding that there was a

TABLE 3 | Results from the meta-analyses.

Outcome	Relative risk (95% CI)	*p*-Value	*N*	Statistical heterogeneity (I^2; Chi^2)
Ra versus non-RA				
Periodontitis (frequency)	1.13 (1.04, 1.23)	0.006	153,277	95%; <0.001
Probing depth (frequency >5 mm)	4.93 (0.84, 28.95)	0.08	339	85%; 0.001
Probing depth (mm)	0.69 (0.26, 1.12)[a]	0.002	1072	98%; <0.001
Plaque index (frequency >0.6)	1.23 (0.81, 1.88)	0.33	379	65%; 0.04
Plaque index (mean)	2.27 (–0.16, 4.70)[a]	0.07	522	99%; <0.001
Any bleeding on probing (frequency)	2.65 (1.00, 7.02)	0.05	792	74%; <0.001
Clinical attachment loss (frequency)	3.63 (0.40, 32.88)	0.25	170	91%; <0.001
Clinical attachment loss (mm)	0.99 (0.38, 1.61)[a]	0.002	958	97%; <0.001
Gingivitis Index (mean)	0.30 (0.20, 0.41)[a]	<0.001	654	72%; 0.03
Loss teeth (mean)	2.46 (0.30, 4.63)[a]	0.03	495	80%; 0.002
Periodontal bone loss (frequency moderate-to-severe)	2.05 (1.40, 2.98)	<0.001	130	N/E
Ra versus OA				
Periodontitis (frequency)	1.10 (0.81, 1.49)	0.54	1344	70%; 0.02
Probing depth (frequency >5 mm)	1.11 (0.95, 1.31)	0.19	617	N/E
Any bleeding on probing (frequency)	0.92 (0.88, 0.97)	0.002	617	N/E
Clinical attachment loss (frequency)	1.04 (0.74, 1.48)	0.81	617	N/E
Loss teeth (mean)	–0.10 (–0.62, 0.42)[a]	0.71	617	N/E

[a]*Mean difference analysis as opposed to relative risk analysis.*

CI, confidence intervals; I^2, inconsistency value; N, number of participants; N/E, not estimatable; OA, osteoarthritis; RA, rheumatoid arthritis.

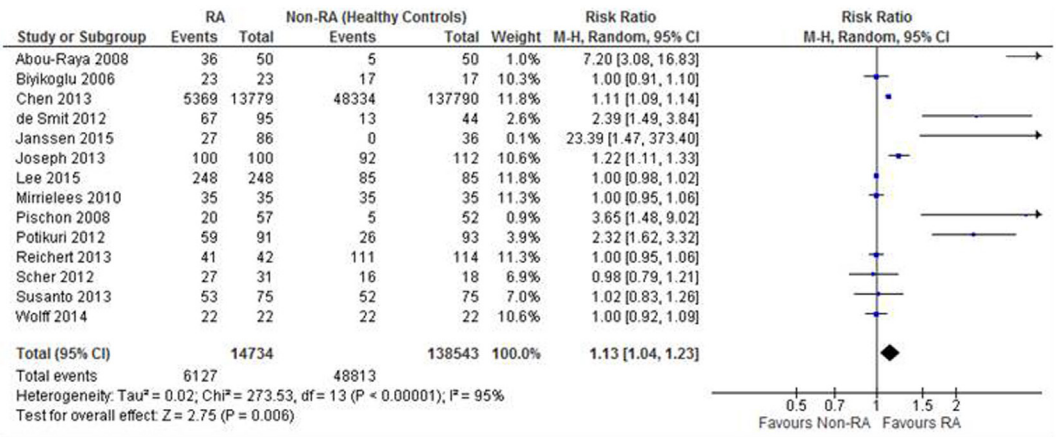

FIGURE 2 | Forest-plot representing risk ratio of periodontitis between patients with RA and healthy control comparisons.

significantly greater risk of periodontitis in people with RA when compared to healthy (non-RA) controls (RR 1.13, 95% CI: 1.04, 1.23; $p = 0.006$). This finding was not demonstrated when comparing the risk of periodontitis in RA and OA controls.

There was no significant difference in probing depths >5 mm in RA compared to healthy controls; however, there was a 0.69-mm greater probing depth in RA. Although the frequency of CAL did not significantly differ between RA and controls; however, the absolute value of CAL demonstrated a MD of 0.99 (95% CI: 0.38, 1.61). There was an increased risk of BOP in RA compared to healthy controls (2.65, 95% CI: 1.00, 7.02; $p = 0.05$) but, interestingly, a reduced risk compared to OA patients (0.92, 95% CI: 0.88, 0.97; $p = 0.002$).

In terms of oral hygiene parameters, gingival index was significantly higher in RA compared to healthy controls; however, this finding was not replicated in plaque index, another parameter of oral hygiene. There was a significantly higher mean loss of teeth in RA compared to controls (MD: 2.46, 95% CI: 0.30, 4.63).

Our primary finding that there is an increased risk of periodontitis in patients with RA compared to healthy controls is in consort with the findings of recent narrative reviews, but our study is the first meta-analysis of its kind interrogating this question. In 2015, Araújo and colleagues published a critical appraisal of studies investigating the relationship between RA and periodontitis (47). Papers published since 2012 were selected including eight epidemiological studies, four periodontal intervention studies,

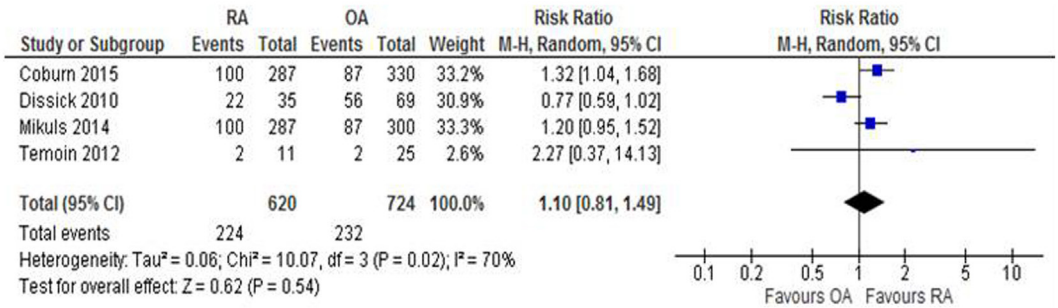

FIGURE 3 | **Forest-plot representing risk ratio of periodontitis between patients with RA and OA participants**.

and five investigating the role of inflammatory mediators in both diseases. They found that 21 studies demonstrated an association though statistical analysis and 3 studies demonstrated an association through descriptive analysis between RA and periodontitis (47). We found an association between RA and periodontitis in our meta-analysis of papers published until October 2015 across an international dataset.

With respect to underlying pathophysiology, CP and RA share many pathological features. These include a number of factors, including oxygen metabolism (41), other shared mechanisms, including active and quiescent inflammatory phases, with the release of several mediators that are common to both conditions, including interleukin 1-beta and prostaglandin E2 (48–50). Similarly, collagenase is a metalloproteinase that specifically degrades collagen. It is not only detected at elevated levels in RA synovial fluid and in the circulation in subjects with RA but also found in gingival crevicular fluid (GCF) and gingival tissue (48). Collagenase activity is higher in GCF from subjects with periodontitis than healthy controls (49). Kobayashi et al. (50) reported that the disease activity of RA correlates with serum levels of IL-6, TNF alpha, and CRP, and the higher cytokine levels may influence BOP depth in RA in patients with moderate to high disease activity. Citrullination of auto-antigens is one of the hallmarks of RA and antibodies to cyclic citrullinated peptides are associated with more aggressive and erosive rheumatoid disease. The main human enzyme causing citrullination is peptidylarginine deiminase (PAD). The only known bacterial enzyme causing citrullination is PPAD, produced by *P. gingivalis*. *P gingivalis* forms part of the red complex of organisms implicated in periodontitis. Several studies worldwide have been conducted to investigate the presence of anti-*P. gingivalis* antibodies. Okada et al. (51) showed significantly higher levels of anti-Pg and anti-CCP antibodies than controls ($p = 0.04$ and $p < 0.0001$). In their study, the investigators also showed a significant association of anti-Pg responses with RA, after adjustment for age, sex, and smoking ($p = 0.005$ and $p = 0.02$), suggesting that serum levels of anti-Pg antibodies are associated with RA and might affect serum levels of RF and periodontal condition in patients with RA.

Study Limitations

The analysis of periodontitis is complicated by differences in the definition of the disease, with some studies defining "mild/moderate/severe" periodontitis (31, 33, 35, 44, 52), others using clinical parameters of periodontitis, including probing depth (10, 29, 38, 40, 53, 54) and CAL (55). In none of the papers, a division of periodontitis into the aggressive and chronic phenotypes of the disease a fact that may be relevant as RA is more classically related to CP was found. Therefore, we recognize that our study has limitations, thus the results should be interpreted with caution.

Heterogeneity of disease is also an issue when considering the RA populations studied with marked variation in the range of disease duration from a mean of 3.4 months (37) to 16.3 years (34). The temporal relationship between RA and periodontitis is yet to be established and one of the difficulties in comparing the outcomes of these two studies would be the markedly different disease populations. The difference in disease activity was most commonly assessed by DAS28 score and varied from a mean score of 2.2 (31) to 7.21 (21), again, representing phenotypically distinct disease that may affect the relationship with periodontal inflammation. However, in terms of meta-analysis, it is useful to have the whole range of the disease spectrum represented.

Treatment regimens were often not stated; however, those that were did vary in terms of the use of DMARDs varying from 0% in the new-onset arthritis patients in Scher et al.'s study (37) and 79% of patients taking at least one DMARD in the study of Janssen and colleagues (31). There is also variation with regard to the type of DMARD used with 74% of participants taking hydroxychloroquine (56), 71% on methotrexate (31) and over half on biological therapy (32). These variations can be accounted for by geographical location and the variation in treatment vogue over the time span of the papers sampled for the meta-analysis. Variation in therapeutics may be relevant as it is possible that RA DMARD therapy could also attenuate periodontitis and thus a seeming reduction in association.

Smoking is a confounder for periodontitis (57) and RA (58) possibly due to the role of cigarette smoke in citrullination and the production of reactive oxygen species. As such it is perhaps limiting, in terms of adjustment for confounding, that seven of the studies selected did not comment on the current smoking status of participants.

The studies included in our analysis were not investigating cause and effect but rather establishing the point prevalence of periodontitis and RA. Further longitudinal trials are required to establish the temporal nature of this association.

Any systematic review investigating this disease association over a long time will be limited by geographical demography, changes in definition of disease, disease severity, and treatment regimens as they are modified over the years, which could lead to complications with the comparison of data from heterogeneous populations. However, within the context of a systematic review, this will simply broaden the relevance of our findings as the association appears to withstand changes in the above variables.

CONCLUSION

We present a systematic review and meta-analysis of the relationship between periodontitis and RA, which demonstrates a significant association between RA and periodontitis. Further studies are required in future to elucidate the mechanism of this association.

AUTHOR CONTRIBUTIONS

NF, TS and NS conceived, analysed and drafted the manuscript. AK conducted literature searches and reviewed the manuscript. All authors approved the manuscript for publication.

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Helicobacter pylori-Mediated Protection from Allergy Is Associated with IL-10-Secreting Peripheral Blood Regulatory T Cells

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Helicobacter pylori infections are usually established in early childhood and continuously stimulate immunity, including T-helper 1 (Th1), Th17, and regulatory T-cell (Treg) responses, throughout life. Although known to be the major cause of peptic ulcer disease and gastric cancer, disease occurs in a minority of those who are infected. Recently, there has been much interest in beneficial effects arising from infection with this pathogen. Published data robustly show that the infection is protective against asthma in mouse models. Epidemiological studies show that *H. pylori* is inversely associated with human allergy and asthma, but there is a paucity of mechanistic data to explain this. Since Th1 and Treg responses are reported to protect against allergic responses, we investigated if there were links between the human systemic Th1 and Treg response to *H. pylori* and allergen-specific IgE levels. The human cytokine and T-cell responses were examined using peripheral blood mononuclear cells (PBMCs) from 49 infected and 58 uninfected adult patients. Concentrations of total and allergen-specific plasma IgE were determined by ELISA and ImmunoCAP assays. These responses were analyzed according to major virulence factor genotypes of the patients' colonizing *H. pylori* strains. An *in vitro* assay was employed, using PBMCs from infected and uninfected donors, to determine the role of Treg cytokines in the suppression of IgE. Significantly higher frequencies of IL-10-secreting $CD4^+CD25^{hi}$ Tregs, but not *H. pylori*-specific Th1 cells, were present in the peripheral blood of infected patients. Total and allergen-specific IgE concentrations were lower when there was a strong Treg response, and blocking IL-10 *in vitro* dramatically restored IgE responses. IgE concentrations were also significantly lower when patients were infected with $CagA^+$ strains or those expressing the more active i1 form of VacA. The systemic $IL\text{-}10^+$ Treg response is therefore likely to play a role in *H. pylori*-mediated protection against allergy in humans.

Keywords: ***Helicobacter pylori*, allergy, regulatory T cells, interleukin-10, IgE**

Abbreviations: BFA, brefeldin A; conA, concanavalin A; CTLA-4, cytotoxic T-lymphocyte antigen 4; ECD, phycoerythrin-Texas Red; GITR, glucocorticoid-induced tumor necrosis factor receptor; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; PC5, phycoerythrin cyanin 5.1; PE, phycoerythrin; PMA, phorbol myristate acetate; TGFβ, transforming growth factor-beta; Treg, regulatory T-cell.

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INTRODUCTION

Helicobacter pylori infection usually becomes established during early childhood (1), when the immune system is developing, and it persists life-long in the absence of effective treatment (2). Peptic ulceration and gastric malignancy may result; however, the infection is asymptomatic in the vast majority of cases. In recent years, there has been considerable interest in the possible beneficial effects of *H. pylori* infection (3–6). Protective associations between the infection and risk of atopy, asthma, and autoimmunity have been reported in epidemiological studies by us and several other groups (7–21). Not all studies have been able to demonstrate such trends, however (22–24). A meta-analysis of 700 cases and 785 controls could not prove a link between *H. pylori* infection and asthma risk (25), and some researchers remain skeptical (26). The most consistently observed protective associations with asthma and atopy, however, are in children (8, 16, 18, 20, 24, 27).

The incidence of atopic disease in developed countries has increased markedly over the past 50 years (28, 29). Although genetic predisposition is very important, genetic changes cannot explain this recent dramatic trend. The worldwide prevalence of *H. pylori* is declining, and fewer children are now infected (6, 30, 31). In developing countries, such as India and Mexico, the infection remains present in over 80% of the population, but in many developed countries, the prevalence of *H. pylori* is now less than 20% and it is expected to decline further (32, 33). A role for *H. pylori* in the "hygiene hypothesis" has been suggested, where childhood exposure to certain infections is needed for development of a healthy immune system (34, 35). Modernization has diminished exposure to many of the immunoregulatory stimuli that humans have co-evolved with, including intestinal parasites, ectoparasites, environmental bacteria, gut commensal organisms, and also *H. pylori* (10, 35–38). It is thought that during the past 60,000 years, human physiology has developed with the continual presence of the bacterium in the stomach (6, 39). There is growing evidence that adverse consequences may arise from a lack of exposure to *H. pylori* (5).

Allergies occur more commonly when certain immunological exposures are absent. Mechanisms include the stimulation of regulatory T cell (Treg) and T-helper 1 (Th1) responses to counterbalance and suppress Th2 activity in atopy (37, 38, 40–42). Although the epidemiological evidence for protective associations of *H. pylori* infection with atopy is compelling, it could be argued that the infection is simply a marker for other exposures with similar risk factors. The first indication of a causal relationship came from finding stronger links between childhood asthma and infection with more pathogenic CagA$^+$ *H. pylori* strains (9). Direct proof of *H. pylori*-mediated protection in humans is still lacking, however. Arnold et al. were the first to demonstrate in a mouse model that *H. pylori* infection is protective against allergic asthma (43). In agreement with the human epidemiological data, these effects were stronger when the mice were infected as neonates. The mechanism was demonstrated to involve dendritic cell-mediated induction of immunosuppressive regulatory T-cells (Tregs), and the *H. pylori* factors important in driving this include vacuolating cytotoxin A (VacA), gamma glutamyl transpeptidase (GGT), and urease (44–47).

There is a wealth of published data demonstrating that *H. pylori* infection induces high-level Treg, Th1, and Th17 responses in both humans and mouse models (48–53). Interferon-gamma (IFNγ)-secreting Th1 cells are associated with increased gastric inflammation and disease, whereas Tregs inhibiting inflammation are associated with reduced incidence of disease and probably contribute to the life-long persistence of *H. pylori* infections (32, 48). They are known to be important for suppressing autoimmunity, allergy, and inflammation (54, 55). There are several different types of Treg cells, commonly characterized as CD4$^+$, FOXP3$^+$, CD127^{low}, and expressing high levels of CD25 (56). Tregs may act *via* a number of mechanisms, including contact-mediated suppression, or secretion of suppressive cytokines, such as interleukin-10 (IL-10), IL-35, or transforming growth factor beta (TGFβ) (57–59). IL-10 is known to be protective against allergy, acting *via* inhibition of antigen presentation, down-regulation of effector T-cell cytokine expression, and inhibition of mast cell degranulation (42, 60–62). TGFβ is also reported to play a role in protection against some animal models of allergy (63, 64). Because of the ontogeny of the immune system, *H. pylori*-infected children tend to have stronger Treg responses than adults (65–67). This aligns with stronger protection in the neonatally infected mouse allergy model (43) and also raises the hypothesis that the elevated frequencies of Tregs present in the circulation of *H. pylori*-infected humans (68) play an important role in protection against allergy.

While a great deal of convincing animal model data exist, it has frequently been difficult to prove protective effects of infections, including parasites, on clinical atopy and asthma (69). For this reason, we decided to look for more subtle effects, studying the IgE responses of non-atopic, non-asthmatic patients with carefully characterized *H. pylori* infection status. As reported previously (68), we found higher frequencies of IL-10-secreting Tregs in the peripheral blood of infected patients. Very low plasma IgE concentrations were present in those with the highest Treg responses, but there were no associations with the Th1 response. This indicated that higher levels of *H. pylori*-induced Tregs are associated with reduced markers of allergy. *In vitro* experiments also confirmed that blocking IL-10 (but not IFNγ or TGFβ) resulted in significantly increased IgE secretion by peripheral blood mononuclear cells (PBMCs) from infected, but not uninfected, donors. These data indicate a role for *H. pylori*-induced IL-10-secreting Tregs in protection against human allergy.

MATERIALS AND METHODS

All reagents were obtained from Sigma-Aldrich Ltd. (Poole, UK) unless otherwise stated.

Ethics Statement

Clinical samples were donated with informed written consent and approval from Nottingham Research Ethics Committee 2 (reference 08/H0408/195).

Volunteers and Clinical Materials

Samples were donated by 107 patients (aged 19–83 years) undergoing a routine upper gastro-intestinal endoscopy at the Queen's Medical Centre, Nottingham, for a variety of indications (most

commonly dyspepsia), but were otherwise healthy. Out of 107 patients, 49 donors were *H. pylori*-positive (14 duodenal ulcer, 9 gastric ulcer, and 26 no ulceration) and 58 were negative (none had peptic ulcers). Male-to-female ratios of these groups were 0.93 and 0.70, respectively; mean ages were 59.7 and 54.6 years, respectively. None of the patients were regularly taking non-steroidal anti-inflammatory drugs or had taken antibiotics or proton pump inhibitor drugs in the preceding 2 weeks. None had clinically diagnosed asthma or allergies.

A series of biopsies were collected from the gastric antrum for determination of *H. pylori* infection status by urease detection, isolation and culture of the organism, and histopathology (48). DNA was purified from *H. pylori* isolates and PCR-genotyping carried out to ascertain *cagA* and *vacA* genotype status (70, 71). The 20-ml peripheral blood samples were collected into EDTA vacutainers. Samples of plasma were separated and stored at −80°C. PBMCs were purified by density gradient centrifugation using Histopaque1077.

Quantification of Treg and Th1-Associated Cytokine Responses of Human PBMCs

Peripheral blood mononuclear cells at 1×10^6/ml in AIM V medium (Invitrogen) were placed 0.2 ml/well into 96-well plates in duplicate. A *H. pylori* lysate antigen prepared from six clinical isolates (48) was added at 25 μg/ml. Negative controls received no antigen, whereas positive controls received 5-μg/ml concanavalin A (conA) before incubation for 48 h at 37°C in 5% CO_2. Commercial ELISA kits (eBioscience, Hatfield, UK) were used to quantify IL-10 and TGFβ1 in culture supernatants, as per the manufacturer's instructions.

Quantification of Treg and Th1 cells in Human PBMCs

As described previously (48, 68, 72), 1×10^6 PBMCs were placed into culture tubes in RPMI1640 with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. *H. pylori* lysate antigen was added at 25 μg/ml, negative controls received no antigen, and positive controls received 20 ng/ml phorbol myristate acetate (PMA) and 1 μmol/l ionomycin. Brefeldin A (BFA) was added to all tubes at 10 μg/ml before 16 h incubation at 37°C in 5% CO_2.

Cells were stained with anti-CD4-phycoerythrin-Texas Red (ECD; Beckman Coulter UK Ltd., High Wycombe, UK) and anti-CD25-PE-cyanin 5 (PC5; Biolegend, Cambridge BioScience Ltd., Cambridge, UK), with either anti-CD127-phycoerythrin (PE; eBioscience, San Diego, CA, USA), anti-CTLA-4-PE (Beckman Coulter), or anti-GITR-PE (Biolegend) antibodies in individual tubes. Fixed and permeabilized cells were stained with anti-IL-10-PE (AbD Serotec, Oxford, UK) and anti-FOXP3-Alexa Fluor®488 antibody conjugate (Biolegend). The gating of $CD25^{hi}$ was set based on the positions of the $FOXP3^+$ and $CD127^{lo}$ populations in $CD4^+$ events. Alternatively, cells were stained with anti-CD4-ECD and fixed in 0.5% formaldehyde before permeabilization and staining with anti-CD69-PC5 and anti-IFNγ-FITC (Beckman Coulter).

Data on 200,000 events were acquired using a Beckman Coulter EPICS Altra flow cytometer and analyzed using Weasel version 3 (73), with reference to appropriate isotype controls.

FOXP3 and *IL10* RT-qPCR

The 5×10^6 PBMCs were purified from fresh blood samples using Histopaque 1077, and total RNA was extracted using an RNeasy minikit (QIAGEN, Crawley, UK), as per the manufacturer's instructions. cDNA was synthesized using SuperScript II reverse transcriptase with an oligo(dT) primer (Invitrogen). Real-time PCR was performed on a Rotor-Gene 3000 (Corbett Research, Cambridge, UK) using primers described in **Table 1**, with a DyNAmo HS SYBR green qPCR kit (New England Biolabs, Ipswich, UK) as previously described (48). Amplification was over 45 cycles of 15 s at 95°C, 30 s at 62°C, and 30 s at 72°C. No template controls were included in each run, and a melting curve analysis was performed. First-stage RT-qPCR samples, produced without reverse transcriptase, were assayed in parallel. Results were analyzed using the Pfaffl method (74). *FOXP3* and *IL10* expression levels were normalized against *GAPDH*, comparing to a pooled reference sample from five *Hp*-negative donors to obtain a fold difference. A commercial pooled human cDNA standard (BD Biosciences) was included as a positive control in all assays.

Human IgE Assays

Plasma IgE concentrations were determined using a Human IgE ELISA Quantification Kit (Bethyl, USA) as per the manufacturer's instructions. Optical densities were measured at 450 nm with a reference wavelength of 595 nm. Concentrations of IgE were determined from a standard curve on each plate. Allergen-specific IgE assays [timothy grass pollen, birch tree pollen, and *Dermatophagoides pteronyssinus* (house dust mite)] were carried out by the Department of Immunology, Queen's Medical Centre, Nottingham, using the ImmunoCAP system (Phadia AB, Uppsala, Sweden).

TABLE 1 | Primer sequences for real-time PCR.

	Primer	
Gene	**Forward**	**Reverse**
GAPDH	CCACATCGCTCAGACACCAT	GGCAACAATATCCACTTTACCAGAGT
FOXP3	CAGCACATTCCCAGAGTTCCT	GCGTGTGAACCAGTGGTAGAT
IL10	GCTGGAGGACTTTAAGGGTTACCT	CTTGATGTCTGGGTCTTGGCT

Sequences are shown in the 5′–3′ orientation.

In Vitro PBMC Culture and Manipulation of the Cytokine Response

A method to induce IgE synthesis in PBMC cultures was adapted from previously published studies (75–77). PBMCs from 5 *H. pylori*-positive and 10 *H. pylori*-negative healthy non-allergic donors were resuspended in RPMI 1640 medium (supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml transferrin, and 4 µg/ml insulin). The 200-µl aliquots containing 3×10^5 cells were added to the wells of a sterile 96-well polystyrene round-bottomed plate (Thermo Scientific). In order to induce Th2-skewed conditions, rIL-4 and sCD40L were added to the cells at final concentrations of 50 µg/ml and 100 ng/ml, respectively (Peprotech). The PBMCs were cultured for 12 days at 37°C and 5% CO_2. Supernatants were then removed and stored at −80°C.

In order to observe the effects of IL-10 and TGFβ on IgE synthesis, on day 0 of the culture, 20-µl recombinant human IL-10 (rIL-10) (AbD Serotec) or rTGFβ (AbD Serotec) were added at various concentrations to appropriate wells in quadruplicate (76). The equivalent volume of medium was added to control wells. Cytokines were blocked by adding anti-IL-10 (rat IgG1 clone JES3-9D7, eBioscience) and anti-TGFβ (mouse IgG1 clone 1D11, Abcam) mAbs at varying concentrations. The respective isotype control antibodies were added at the equivalent concentrations. The 20-µg/ml JES3-9D7 has previously been shown to efficiently neutralize endogenously produced IL-10 in a similar culture system (77). The neutralization dose (ND50) of 1D11 antibody is typically 0.25–1.25 µg/ml in the presence of 1 ng/ml recombinant human TGFβ1 (78). The PBMCs were then cultured for 12 days at 37°C with 5% CO_2 before collection and assay of the supernatants for IgE.

Statistical Analysis

Statistical analyses were carried out using Prism 6.00 (GraphPad Software, CA, USA). Since the data were not normally distributed, non-parametric analysis methods were used. Statistical tests of paired data were carried out using the Wilcoxon signed rank test. For unpaired data, the Mann–Whitney *U*-test was used. A significant difference was taken at $p \leq 0.05$.

Box and whisker plots depict the medians and interquartile ranges in the boxes, with whiskers extending to the maximum and minimum points in the data.

RESULTS

Peripheral Blood Th1 and Treg Responses in Infected and Uninfected Patients

Previous work showed a high-level Treg and Th1 response in the *H. pylori*-infected human gastric mucosa and significantly increased numbers of $CD4^+CD25^{hi}$ Tregs in peripheral blood (48, 68). We further investigated the circulating Treg population and determined if higher numbers of Th1 cells were also present. We began by quantifying the cytokines IFNγ, IL-10, and TGFβ1 in PBMC culture supernatants from infected and uninfected patients, and comparing the frequencies of Treg and Th1 cells by flow cytometry. PBMCs from all patients secreted IFNγ when stimulated with *H. pylori* lysate antigen (*Hp*) (**Figure 1A**) or the positive control mitogen conA; however, the concentrations were higher (median difference: 3.6-fold) among the *Hp*-stimulated supernatants from infected compared to uninfected individuals ($p = 0.05$). This is indicative of a systemic Th1 response to *Hp* infection. Similarly, there were slightly higher concentrations of IL-10 (median: 1.3-fold, $p = 0.02$), but there were no differences in TGFβ1 levels.

Using flow cytometry to quantify *Hp*-specific Th1 cells, we immunostained for the early activation marker CD69 (72) and analyzed cells that were activated by *Hp* lysate antigen (**Figure 1B**). Higher frequencies of $CD69^{hi}CD4^+$ events were obtained from the *Hp*-stimulated PBMCs of infected compared to uninfected donors (median: Hp^+ 10.1%, Hp^- 4.9%; $p = 0.001$), demonstrating a higher frequency of *Hp*-specific systemic T-cells during infection (data not shown). When cells from both infected and uninfected donors were stimulated with *Hp*, there was a significantly increased frequency of $CD69^{hi}CD4^+IFN\gamma^+$ events compared to unstimulated PBMCs (paired analyses: infected $p = 0.005$; uninfected $p = 0.001$; **Figure 1C**), and similar statistically significant trends were found following PMA/ionomycin stimulation. No significant differences in the responses of cells from infected and uninfected donors were found; therefore, we could not confirm an increased frequency of Th1 cells in the peripheral blood of the infected patients. There were also no differences according to the *cagA* virulence genotype of the colonizing bacterial strains (not shown).

To quantify the Treg response, $CD25^{hi}$ staining was taken as a higher intensity of fluorescence compared to that observed for CD4 negative cells (**Figure 2A**), included the $FOXP3^+$ and $IL\text{-}10^+$ populations (**Figures 2B,C**), and corresponded with the CD25 staining intensity in the $CD127^{lo}$ population (**Figure 2D**). As expected, not all $CD4^+CD25^{hi}$ events expressed FOXP3. 48–81% of gated $CD4^+CD25^{hi}IL\text{-}10^+$ events were $FOXP3^+$ (**Figure 2E**). A 2.5-fold higher median frequency of $CD4^+CD25^{hi}$ cells ($p = 0.002$) was present in PBMCs from infected patients (**Figure 3A**). There was also a 2.5-fold increased level of $IL\text{-}10^+$ $CD4^+CD25^{hi}$cells ($p = 0.007$; **Figure 3B**). There was a modest 1.7-fold increase in the frequencies of $CD4^+CD25^{hi}CTLA\text{-}4^+$ cells ($p = 0.007$, data not shown), but there was no difference in the frequencies of $CD4^+CD25^{hi}GITR^+$ cells (not shown). The proportion of $FOXP3^+$ events among $CD4^+CD25^{hi}$ cells was not different between infected and uninfected donors, or with/without antigen stimulation (**Figure 3C**), and *FOXP3* mRNA levels were no different when assaying PBMCs by RT-qPCR (**Figure 3D**). The variation in $FOXP3^+$ cell frequencies was extremely wide within both the infected and the uninfected groups, which hampered our ability to detect statistically significant differences. The frequencies of $IL\text{-}10^+$ and $FOXP3^+$ $CD4^+CD25^{hi}$ cells and the corresponding *IL10* and *FOXP3* transcript levels did not vary significantly according to the age or gender of the patient groups; however, those with gastric or duodenal ulcers had significantly lower frequencies of $CD4^+CD25^{hi}$ cells than who only had gastritis (medians: 1.80 and 3.89%, respectively; $p = 0.047$). Similar findings were obtained when analyzing $CD4^+CD25^{hi}IL\text{-}10^+$ data (medians: 0.10 and 0.17%, respectively; $p = 0.041$). In contrast, the proportion of $FOXP3^+$ events among $CD4^+CD25^{hi}$ cells was not significantly different with respect to peptic ulcer disease status among the infected group. Stimulating PBMCs from infected patients with

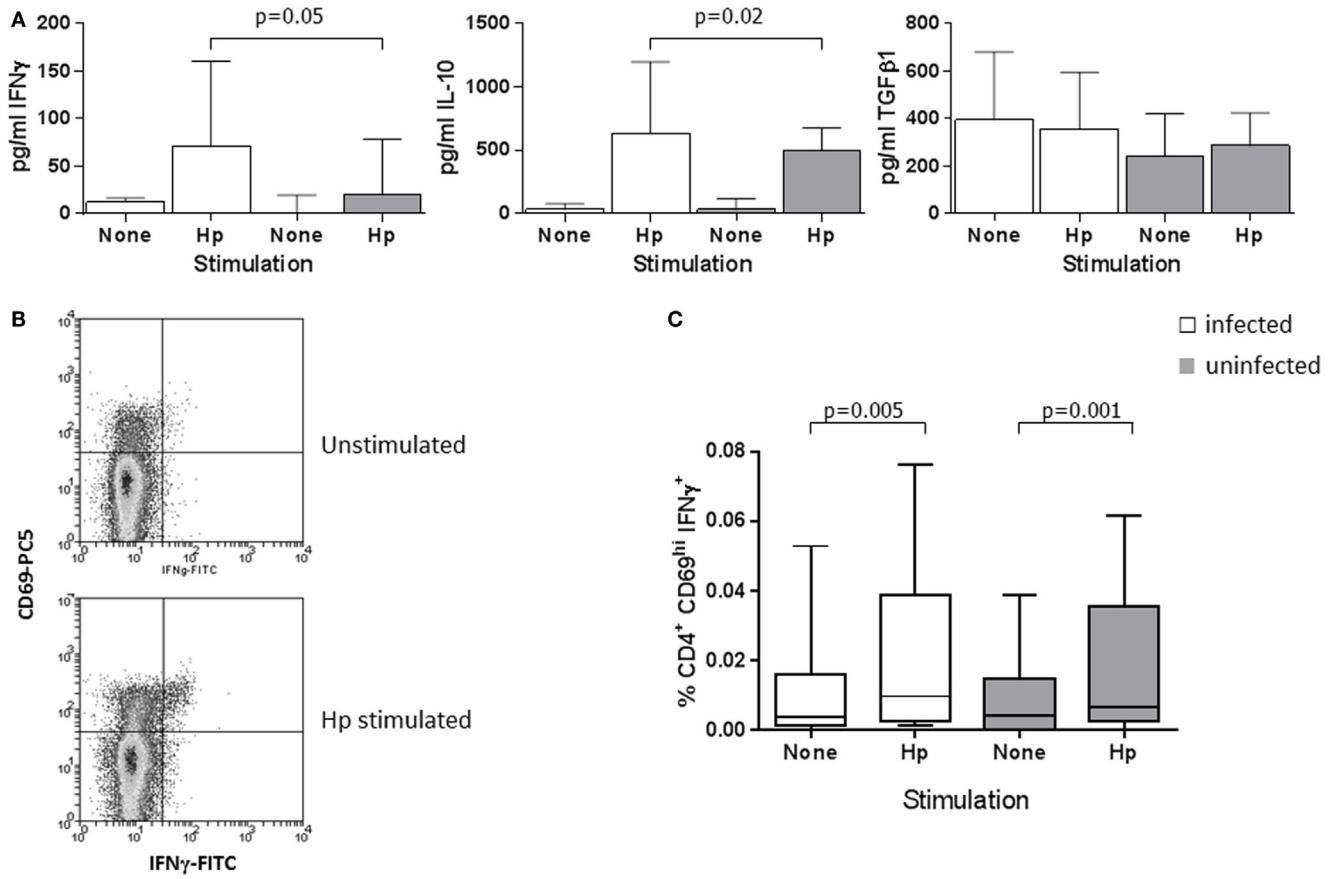

FIGURE 1 | **Peripheral blood Th1 and Treg cytokine responses in *H. pylori*-infected and uninfected patients**. **(A)** PBMCs from 13 infected patients (white bars) and 11 uninfected patients (gray bars) were cultured for 48 h in the presence of 25 μg/ml *H. pylori (Hp)* lysate, with medium alone as a negative control or with 20 μg/ml conA as a positive control stimulus. IFNγ, IL-10, and TGFβ1 concentrations were determined using commercial ELISA kits. Bars depict the median and error bars show the 95% confidence interval. Following conA stimulation, median concentrations from the PBMCs of infected and uninfected patients were, respectively, 1885 and 2035 pg/ml IFNγ, 375 and 393 pg/ml IL-10, and 352 and 306 pg/ml TGFβ1. Th1 cells were quantified in PBMCs from 34 infected and 24 uninfected patients using flow cytometry. **(B)** Example flow cytometry dot plots of gated $CD4^+$ cells from an infected donor, stained for CD69 and IFNγ after culture of PBMCs in medium alone, or with *Hp* lysate antigen. Quadrant indicates the position of $CD69^{hi}$ $IFN\gamma^+$ events, with the lower left quadrant containing both CD69-negative and -positive events. **(C)** The percentage of $CD4^+CD69^{hi}$ $IFN\gamma^+$ events among PBMC lymphocytes is shown following culture in medium alone (unstimulated) or with *Hp* lysate antigen. When stimulated with PMA and ionomycin as a positive control stimulus, the median percentages of $CD4^+CD69^{hi}IFN\gamma^+$ events in PBMCs from infected and uninfected donors were 1.13 and 1.17%, respectively. The percentages of $CD4^+CD69^{hi}IFN\gamma^+$ events were not significantly different between *Hp*-stimulated samples from infected and uninfected donors.

Hp antigen resulted in a significant increase in the proportion of Tregs secreting IL-10 ($p = 0.01$), but there was no significant effect on cells from uninfected patients (**Figure 3E**). This indicates that *H. pylori* infection induces a specific IL-10-secreting peripheral blood Treg response. *IL10* mRNA levels in freshly isolated PBMCs from infected patients were 3.8-fold higher than the uninfected group ($p = 0.001$; **Figure 3F**), and there was a 4.8-fold higher mRNA level in the samples from infected patients without peptic ulcer disease compared to those who had ulcers ($p = 0.017$). These data confirm the presence of increased numbers of $IL\text{-}10^+$ cells in the circulation of *H. pylori*-infected patients, particularly when peptic ulceration is absent. Since there was an elevated systemic IL-10-secreting Treg response in infected patients, we hypothesized that as in the mouse, this could be an important mechanism behind the protective associations with allergy.

Association between the Human Regulatory T-Cell and IgE Responses

Total and allergen-specific IgE concentrations in the plasma of 49 infected and 46 uninfected patients were not significantly different (**Table 2**); however, among the infected group, there were differences in total IgE according to the virulence genotype of the colonizing *H. pylori* strains. This was important to evaluate, since presence of a strain expressing CagA has previously been reported to be associated with greater protection against asthma (9). VacA is known to be protective against allergy in mice (45). Since $cagA^+$ strains usually also express the most active i1 form of *vacA* (71), we investigated how IgE levels related to these virulence factors. Apart from in five cases, the 29 $cagA^+$ strains were also of the more active *vacA* i1 type. All but 5 of the 20 less pathogenic $cagA^-$ strains were typed as having the

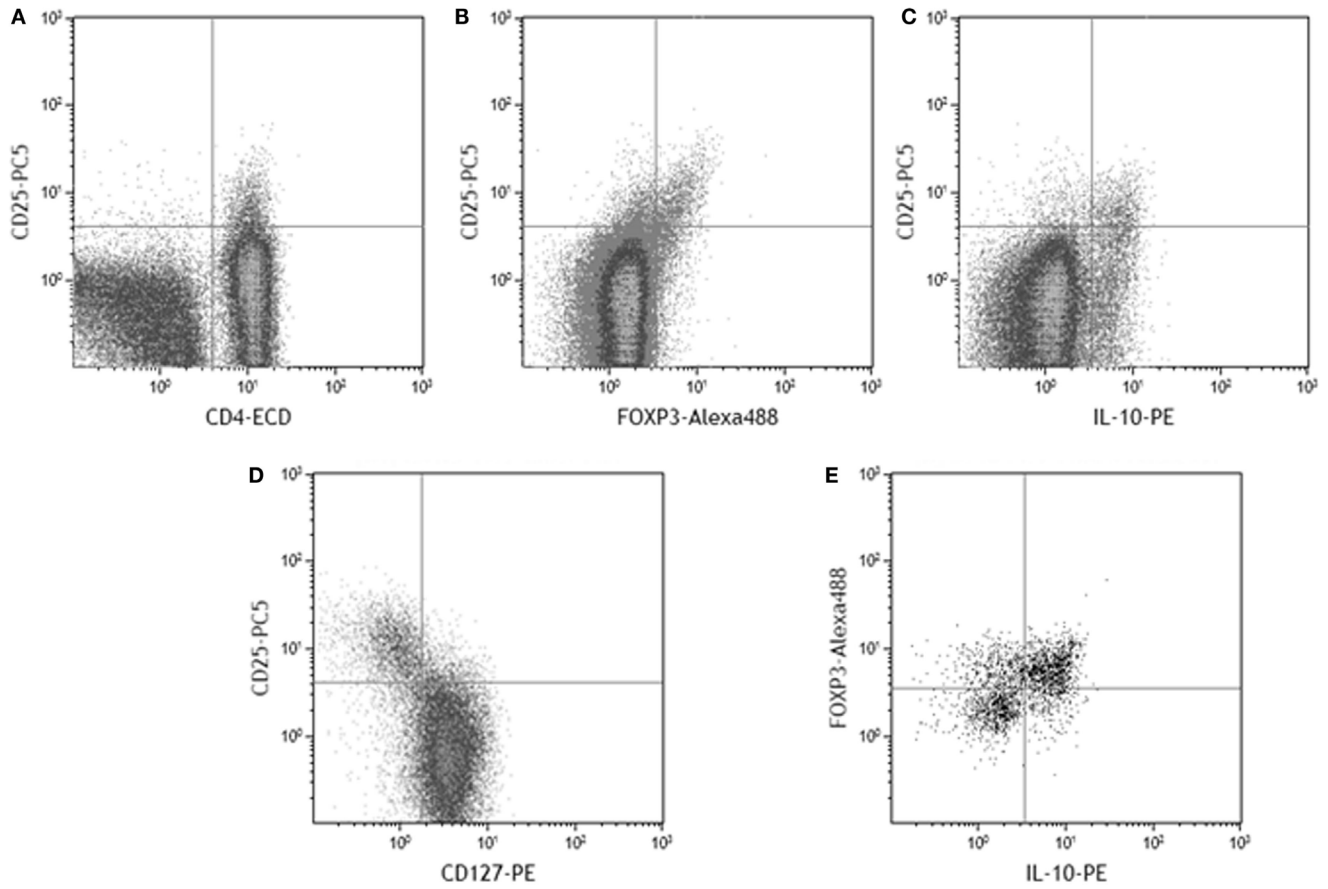

FIGURE 2 | Example flow cytometry dot plots to display the gating and designation of CD25^{hi} cells among PBMC lymphocytes. CD25^{hi} events were gated on the basis that expression is of higher intensity in CD4^{+} events **(A)**. Among gated CD4^{+} events, the CD25^{hi} population contains FOXP3^{+} events **(B)**, IL-10^{+} events **(C)**, and corresponds to the CD127^{lo} population **(D)**. Among gated CD4^{+}CD25^{hi} events, the majority of IL-10^{+} events were also FOXP3^{+} **(E)**.

less active i2 form of *vacA*. We were therefore unable to pick apart the roles of these factors individually. The patients infected with the more virulent *cagA*$^{+}$ or *vacA* i1 strains had threefold lower concentrations of IgE compared to those with *cagA*$^{-}$ or *vacA* i2 strains (**Figure 4**; $p = 0.005$ and $p = 0.029$, respectively). Surprisingly, however, there were no significant differences in Treg frequencies or IL-10 transcripts between those with *cagA*$^{+}$ versus *cagA*$^{-}$ strains, and the same result was found for *vacA* genotypes (data not shown).

When comparing the magnitude of the Treg response with IgE levels, we observed some interesting trends. Patients with the highest frequencies of CD4^{+}CD25^{hi} PBMCs had the lowest total IgE concentrations. The effect was stronger among the samples from *H. pylori*-positive patients (**Figures 5A,C**), where having a high-level Treg response was more common. The data were stratified according to whether or not CD4^{+}CD25^{hi} cell frequencies were above or below 10%, which equated to the mean + 1 SD of all samples tested and was well above the normal range for frequencies of these cells in peripheral blood as previously reported in other studies (79, 80). Among the samples from infected patients with high CD4^{+}CD25^{hi} cell frequencies, there was a fivefold lower median IgE concentration, compared to those with frequencies below 10% ($p = 0.001$) (**Figure 5C**). Among the samples from infected patients with high IL-10^{+} CD4^{+}CD25^{hi} cell frequencies (>0.4%, mean + 1 SD), similarly there was a fivefold lower median IgE concentration, compared to those with low IL-10^{+} Treg frequencies ($p = 0.01$) (**Figure 5B,D**). There was no significant difference in the total plasma IgE concentrations of uninfected patients with high and low Treg frequencies (**Figures 5C,D**).

A significantly reduced grass ($p = 0.004$) and birch ($p = 0.002$) pollen-specific IgE concentration was present among infected patients with high frequencies of peripheral blood Treg cells (**Table 3**), but there were no significant differences among the uninfected group, or with regard to house dust mite-specific IgE. Similar trends were observed when comparing IgE concentrations with frequencies of IL-10^{+}CD4^{+}CD25^{hi} PBMCs (data not shown). There were no significant trends when PBMC Th1 responses were compared with IgE levels (data not shown). Together, these data indicate that *H. pylori* infection is associated with unusually high circulating Treg levels, which could perhaps directly or indirectly suppress IgE production and influence the development of allergy.

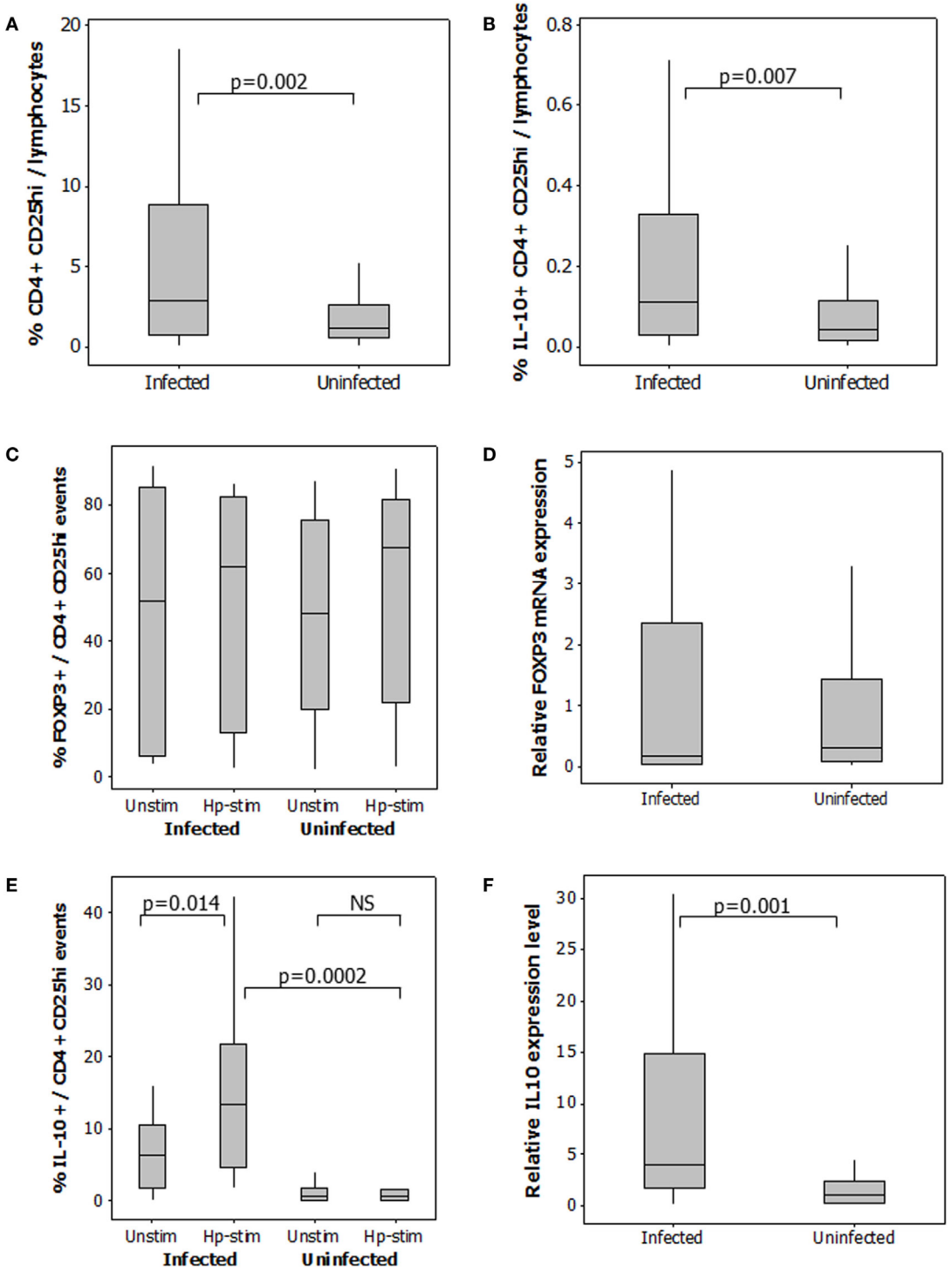

FIGURE 3 | Regulatory T-cells in the peripheral blood of 49 *H. pylori*-infected and 58 uninfected patients. The percentage of $CD4^+CD25hi$ events among gated lymphocytes **(A)**. For determination of IL-$10^+CD4^+CD25^{hi}$ Tregs **(B)**, PBMCs were cultured in the presence and absence of *Hp* lysate antigen. Median frequencies of IL-$10^+CD4^+CD25^{hi}$ events among unstimulated cells were 0.04% (uninfected donors) and 0.04% (infected donors). The proportion of $CD4^+CD25^{hi}$ cells expressing FOXP3 **(C)** and IL-10 **(E)** was assessed in PBMCs cultured with and without *Hp* lysate antigen (Unstim and Hp-stim, respectively). RT-PCR was performed on freshly isolated PBMCs from 48 infected and 26 uninfected patients to quantify *FOXP3* **(D)** and *IL10* **(F)** mRNA expression levels. Data were normalized relative to *GAPDH* expression. NS = no significant difference.

TABLE 2 | Total and allergen-specific IgE levels in the plasma of 49 infected and 46 uninfected patients.

IgE measurement	Median concentration and interquartile range	
	***H. pylori*-positive**	***H. pylori*-negative**
Total (ng/ml)	58.7 (22.1–140.1)	31.4 (9.61–133.3)
House dust specific (kUA/l)	0.05 (0.03–0.13)	0.04 (0.02–0.21)
Grass pollen specific (kUA/l)	0.00 (0.00–0.03)	0.00 (0.00–0.03)
Birch pollen specific (kUA/l)	0.01 (0.00–0.02)	0.00 (0.00–0.01)

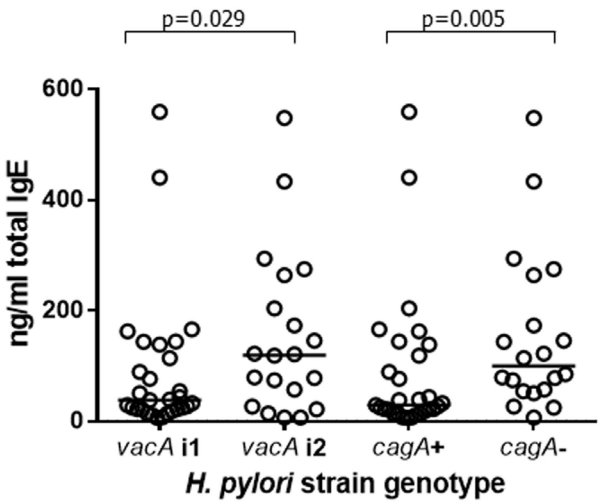

FIGURE 4 | Plasma IgE concentrations from 49 patients infected with *H. pylori* strains of differing virulence genotypes. Of the 29 *cagA*$^+$ strains, 24 were of the i1 *vacA* genotype and 5 were *vacA* i2. Of the 20 *cagA*$^-$ strains, 15 were *vacA* i2 and 5 were of the *vacA* i1 genotype. Points represent the results from individuals and horizontal lines depict the median.

Mechanistic Experiments to Investigate the Role of Th1 and Treg Cytokines in the Suppression of IgE

To test the previously observed associations between *H. pylori* infection, serum IgE levels and abundance of peripheral blood Treg cells, an *in vitro* culture system was employed. PBMCs from 5 infected and 10 uninfected healthy donors (all without ulcers) were cultured under Th2-skewing conditions [with IL-4 and rCD40L (75)] for 12 days. Total IgE, IL-10, and TGFβ1 concentrations in the culture supernatants were then measured by ELISA. These culture conditions were used to investigate the hypothesis that PBMCs from *H. pylori*-positive donors would be inherently less able to respond to the Th2 stimulation compared to those from *H. pylori*-negative donors, possibly due to IL-10 or TGFβ inhibition of IgE production *in vitro.* The culture system provided an opportunity to investigate the consequence of adding or selectively blocking IL-10 and TGFβ. From the *in vivo* data, we hypothesized that blocking IL-10 would increase IgE production more markedly when the PBMCs were from *H. pylori*-positive donors. As anticipated, IgE concentrations were lower for the infected donors compared to those who were not infected (medians 25.8 and 34.2 pg/ml, respectively; $p = 0.013$; **Figure 6A**). The concentrations of IL-10 were fivefold higher in the supernatants of PBMCs from the infected donors ($p = 0.055$; **Figure 6B**), but there were no differences in the concentrations of TGFβ1 (**Figure 6C**).

The cultures were also performed in the presence or absence of varying concentrations of recombinant IL-10, TGFβ1, or antibodies known to block the function of these cytokines. A range of concentrations was used so that we could investigate if there was a dose–response effect. Comparing each treatment to the non-treated control for each donor, the percentage decrease in total IgE resulting from the addition of recombinant cytokines was calculated. Adding the recombinant cytokines, at a range of concentrations reported previously to potently interfere with human T-helper cell activity (76, 81), caused a dose-dependent reduction in IgE secretion by PBMCs from both *H. pylori*-positive and -negative donors (**Figures 7A,B**). rIL-10 appeared to have a more potent effect than rTGFβ. Adding as little as 10 ng/ml rIL-10 resulted in a median 31% reduction in IgE concentration in the cultures compared to controls without rIL-10 ($p = 0.020$). Adding 20 ng/ml rIL-10 resulted in a median 52% reduction in IgE ($p = 0.001$). In contrast, a significantly reduced IgE concentration could only be achieved using the highest concentration of 100 ng/ml rTGFβ (5% reduction compared to no rTGFβ controls, $p = 0.041$). No differences in IgE response could be found between the PBMCs from *H. pylori*-positive and -negative donors in these assays however.

Adding IL-10 mAb, at a range of concentrations reported previously to block cytokine activity (77, 78), resulted in increased IgE production by PBMCs from both groups (*H. pylori*-positive and -negative donors), compared to cultures with the equivalent concentration of isotype control antibodies (**Figure 7C**). Adding anti-TGFβ had very little effect (**Figure 7D**). The difference in the size of the effect from IL-10 blockade between *H. pylori*-positive and -negative donors was statistically significant (20 μg/ml: 24%, $p = 0.02$; 40 μg/ml: 53%, $p = 0.006$). Therefore, we had confirmation that blocking IL-10, which was present at higher concentrations in the isotype control cultures of PBMCs from *H. pylori*-positive donors (**Figure 6B**), had a pronounced suppressive influence on IgE production *in vitro*.

DISCUSSION

This study aimed to further investigate the inverse association between *H. pylori* infection and allergy in humans, and fill an important gap in current literature by exploring the potential mechanisms behind this. We were able to demonstrate a link between IL-10-secreting peripheral blood Treg cell numbers and the concentration of IgE in the plasma of infected patients, and we also showed that IL-10 produced by leukocytes from those with *H. pylori* played an important role in controlling IgE production *in vitro*.

Our previous work found an increased Treg and Th1 response in the *H. pylori*-infected human gastric mucosa (48) and higher frequencies of Tregs in the peripheral blood of infected patients (68). These cell types have previously been implicated in the suppression of Th2 responses and allergy. We hypothesized that increased levels of Treg cells and/or Th1 cells in the circulation

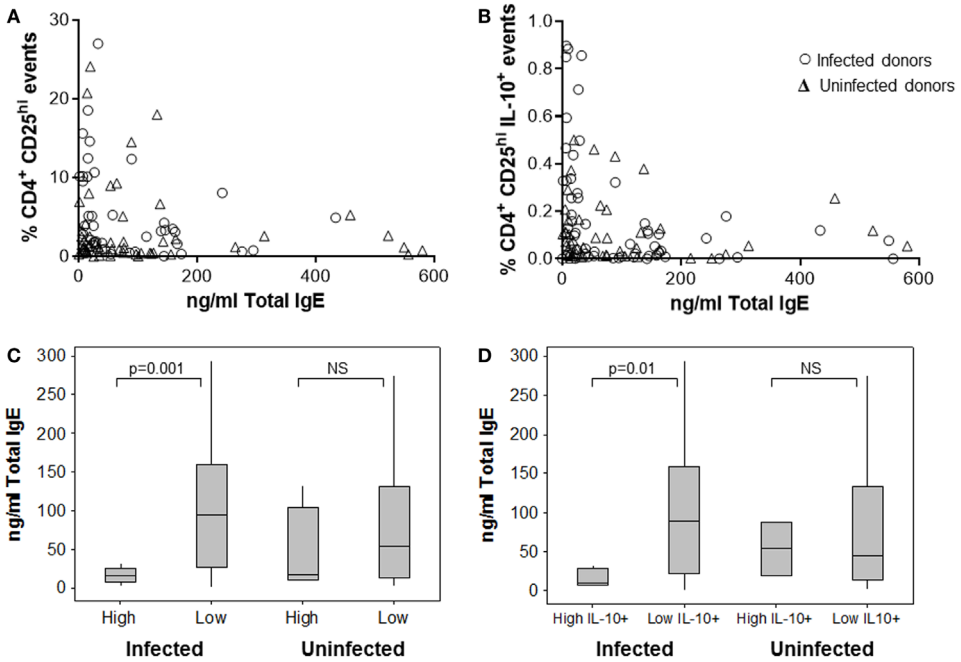

FIGURE 5 | Association between human peripheral blood regulatory T-cells and the IgE response. The frequency of $CD4^+CD25^{hi}$ **(A)** and IL-10^+ $CD4^+CD25^{hi}$ Tregs **(B)** in PBMCs from 49 infected and 46 uninfected donors was assessed by flow cytometry after 15 h culture with *Hp* lysate antigen. This was compared to the total plasma IgE concentration for each patient. The data were divided according to whether there were high (>10%) or low frequencies of $CD4^+CD25^{hi}$ lymphocytes **(C)** and high (>0.4%) or low frequencies of IL-$10^+CD4^+CD25^{hi}$ events **(D)** to further examine associations with total IgE concentrations. NS = no significant difference.

TABLE 3 | Allergen-specific IgE levels in the plasma of 49 infected and 46 uninfected patients with high and lower peripheral blood $CD4^+CD25^{hi}$ cell frequencies.

	Median concentration (kUA/l) and interquartile range		
	House dust	**Grass pollen**	**Birch pollen**
***H. pylori* infected**	NS	$p = 0.004$	$p = 0.002$
High Treg frequencies	0.06 (0.03–0.14)	0.00 (0.00–0.00)	0.00 (0.00–0.00)
Lower Treg frequencies	0.05 (0.04–0.13)	0.02 (0.00–0.18)	0.01 (0.00–0.02)
Uninfected	NS	NS	NS
High Treg frequencies	0.03 (0.03–5.74)	0.00 (0.00–0.04)	0.00 (0.00–0.02)
Lower Treg frequencies	0.04 (0.02–0.09)	0.00 (0.00–0.03)	0.00 (0.00–0.02)

Allergen-specific IgE data were stratified according to whether the patient had a high Treg response (>10% $CD4^+CD25^{hi}$ PBMCs) or if the frequency was below this level. A Mann–Whitney U-test analysis was performed to examine differences in the IgE responses of these groups. NS = no significant difference.

influence immune responses at extra-gastric sites in the body and may play a role in preventing an elevated IgE response. Initially, we compared the IFNγ, IL-10, and TGFβ1 responses of PBMCs to stimulation with *Hp* lysate antigen. Enhanced concentrations of IFNγ [as shown previously (82)] and IL-10 were found in the PBMC supernatants from infected patients, indicating that peripheral blood Th1 and Treg responses were increased. Cells from both *H. pylori*-positive and -negative patients secreted IFNγ and IL-10 in response to *Hp* lysate antigens, indicating that the response was not antigen specific. Flow cytometry was therefore used to determine whether increased frequencies of Th1 and Treg cells were present in the blood of infected patients.

Our experiments employed detection of CD69 expression, an early activation marker (83), as a tool to identify IFNγ$^+$CD4$^+$ Th1 cells that were responsive to *Hp* stimulation. This could be a consequence of antigen-specific reactivation of memory cells or T-cell receptor-independent immune activation. In agreement with the work of Lundgren et al. (84), peripheral blood $CD4^+$ cells secreted IFNγ when stimulated with *Hp* antigen; however, there were no differences between the samples from infected and uninfected donors. The response is therefore unlikely to be due to reactivation of *H. pylori*-specific memory cells, but could arise because of shared or cross-reactive antigens with other bacteria, or because ligands for pattern recognition receptors were present in the antigen preparation (85).

In agreement with our previous work, and that of others, we found higher levels of $CD4^+CD25^{hi}$ lymphocytes among PBMCs from the infected donors (68, 84). It was necessary to determine if these populations could be classed as Tregs by using a selection of cellular markers, and a large proportion of the $CD4^+CD25^{hi}$ events expressed FOXP3 and/or IL-10. Since enhanced FOXP3$^+$ Treg levels are present in the infected human gastric mucosa (48, 49, 51), we were surprised to find no difference in the frequencies

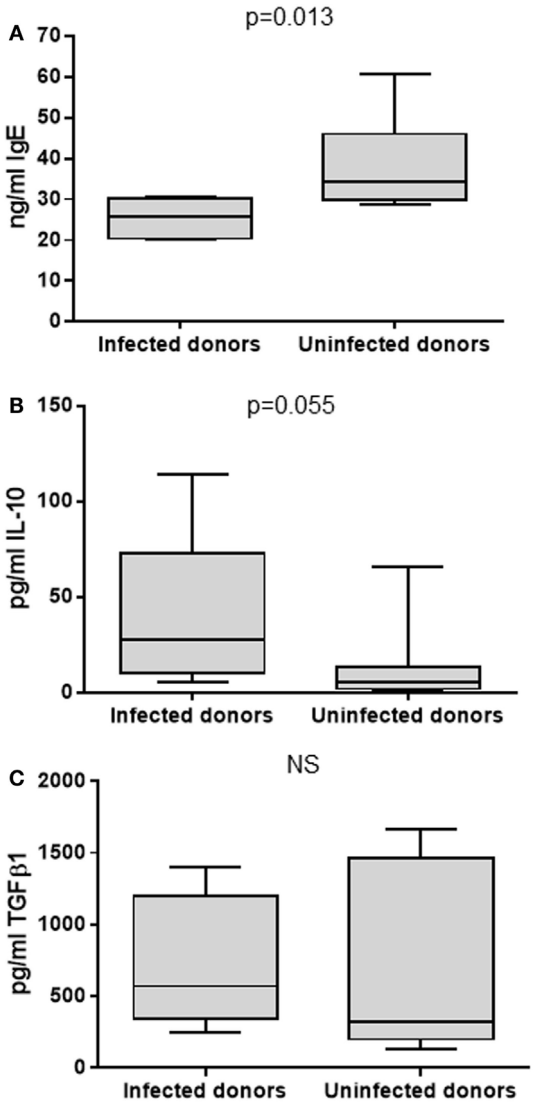

FIGURE 6 | Culture of PBMCs under Th2-skewing conditions. PBMCs from 5 infected and 10 uninfected donors were cultured for 12 days before assaying the supernatants for total IgE **(A)**, IL-10 **(B)**, and TGFβ1 **(C)** by ELISA. NS = no significant difference.

of FOXP3$^+$CD4$^+$CD25^{hi} PBMCs, but this result was confirmed using RT-qPCR. FOXP3 is not a completely reliable marker for human Tregs, since its expression can be induced in activated cells that lack suppressive function, and not all Treg populations are FOXP3$^+$ (56, 86). Interestingly, we were able to show increased levels of CD4$^+$CD25^{hi}IL-10$^+$ lymphocytes in the PBMCs of infected patients. When stimulating PBMCs with *Hp* lysate antigen, a higher proportion of the Tregs from *H. pylori*-positive patients expressed IL-10. There was no effect on the CD4$^+$CD25^{hi} population from uninfected patients, indicating the presence of a *H. pylori*-specific circulating Treg response. We were also able to confirm this by RT-qPCR, where higher levels of *IL10* mRNA were found in PBMCs from the infected patients. This agrees with several previous reports of *H. pylori*-specific IL-10-secreting CD4$^+$ cells in the blood (87–89). We previously found increased levels of IL-10$^+$FOXP3$^+$ Tregs in the *H. pylori*-infected human gastric mucosa, cellular FOXP3 expression was of high level and could be confirmed by RT-qPCR (48). Our data suggest that slightly different Treg populations may be present in the blood, and these could include a higher proportion of FOXP3$^-$ subtypes, such as Tr1 cells (which are IL-10$^+$) (90) and FOXA1$^+$ cells (associated with IFNβ responses) (91), since *H. pylori* stimulates the production of IFNβ by gastric epithelial cells (92). Recently, we were able to show that significantly higher proportions of peripheral blood Tregs from infected patients expressed the chemokine receptor CCR6 and that high concentrations of its ligand, CCL20, were present in infected human gastric mucosal tissue. The CCL20–CCR6 axis was shown to play an important role in the migration of Tregs to the infected gastric mucosa (68). Some studies have suggested that CCL20/CCR6 interactions are important in the development of Th2 responses and allergy (93, 94). It may therefore be the case that CCR6$^+$ Tregs in the circulation of those infected with *H. pylori* are more capable of migration to the site of allergic responses.

Increased concentrations of IL-10 (but not IFNγ) are present in the serum of *H. pylori*-infected patients (95), and we have found high levels of *Hp*-specific IL-10-secreting Tregs in the circulation. This makes it a likely possibility that such cells could cause bystander suppression of other unrelated extra-gastric immune responses *via* IL-10 secretion. Since the major function of Tregs is to prevent autoimmune disease caused by an overzealous immune response (58), we hypothesized that the *H. pylori*-induced Treg response is involved in protection against allergy. Increased IL-10 responses have been postulated as a mechanism behind *H. pylori*-mediated protection from allergy in humans (96); however, Cam et al. found that PBMC IL-10 production was not influenced by *H. pylori* status or presence of atopy in children (97). Because of these controversies, we examined the relationship between plasma IgE concentrations and the frequency of IL-10$^+$ CD4$^+$CD25^{hi} Tregs among PBMCs in infected and uninfected individuals. No significant differences in IgE concentrations (total or antigen specific) were found between the groups of infected and uninfected donors. This may be due to collecting samples from patients with GI symptoms, where only a small proportion had concentrations of serum IgE above the normal range, and none had reported that they suffered from allergies or asthma. When associations between Treg frequency and plasma IgE concentration were examined, as anticipated from the literature (76, 98), higher Treg responses coincided with low IgE levels. This trend was exaggerated among the *H. pylori*-positive group, which contained more individuals with high Treg frequencies accompanied by lower concentrations of total, grass pollen-, and birch pollen-specific IgE. The trend was not statistically significant among the uninfected group, indicating that stronger Treg responses in *H. pylori* infection may suppress IgE levels. We acknowledge that as the samples were collected throughout the year, the data are likely to be confounded by seasonal variation in exposure to pollens. Regardless of this, we were able to detect significant differences in the relationship between IgE and circulating Tregs between the infected and uninfected groups.

Blaser et al. reported stronger protective associations between childhood asthma and more virulent CagA$^+$ *H. pylori* infections (7, 9). Our data supported this finding, since those infected with

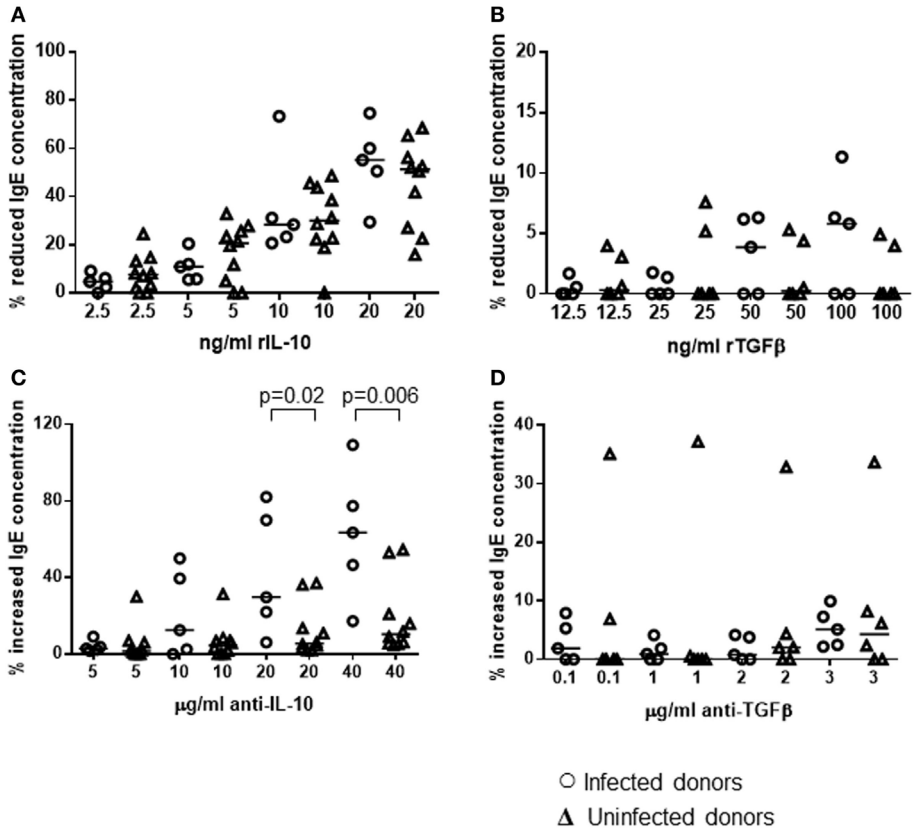

FIGURE 7 | Influence of recombinant IL-10 and TGFβ or their blocking antibodies on the *in vitro* IgE response of PBMCs from *H. pylori*-infected and -uninfected donors. PBMCs from 5 infected and 10 uninfected donors were cultured for 12 days under Th2-skewing conditions, with the addition of rIL-10 **(A)**, rTGFβ **(B)**, anti-IL-10 **(C)**, or anti-TGFβ **(D)** blocking monoclonal antibodies. Equivalent volumes of medium were added to further wells as controls for recombinant cytokines; equivalent concentrations of isotype control antibodies were added as controls for the cytokine-blocking antibodies. Total IgE concentrations were assayed by ELISA and the percentage change was calculated for each individual with respect to the control cultures. Points represent the results from individuals and horizontal lines depict the median.

cagA$^+$ strains had threefold lower plasma IgE concentrations. A number of *H. pylori* virulence factors have been reported to influence the development of allergy in mouse models, including *H. pylori* neutrophil-activating protein (HP-NAP) (a potent stimulator of Th1 responses) and VacA (known to interfere with antigen presentation and to inhibit T-cell activation; also proposed to stimulate Tregs) (45, 99, 100). Strains which express the active i1 form of VacA are usually also *cagA*$^+$ and those expressing the i2 form of VacA are usually *cagA*$^-$ (101). This was indeed true in 39/49 of our clinical isolates and, since the *vacA* types also followed the same trend in the IgE data, we consider it likely that the reported stronger protection from CagA$^+$ strains against childhood asthma could actually have been driven by VacA, with CagA acting as a marker for this. We previously found elevated IL-10 responses in the *cagA*$^+$-infected gastric mucosa (48), but in the present study, we could not find a corresponding significant difference in the peripheral blood (data not shown). It has been suggested that *cagA*$^+$ strains may elicit an increased gastric Treg response to modulate the heightened inflammation, but that there is also a pronounced effect on the migration of Tregs, which may have an impact on the populations found in peripheral blood (68, 102).

We performed experiments with an *in vitro* culture system to investigate the impact of IL-10 and TGFβ on IgE production in a well-controlled manner. The healthy donors who provided us with blood samples did not have different plasma IgE concentrations according to their *H. pylori* status (data not shown); therefore, these experiments allowed us to investigate the potential for their PBMCs to be influenced by a Th2-skewing treatment and produce IgE *in vitro*. The cells from the uninfected donors secreted significantly higher IgE concentrations, while producing markedly lower levels of IL-10. This implies that there were marked differences in the PBMC cell populations of infected and uninfected donors and is in agreement with the increased frequencies of IL-10-secreting Tregs among those with *H. pylori*. When rIL-10 was added to the cultures, in accordance with previous data, the PBMCs produced lower amounts of IgE (76). This occurred whether the cells were from infected or uninfected donors. When IL-10 blocking antibody was added, however, there was a significantly increased IgE response, which was especially marked when PBMCs were from infected donors. There were minimal effects from rTGFβ1 or TGFβ antibody blockade. These experiments demonstrate the important role of the *H. pylori*-induced IL-10 response in controlling IgE production.

We acknowledge that a weakness in this study comes from the fact that the blood samples did not come from allergic or atopic patients; however, we have gathered some interesting novel data that confirm and extend previous research on this topic. It is not possible to use an *in vivo* interventional approach in humans to study the role of IL-10 in the protective mechanism behind *H. pylori*-mediated protection from allergy. Our *in vitro* culture system provided some important clues, however, and enables us to move the project forward. Since we wish to understand the effects of *H. pylori* in protection against allergy, the best approach would be to perform an interventional study investigating the effects of antibiotic eradication therapy on allergic parameters and peripheral blood Treg frequencies. This would include looking for differences in IL-10-expressing populations in particular.

In summary, we demonstrated the presence of an increased frequency of regulatory T-cells in the peripheral blood of *H. pylori*-infected individuals. These cells tended to express IL-10, and high-level responses were associated with reduced plasma IgE concentrations. Using an *in vitro* culture system, we showed for the first time that PBMCs from *H. pylori*-infected donors had an enhanced ability to minimize IgE responses, acting in part *via* IL-10 since there was a 50% increase in IgE when IL-10 was blocked. Further *in vitro* work (not shown) found that $CD4^+CD25^{hi}$ cells, purified from PBMCs of infected and uninfected donors using magnetic bead separation, were able to suppress proliferation and IFNγ production by $CD4^+CD25^-$ effector T cells stimulated with anti-CD3/28 beads. This confirms that the cells have a regulatory function, and we are currently working to evaluate whether Tregs from infected individuals have any enhancement in suppressive activity. We also aim to carry out further experiments to elucidate the mechanisms, investigating the types of Tregs responsible (e.g., Tr1 and $FOXA1^+$ cells), and are planning to investigate whether *H. pylori* exerts a long-lasting influence on the Treg and IL-10 response from early life, or if the immunological effects require continual life-long presence of the infection in the gastric mucosa. Understanding these concepts is going to be of paramount importance, considering the diminishing prevalence of *H. pylori* around the world, and newly reported hopes for an effective vaccine (103, 104).

AUTHOR CONTRIBUTIONS

KH, DL, AG, RK, JA, and KR designed the experiments; KH, DL, AG, RK, JW, WT, JR, ES, and KR carried out the experimental work; KH, DL, AG, RK, ES, KK, and KR analyzed the data; and KH, DL, KK, and KR wrote the manuscript.

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REVIEW
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Innate Sensing of the Gut Microbiota: Modulation of Inflammatory and Autoimmune Diseases

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The mammalian gastrointestinal tract harbors a diverse microbial community with which dynamic interactions have been established over millennia of coevolution. Commensal bacteria and their products are sensed by innate receptors expressed in gut epithelia and in gut-associated immune cells, thereby promoting the proper development of mucosal immune system and host homeostasis. Many studies have demonstrated that host–microbiota interactions play a key role during local and systemic immunity. Therefore, this review will focus on how innate sensing of the gut microbiota and their metabolites through inflammasome and toll-like receptors impact the modulation of a distinct set of inflammatory and autoimmune diseases. We believe that a better understanding of the fine-tuning that governs host–microbiota interactions will further improve common prophylactic and therapeutic applications.

Keywords: microbiota, toll-like receptors, inflammasome, autoimmunity, inflammatory diseases

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INTRODUCTION

The mammalian gastrointestinal (GI) tract harbors more than 500 bacterial species that have protective, metabolic, and trophic roles and are a constant source of stimulation for the immune system (1). Surveillance mechanisms of the innate immune system control the communication of the gut microbiota with the internal environment, preventing their penetration and systemic spread, and maintaining intestinal homeostasis.

The GI innate immune system consists primarily of a physical barrier, which is composed of intestinal epithelial cells (IECs), represented by absorptive enterocytes, mucus-producing goblet cells, hormone-producing enteroendocrine cells, and Paneth cells, which produce antimicrobial peptides and lectins, among other molecules. The selective permeability of the epithelial barrier allows nutrients absorption while contributing to the immune responses by providing microbial products, which can be recognized through pattern recognition receptors (PRRs), including toll-like receptors (TLRs), nucleotide-binding domain leucine-rich repeat-containing receptors (NLRs), RIG-I like receptors (RLRs), C-type lectin family, and AIM2-like receptors (ALRs) (2), triggering different intracellular signaling cascades against pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs).

The relationship between the intestinal epithelium and the gut microbiota is not restricted to prevent host invasion by commensals. Bacteria-mediated fermentation of food that cannot be fully

digested in the mammalian intestine releases products such as short-chain fatty acids (SCFAs), which are used as fuel by IECs. In turn, the host provides a stable, nutrient-rich ecosystem for commensals to thrive. The microfold (M) cells are specialized epithelial cells of the mucosa-associated lymphoid tissue found in the follicle-associated epithelium (FAE) that mediate the uptake and transepithelial transport of luminal antigens to intraepithelial immune cells, such as macrophages and dendritic cells, which are responsible for presenting antigens to lymphocytes (3). In the intestinal lamina propria, macrophages and dendritic cell-mediated antigen uptake, either directly from the intestinal lumen by extending projections between IECs or indirectly by M cells, results in cytokine production, which drives either inflammatory Th17 or regulatory T cells, and T-cell-dependent and -independent IgA class-switching responses (4–6).

In this review, we focused on the interactions between the gut microbiota and two distinct groups of molecules, the innate sensors, namely, TLRs and the inflammasomes, which are typically composed of the innate sensors NLRs and ALRs, the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and the proinflammatory caspase-1. Increasing efforts have been pointed out toward the understanding of how innate sensing of commensals promotes homeostasis and immunity. Here, we review the impact of innate sensing of the gut microbiota on inflammatory and autoimmune diseases' outcomes.

TOLL-LIKE RECEPTORS

Toll-like receptors are type I transmembrane proteins with an extracellular horseshoe-shaped domain-containing leucine-rich repeats (LRRs) and a cytoplasmic tail containing a toll/IL-1 receptor (TIR) domain (7). They correspond to one of the five main families of PRRs, which recognize a variety of PAMPs and DAMPs.

Toll-like receptors are capable of forming both homodimers and heterodimers to detect different types of ligands. TLR2/TLR1 heterodimers recognize triacylated lipopeptides (8), and it has been recently demonstrated that they can also be activated by α-synuclein, a protein associated with Parkinson's disease (9). On the other hand, diacylated lipopeptides are recognized by TLR2/TLR6 heterodimers (8).

A recent study has shown that human immunodeficiency virus (HIV-1) structural proteins can activate TLR2- and TLR2-related heterodimers (10). A variety of viral proteins from measles virus (MV), hepatitis C virus (HCV), human cytomegalovirus (hCMV), and herpes simplex (HSV) have also been indicated as TLR2 activators (11–14). Lipoteichoic acid (LTA), zymosan, and peptidoglycan also trigger TLR2 signaling when TLR2/TLR6/CD14, TLR2/Dectin-1, and TLR2/CD14 are assembled, respectively (15–20).

TLR3 recognizes viral double-stranded RNA (dsRNA) (21) and is also capable of recognizing DAMPs released from damaged tissues (22), while TLR7 and TLR8 detect single-stranded RNA (ssRNA) found during viral replication (23–25). TLR4 is the main sensor for lipopolysaccharides (LPS) from Gram-negative bacteria (26) and also detects viral motifs (27). Flagellin is sensed by TLR5 (28), and TLR9 detects unmethylated CpG sequences in DNA molecules of bacterial and viral genomes (29). TLR10 is expressed in humans, but not in mice, making the search for a specific ligand experimentally difficult. It has been recently shown that TLR10 expressed in IECs responds to an unidentified component of *Listeria monocytogenes*, but it requires the presence of TLR2 for inducing NFκB activation (30). This opens the possibility that TLR10 acts as a coreceptor of TLR2, as it is phylogenetically related to TLR1 and TLR6 (31). Furthermore, other authors have demonstrated anti-inflammatory properties of TLR10 expressed in human peripheral blood mononuclear cells (PBMCs) (32).

TLR11 is expressed in mice and as a non-functional pseudogene in humans and has been related to prevention of *Salmonella* infection, the detection of uropathogenic *Escherichia coli*, and the recognition of a profiling-like molecule from *Toxoplasma gondii* (33–35). TLR12 and TLR13 are not expressed in humans but are expressed in mice and are capable of detecting the profiling-like molecule of *T. gondii* and 23S rRNA, respectively (36, 37). Besides PAMPs, endogenous ligands activate TLRs in the absence of infection (38).

Toll-like receptor–ligand interactions lead to dimerization of extracellular domains and results in dimerization of their cytoplasmic TIR domains. This structural modification is recognized by TIR domains on the adaptor proteins myeloid differentiation primary response gene 88 (MyD88), MyD88 adapter-like (MAL)/TIR domain-containing adapter protein (TIRAP), TIR domain-containing adapter-inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM), triggering downstream signaling pathways, which culminate in the expression of inflammatory cytokines, several anti-viral and anti-pathogen proteins, leading to the initiation of the adaptive immune response. The Myd88-dependent signaling pathway is common to all TLRs, except TLR3, which is dependent on TRIF. TLR4 uses both MyD88- and TRIF-dependent signaling pathways (7).

The detection of common molecules shared by pathogenic and non-pathogenic bacteria should trigger the same inflammatory response, but paradoxically, the recognition of commensal bacteria by intestinal receptors is somehow regulated to not result in inflammatory responses but to induce tolerance. Moreover, these TLR-mediated signals contribute to the intestinal homeostasis by regulating IECs proliferation and epithelial integrity (39–42). Interestingly, the localization, distribution, and expression of TLRs in the intestinal epithelium seem to be directly related to their role in maintaining homeostasis (43). IECs have a polarized organization, with an apical pole facing the intestinal lumen and a basolateral one that communicates with other IECs and the lamina propria. TLRs are differentially distributed through the polarized organization of IECs and in different quantities along the entire GI tract.

The human esophagus expresses TLR4, low levels of TLR1 and TLR5, and high levels of TLR2 and TLR3 mRNA in epithelial cells (44, 45). In gastric epithelial cells, it is possible to find expression of TLR2, TLR4, and TLR5, as both mRNA and protein (46). In the human small intestine, it is possible to find mRNA expression from the TLRs 1–10 (47). Under homeostatic conditions, enteroendocrine IECs from intestinal crypts express TLR1, TLR2, and

TLR4 (48), and IECs from human colon express relatively high levels of mRNA for TLR3 and less for TLR2 and TLR4 (49).

Colonic IECs are hyporesponsive to LPS, and it may be due to epigenetic mechanisms that downregulate MD-2 and TLR4 expression (50). Moreover, TLR3 in murine IECs seems to have an age-dependent expression related to an enhanced response to rotavirus in adult mice (51). On the other hand, the high basolateral expression of TLR5 is directly induced by administration of flagellin to IECs (52). TLR9 apical expression in colonic IECs has been related to the maintenance of the intestinal homeostasis (53).

The FAE presents a differential expression of TLRs in comparison with villous IECs. The murine small intestine expresses TLR2 and TLR9 in both apical and basolateral sides of the FAE, whereas IECs in the villi shows apical expression only. The TLR5 is abundantly expressed in the apical pole of villous IECs and FAE, whereas TLR4 expression is low (54).

It is conceived that TLR-mediated sensing of microbial products has a dual role in promoting the fine-tune balance between proinflammatory and pro-regulatory immune responses. A better understanding of how these molecules are regulated in face of commensals and pathogenic microorganisms will benefit the design of prophylactic and therapeutic approaches.

THE DUAL ROLE OF TLRs IN TRIGGERING INFLAMMATORY AND AUTOIMMUNE DISEASES

Bearing in mind the importance of the gut microbiota for the development of the immune system, one might speculate that microbial components could also modulate immunity and trigger inflammatory and autoimmune diseases. Several studies have shown correlations between altered expression of TLRs and inflammatory/autoimmune conditions, and increasing evidence suggests the participation of the gut microbiota during TLRs abnormal signaling and the development of diseases, including type 1 diabetes (T1D), multiple sclerosis (MS), rheumatoid arthritis (RA), and inflammatory bowel diseases (IBDs) (**Table 1**) (55–58).

Rheumatoid arthritis, IBDs, T1D, and MS are classified as multifactorial diseases with genetic and environmental factors contributing to their pathogenesis. Among the environmental factors, the exposition to microbial components has been associated with their development (55, 59–62). The concept called "molecular mimicry" was proposed to explain the role of microbial agents in inflammatory and autoimmune diseases, and according to this concept, cross-reactivity between epitopes from microbes and self-proteins can cause deregulated immune responses, leading to autoantibody production and activation of effectors cells (63).

The major organs exposed to microbial agents and their components are the skin and gut. In the gut, sensing of microbial antigens by innate immune cells and non-immune cells, such as IECs and stromal cells, is mediated by PRRs that recognized PAMPs. It is possible that bacterial components stimulate the mucosal immune cells by penetrating a damaged epithelial barrier or *via* a paracellular pathway; alternatively, microbial antigens may interact with TLRs at the apical surface of IECs, inducing inflammatory activation of the mucosal immune system. In addition, innate immune cells, such as dendritic cells and macrophages, can also sense PAMPs through TLRs initiating rapid and effective inflammatory responses against microbial invasion (57, 64). As mentioned above, the commensal microbiota is necessary for the constant stimulation of the immune system (1) and TLR-mediated sensing of these microorganisms may play a dual role in disease development as a source of both inflammatory and regulatory signals.

Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation in which cartilage and bone of the affected subjects are progressively destroyed in multiple joints (55). Several immune and resident cells, such as chondrocytes and fibroblasts, contribute to the development and progression of RA. Release of proinflammatory cytokines, such as tumor-necrosis factor (TNF)-α and interleukin-1 (IL-1), mainly by macrophages, activation of Th17 lymphocytes, and production of autoantibodies are suggested to play an important role in the disease onset (65). Studies showing the presence of bacterial cell wall components in the joints of RA patients accompanied by changes in their gut microbiota support the idea that commensal bacteria may initiate inflammation in genetically susceptible individuals (66, 67).

Abdollahi-Roodsaz et al. (56) using the IL-1 receptor antagonist-deficient (*IL1rn*$^{-/-}$) mice that spontaneously develop T-cell-mediated autoimmune arthritis showed that germ-free (GF) *IL1rn*$^{-/-}$ presented no signs of arthritis during 20 weeks of follow-up, whereas matched non-GF animals started to develop the disease from the age of 5 weeks. The authors also demonstrated that the activation of TLRs was dependent on the microbial status of the mice and that TLR2 deficiency in *IL1rn*$^{-/-}$ mice led to less Foxp3^{+} expression and reduced suppressive activity of Tregs, resulting in an enhancement of clinical and histopathological scores of arthritis. By contrast, *IL1rn*$^{-/-}$*Tlr4*$^{-/-}$ mice were protected against severe disease. It has been shown that TLR4 contributes to more severe arthritis by modulating IL-17 production and Th17 cell expansion (56, 68). In fact, gut-residing segmented filamentous bacteria (SFB) have been shown to drive both IL-1β and IL-6 production, and Th17 development, which promotes arthritis (69, 70). Therefore, one may conceive that a delicate equilibrium in TLR-mediated sensing of the gut microbiota is necessary to maintain homeostasis and prevent certain autoimmune diseases.

Although the gut microbiota has been extensively explored as an etiologic factor of RA, less is known about oral commensal microorganisms. In this context, the Gram-negative commensal bacterium *Porphyromonas gingivalis* has been a major focus of investigation as it provides a direct link between a specific microorganism and an autoimmune disease (71, 72). In fact, individuals with periodontitis present an increased proportion of *P. gingivalis* and specific antibodies to this bacterium, which has been linked to RA (73). A recent study showed that periodontal disease (PD) induced by *P. gingivalis* increases the severity of experimental arthritis through Th17 induction. This periodontal pathogen induces IL-17-producing T cells through TLR2 activation on

TABLE 1 | Toll-like receptors and related molecules involved in inflammatory and autoimmune diseases.

Molecule	Disease	Model	Evidence	Reference
IL-1 receptor, TLR2, TLR4	Rheumatoid arthritis (RA)	*IL1rn*$^{-/-}$ Germ-free mice	TLR2 deficiency in *IL1rn*$^{-/-}$ mice led to less Foxp3 expression and reduced suppressive activity of Tregs; *IL1rn*$^{-/-}$*Tlr4*$^{-/-}$ mice were protected	(56)
TLR2		DBA-1 mice	Repeated oral inoculations of the periodontal pathogens *Porphyromonas gingivalis* and *Prevotella nigrescens*-induced Th17-mediated periodontitis in mice, which was dependent on TLR2-expressing APCs	(74)
TLR2, MyD88	Inflammatory bowel disease (IBD)	C57Bl/6 mice	Variants of the MDR1/ABCB1 gene have been associated with susceptibility to UC. TLR2/MDR1A double-knockout mice presented exacerbated colitis score, which could be inhibited by treatment with IL-1R antagonist; intestinal CD11b^{+} Ly6C^{+}-derived IL-1β production and inflammation was dependent on MyD88	(77)
TLR2, TLR4, MyD88		129/ SvJ × C57Bl/6 mice	TLR2$^{-/-}$, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice showed increased susceptibility to colonic injury than WT. Antibiotic treatment increased mortality and morbidity, and abrogated the production of cytoprotective and reparative factors	(39)
TLR2, TLR3, TLR4, TLR5		Human; adults	TLR2 and TLR5 expression on IECs remain unchanged in active IBD; upregulation of TLR2 was observed in inflammatory cells from the lamina propria; UC and CD patients showed differential expression of TLR3 and TLR4, which occurred on basolateral and apical surfaces of IEC	(81)
TLR4		Human; adults	DCs from UC and CD patients showed increased TLR4 expression and the uptake of LPS started earlier than in controls; stimulated DCs secreted high amounts of TNF-α and IL-8	(83)
TLR9		C57Bl/6 mice	Apical TLR9 stimulation on IECs conferred intracellular tolerance to subsequent TLR challenges; IECs from TLR9-deficient mice displayed lower NF-κB activation threshold, and these mice were highly susceptible to experimental colitis	(53)
TLR9, TLR3	Type 1 diabetes (T1D)	BioBreeding Diabetes Resistant (BBDR) rats	ssDNA parvovirus Kilham rat virus (KRV) acts as a TLR9 ligand to upregulate proinflammatory cytokines and induce islet destruction; pretreatment with poly I:C acts synergistically with KRV to induce diabetes in 100% of infected rats	(90)
TLR2, TLR4		NOD mice	Apoptotic β-cell is sensed by APCs through TLR2, which could stimulate the priming of diabetogenic T cells	(91)
MyD88		NOD mice Germ-free mice	MyD88-deficient NOD mice did not develop T1D Germ-free (GF) MyD88-deficient NOD mice developed T1D; colonization of GF MyD88-deficient NOD mice with the bacterial community present in healthy mouse gut-attenuated symptoms	(58)
MyD88, TRIF, TLR2, TLR4		NOD mice Germ-free mice	TRIF deficiency did not promote T1D development in MyD88 sufficient NOD mice; only double-deficient mice were susceptible to T1D; reduction in disease incidence caused by TLR2 deletion was reversed in GF TLR2-deficient mice	(92)
TLR4, TLR9, MyD88	Multiple sclerosis (MS)	C57Bl/6 mice	LPS- and CpG-stimulated B cells produce IL-10 in a MyD88-dependent manner; DCs produce less IL-12 and restrain Th1 differentiation	(102)
TLR4		C57Bl/6 mice	TLR4 is highly expressed in Th17 cells and LPS directly stimulated Th17 differentiation *in vitro*; subcutaneous injection of LPS increased the frequency of IL-17 producing cells worsening experimental autoimmune encephalomyelitis (EAE)	(100)

IL1rn, IL-1 receptor antagonist-deficient mice; MDR1, multidrug resistance gene; UC, ulcerative colitis; IECs, intestinal epithelial cells; CD, Crohn's disease.

APCs (74), which suggests that the same TLR molecule may play different roles in RA onset, depending on where the molecule is expressed and with which microorganism the interaction occurs. Thus, more studies are still necessary to better determine the role of TLR-mediated sensing of the microbiota and/or pathogens in autoimmune diseases, such as RA. Mice models with organ-specific TLR expression could be used to shed light on how TLRs contribute to disease onset depending on their localization.

Inflammatory bowel diseases have been extensively studied in regarding of the gut microbiota–immune system interactions. Two major forms of IBD have been investigated: the ulcerative colitis (UC) and Crohn's disease (CD). Distinctive and complex chronic inflammatory processes characterize both disorders (61, 62), and it has been demonstrated that the intestinal microbiota of patients differ from healthy controls, showing an increase of *Enterococcus* spp. and *Bacteroides* spp. accompanied by a decrease of *Bifidobacterium* spp. and *Lactobacillus* spp. levels (75, 76), suggesting that the gut microbiota may play a pivotal role in intestinal inflammatory diseases.

Increased susceptibility to severe UC has been associated with variants of the multidrug resistance gene (MDR1/ABCB1). Deletion of TLR2 in MDR1A deficiency resulted in fulminant pancolitis, characterized by expansion of CD11b^{+} myeloid cells, and a shift toward Th1 immune responses in the lamina propria. An unaltered microbiota was required for colitis exacerbation in TLR2/MDR1A double-knockout mice once protection from

colitis was observed upon antibiotic treatment (77). Treg-specific deletion of MyD88 has been recently shown to culminate in deficiency of Treg cells, increase in Th17 cells, and exacerbated experimental colitis. An overgrowth of SFB and increased bacterial translocation was reported, and also impaired antimicrobial IgA responses (78), suggesting that the TLR-mediated sensing of the gut microbiota contributes to Treg cell-dependent maintenance of the intestinal homeostasis. On the other hand, it has been shown that gut microbiota-mediated triggering of intestinal epithelial TLRs is not critical for promoting intestinal inflammation once mice lacking TNF receptor-associated factor 6 (TRAF6), but not MyD88/TRIF, were protected from dextran sodium sulfate (DSS)-induced colitis (79). Therefore, it is becoming clear that not only the microbiota is important during TLRs signaling through colitis development but also where these molecules are expressed.

Apart from the alterations in the gut microbiota composition, Cario and Podolsky have studied TLRs expression in CD and UC patients and showed variations in the expression of some receptors. During homeostasis, TLR2 and TLR4 are presented in small amounts on IECs and lamina propria cells to minimize microbiota recognition and to maintain tolerance (80, 81). So, signaling through those PRRs may determine the balance between immunity and tolerance. The authors observed that TLR2 and TLR5 are expressed on IECs from non-IBD subjects and remain unchanged in active IBD. Upregulation of TLR2 was observed in inflammatory cells from the lamina propria of active IBD patients; differential expression of TLR3 was verified in UC patients, which presented a basolateral expression on IECs; both UC and CD patients showed an abundant TLR4 expression on basolateral and apical surfaces of IECs, respectively, and enhanced expression of TLR4 was also present in the lamina propria of IBD individuals (80).

In DCs, PRRs stimulation induces IL-23 releases, which is an important component of antimicrobial defense, but when excessively produced it favors proinflammatory T-cell response and reduces $Foxp3^+$ T cell differentiation. In this way, DCs may act as a key player in the initiation, continuation, and control of IBD (82). Conventional DCs (cDC) from UC and CD patients during remission phase showed an increased TLR4 expression. After stimulation, these cells secreted higher amounts of TNF-α and IL-8, and the uptake of LPS started earlier and was higher than in controls (83). These data suggest that an aberrant TLR4 signaling in cDC of IBD patients may result in an inflammatory phenotype during the acute phase.

Paradoxical effects of TLRs activation may be present in IBD. Bacterial DNA induces strong Th1 immune responses with high production of TNF-α and IL-8, which is also found in experimental and human IBD (84). On the other hand, bacterial DNA stimulation of IECs *via* apical TLR9 may result in anti-inflammatory effects, with the inhibition of TNF-α and IL-8 secretion, as well as NFκB activation, reducing colitis severity (53). Furthermore, it has been observed in a $CD4^+$ T-cell-dependent Severe combined immunodeficiency (SCID) transfer model of colitis that pretreatment of donor mice with CpG completely abolished colitis development in SCID recipients in a CpG–TLR9-mediated modulation of T-cell function (85).

Another important protective effect promoted by TLR signaling was recently elucidated by Kawashima et al. (86) showing that dsRNA of lactic acid bacteria (LAB), one major commensal bacteria, triggered interferon-β (IFN-β) production through TLR3 activation pathway and protected mice from experimental colitis. On the other hand, pathogenic bacteria induced much less IFN-β and contained less dsRNA than LAB, indicating that dsRNA was not involved in pathogen-induced IFN-β induction. These results point toward TLR3 as a sensor to commensal bacteria and suggest a mechanism by which this endosomal receptor contributes to anti-inflammatory and protective immune responses.

Paradoxical effects of TLRs participation are also observed in T1D, an organ-specific autoimmune disease in which insulin-producing β cells are mainly destroyed by not only Th1 but also Th17 lymphocytes (87). The autoreactive $CD4^+$ T cells that infiltrate the pancreas present a proinflammatory phenotype, characterized by IFN-γ secretion (88), and support cytotoxic T lymphocytes (Tc), which are responsible for the progressive destruction of β cells (55). Although the later steps of T1D development are well known, the initial steps remain unclear.

The role of TLRs–PAMP interactions during T1D onset has been investigated in several animal models. Studies performed in BioBreeding Diabetes Resistant (BBDR) rats infected with the ssDNA parvoviruses Kilham rat virus (KRV) showed that KRV acts as a TLR9 ligand by upregulating proinflammatory cytokines in pancreatic lymph nodes, thus inducing islet destruction (89). Pretreatment with poly I:C, a TLR3 ligand, acts synergistically with KRV to induce diabetes in 100% of infected rats (90). Also, it has been demonstrated that the activation of APCs through TLR2-mediated sensing of β cells death contributes to T1D initiation in non-obese (NOD) mice (91). In contrast with these findings, Wen et al. (58) investigating the effects of *MyD88* gene disruption on disease incidence and progression in NOD mice showed that MyD88-deficient NOD mice did not develop T1D, and that the observed protection was dependent on commensal microorganisms once GF MyD88-deficient NOD mice developed disease. The authors also demonstrated that the gut microbiota composition was changed by MyD88 deficiency and colonization of GF MyD88-deficient NOD mice with the bacterial community termed "altered Schedler's flora" (ASF), normally present in healthy mouse gut, attenuated T1D. Thus, it can be suggested that in the absence of TLRs signaling, some bacterial groups predominate and induce tolerogenic responses. However, the receptors and signaling pathways involved in microbiota-dependent protection against T1D development remain unclear.

Some protective signals against T1D development triggered by the gut microbiota have been revealed through studies with NOD mice lacking MyD88 crossed with mice deficient for other components of the innate immune response. As ASF bacteria colonization has reduced T1D in GF MyD88-deficient NOD mice, it is possible that several signaling pathways activated by TLR agonists could contribute to protection when MyD88 signaling is absent. In fact, stimulation of TLR3 and TLR4 induces the activation of a MyD88-independent, TRIF-dependent signaling pathway (55), which has been pointed out as a negative regulator of immunity. In this context, Burrows and colleagues (92) have demonstrated that TRIF deficiency did not

affect T1D development in Myd88 sufficient NOD mice, while double-deficient mice were more susceptible to T1D. Thus, it could be suggested that TLR3- and TLR4-mediated sensing of the gut microbiota participates in the protection against T1D development in MyD88-deficient NOD mice through tolerating mechanisms involving TRIF signaling.

Protective effects from TLR–microbiota interaction have also been demonstrated in MS studies. The MS is an immune-mediated chronic disease in which demyelination and axonal damage are caused by infiltration of myelin-specific autoreactive Th1 and Th17 cells into the central nervous system (CNS) in either relapsing/remitting or progressive condition (93, 94). CNS-derived peptides, such as the melanocortin antagonist agouti-related peptide (AgRP) and neuropeptide Y (NPY), are capable of modulating food intake and physiological processes that control nutrient absorption, which may influence the gut microbiota composition. In turn, gut microbiota modulates brain functions by releasing SCFAs and antigens, such as lipopolysaccharide (LPS), polysaccharide A (PSA), and LTA (95).

Miyake and colleagues (96) have investigated whether the gut microbiota was altered in MS by comparing the gut microbiota of 20 patients with relapsing–remitting (RR) MS with that of 40 healthy subjects. They found differences in the relative abundance of both archaea and butyrate-producing bacteria when comparing MS patients and healthy individuals and a significant reduction of clostridial species in MS patients. Interestingly, none of the clostridial species that were significantly reduced in the gut microbiota of MS patients overlapped with other spore-forming clostridial species capable of inducing colonic regulatory T cells, which have been associated with protection from autoimmunity and allergies (97). However, it has been shown that reconstitution of the gut microbiota from antibiotic-treated mice with *Bacteroides fragilis* protected against experimental autoimmune encephalomyelitis (EAE) by a mechanism dependent on PSA-induced IL-10-producing Treg cells, which may rely on TLR2 signaling (98). In fact, *B. fragilis*-derived PSA converts effector $CD4^+$ T cells into IL-10-producing T cells *in vitro* by a TLR2-dependent mechanism (99). On the other hand, TLR4-mediated sensing of LPS has been shown to stimulate *in vitro* differentiation of Th17 cells and subcutaneous LPS injection increased the frequency of IL-17-producing cells in inguinal lymph nodes, worsening EAE (100).

Numerous studies have focused on the role of B cells during MS pathogenesis (101, 102). B cell-deficient C57Bl/6 mice suffer an exacerbate EAE form and transfer of IL-10-producing B cells into IL-10-deficient mice protected them from disease (101). In fact, it has been recently shown that microbiota-driven IL-1β and IL-6 production promotes regulatory B cells differentiation (103). Lampropoulou et al. (102) showed that TLR-activated B cells produce IL-10 in a MyD88-dependent manner by stimulating TLR4 and TLR9 with LPS and CpG oligonucleotides, respectively. These B cells limit the capacity of DC to produce IL-12 and restrain Th1 differentiation. Therefore, new studies are fundamental to elucidate the role of different TLRs in the context of EAE modulation, which seems to depend on microbiota-driven inflammatory and regulatory immune cells.

INFLAMMASOMES IN THE GUT

Inflammasomes are multimeric protein complexes typically composed of a sensor protein, the adaptor protein ASC, and the proinflammatory caspase-1, which can be triggered by a variety of stimuli associated with infection and cellular stress (104). Inflammasome activation results in recruitment of ASC, proteolytic cleavage, and activation of caspase-1, which leads to process and release of the proinflammatory cytokines IL-1β and IL-18 (105). The majority of inflammasomes contain a NLR sensor molecule, namely NLRP1 (NOD-, LRR-, and pyrin domain-containing 1), NLRP3, NLRP6, NLRP7, NLRP12, or NLRC4 (NOD-, LRR-, and CARD-containing 4). However, other two inflammasomes have been described containing the pyrin and HIN domain-containing protein (PYHIN) family members absent in melanoma 2 (AIM2) and IFN-γ-inducible protein 16 (IFI16) as sensor molecules (106). A non-canonical activation pathway of inflammatory caspases 4/5 in humans and caspase-11 in mice has also been described and depends on intracellular sensing of lipopolysaccharide (LPS) released upon Gram-negative bacteria escape from vacuoles (107).

Inflammasome signaling has been extensively studied in macrophages in different contexts (104, 108), but little is known about inflammasome expression and function in cells located in the gut. Intestinal $CD11b^+F4/80^+$ mononuclear phagocytes that normally reside in the lamina propria were shown to be anergic to ligands for TLRs or commensals but to produce IL-1β upon NLRC4 activation after infection with pathogenic *Salmonella* or *Pseudomonas* (109). More recently, *Enterobacteriaceae* and the pathobiont *Proteus mirabilis* were shown to induce robust IL-1β production through NLRP3 activation in newly recruited intestinal $Ly6C^{high}$ monocytes upon epithelial injury (110). IECs, the first cellular barrier toward the gut lumen, were also shown to express a variety of inflammasome components such as NAIPs 1, 2, 5, and 6 in mice (hNAIP in humans), NLRC4, NLRP1, NLRP6, AIM2, caspase-1, caspase-11 (-4, in humans), ASC, and IL-18. These inflammasome components contribute to intestinal homeostasis by regulating commensals' ecology, by restricting pathogens, and by restoring epithelial barrier integrity (105). However, further studies are still necessary to shed light on how inflammasomes are regulated in both hematopoietic and non-hematopoietic intestinal cells in face of commensal microorganisms, diet-derived antigens, and pathogens to maintain homeostasis and systemic immunity.

INFLAMMASOME-MEDIATED SENSING OF THE GUT MICROBIOTA AND INFLAMMATION

The NLRP6 inflammasome has been shown to regulate intestinal microbiota ecology as metagenomic analysis has revealed pronounced dysbiosis in NLRP6-deficient mice (111, 112). Changes in the biogeographical distribution of microbiota are also observed in NLRP6 deficiency, leading to accumulation of commensal microorganisms in the colonic crypts. Reduced IL-18 levels and increased relative abundance of bacterial phyla

Bacteroidetes (*Prevotellaceae*) and TM7 have been observed in NLRP6-deficient mice, which present spontaneous intestinal hyperplasia, chemokine (C–C motif) ligand 5 (CCL5)-mediated inflammatory cell recruitment, and exacerbation of DSS-induced colitis. Cross-fostering and cohousing experiments were sufficient to transfer dysbiotic microbiota to neonatal and adult wild-type (WT) mice, leading to increased susceptibility to DSS-induced colitis. Although deficiency in other inflammasome components, including ASC, caspase-1, and IL-18, also led to dysbiosis and exacerbated colitis, deficiency in NLRC4, NLRP10, NLRP12, and AIM2 had no impact on the susceptibility of WT mice to colitis upon cohousing (111). These mice were shown to have a distinct configuration of their microbiota when compared to NLRP6-deficient mice, which might explain the different results and suggests that although inflammasomes share many of their effector molecules, different impacts on microbiota ecology can be observed.

It has been recently shown that mice deficient in NLRP6 and the inflammasome components ASC and caspase-1 lack a thick continuous overlaying inner mucus layer in the gut due to abrogated mucus secretion by IECs and are unable to clear enteric pathogens from mucosal surface (113). As mucus has an important role in regulating host–microbial interactions, it is likely that dysbiosis and increased intestinal inflammation observed in NLRP6 mice are consequences of this reduced mucus secretion by epithelial cells. Deficiency in NLRP6 and IL-18 has also been linked to colitis-related colorectal cancer (CRC) development. Enhanced tumorigenesis in these mice was dependent on microbiota-induced CCL5-driven inflammation and local IL-6 production and could be transferred to WT mice upon cohousing (114).

Corticotropin-releasing hormone (CRH)-mediated reduction of intestinal NLRP6 expression in mice exposed to water-avoidance stress (WAS) has been shown to result in altered gut microbiota and acute small intestinal inflammation. These mice presented intestinal erythema, leukocyte infiltration, increased intestinal permeability, and increased mucosal expression of IL-17 and IL-6. Other inflammasome components that had their expression partially inhibited by WAS were ASC, caspase-1, IL-1β, and IL-18, while no significant impact was observed on NLRP3 expression. As observed for colitis and colitis-related CRC, non-stressed mice developed enteritis upon cohousing with WAS-exposed mice and probiotic therapy prior to WAS reduced intestinal inflammation and prevented WAS-mediated dysbiosis (115).

NLRP3-deficient mice have also been shown to be more susceptible to DSS-induced colitis, to have a dysbiotic microbiota, and to present altered colonic β-defensin expression and decreased antimicrobial capacity. Their neutrophils exhibited impaired chemotaxis and enhanced spontaneous apoptosis (116). Modulation of NLRP3 in the gut epithelium by high-fiber feeding has been suggested to contribute to intestinal homeostasis and protection from colitis through maintenance of a healthy microbiota and due to SCFA-mediated sensing by G-protein-coupled receptors GPR43 and GPR109A and IL-18 release (117). However, upon epithelial injury, members of the gut microbiota, such as *Enterobacteriaceae*, and in particular the pathobiont *P. mirabilis* induce NLRP3-mediated IL-1β release by Ly6C^{high} monocytes, leading to intestinal inflammation (110).

The influence of inflammasome-mediated sensing of the microbiota goes beyond inflammatory processes in the gut. NLRP3, NLRP6, and inflammasome components ASC, caspase-1, and IL-18-deficiency-associated dysbiosis leads to exacerbated hepatic steatosis and inflammation through influx of TLR4 and TLR9 agonists into the portal circulation, which results in enhanced hepatic TNF-α expression and non-alcoholic steatohepatitis (NASH)/non-alcoholic fatty liver disease (NAFLD) progression. Cohousing experiments were sufficient to exacerbate NASH in WT mice, suggesting that intestinal dysbiosis may govern initial steps of systemic autoinflammatory disorders (118).

It has been shown that Pstpip2^{cmo} mice, which are prone to develop osteomyelitis, present dysbiotic intestinal microbiota characterized by *Prevotella* outgrowth. Interestingly, these mice were protected from inflammatory bone disease and bone erosion when fed with high-fat diet (HFD), which had a marked impact on intestinal *Prevotella* reduction and significantly reduced pro-IL-1β expression in neutrophils. Antibiotic treatment was also efficient in reducing pro-IL-1β expression in Pstpip2^{cmo} mice, but cohousing experiments and fecal microbiota transplantation from Pstpip2^{cmo} mice failed to cause disease in WT mice. However, fecal microbiota transplantation from low-fat diet (LFD)-fed diseased Pstpip2^{cmo} mice to young LFD-fed Pstpip2^{cmo} mice by oral gavage promoted the expansion of *Prevotella*, and significantly accelerated the development of osteomyelitis, whereas transplantation of fecal microbiota from HFD-fed Pstpip2^{cmo} mice to young LFD-fed Pstpip2^{cmo} mice limited *Prevotella* outgrowth and significantly protected mice from developing osteomyelitis. Caspase-1/8-mediated processing of microbiota-induced pro-IL-1β was necessary to promote autoinflammatory disease in these susceptible mice (119).

Extraintestinal inflammatory processes have also been shown to be partially dependent on inflammasome-mediated sensing of the microbiota metabolite acetate. GF, antibiotic-treated, and GPR43-dificient mice are protected from joint inflammation upon injection of monosodium urate monohydrate (MSU) crystals. It was demonstrated that microbiota reconstitution or acetate administration to GF mice restored MSU crystals-induced inflammation and that GPR43 is at least partially necessary to adequate inflammasome assembly and IL-1β production in response to acetate (120). Therefore, one may suggest that inflammasome-mediated sensing of both intestinal microbiota and their products contributes to control local and systemic inflammatory disorders, which should be considered when designing prophylactic and therapeutic applications.

INFLAMMASOME AND TLR INTERACTIONS

Many studies have focused on the interactions between inflammasomes and TLRs. In fact, it has been shown that the enteric pathogens *E. coli*- and *Citrobacter rodentium*-induced TLR4/TRIF-dependent synthesis of caspase-11 and activation of NLRP3 inflammasome in macrophages (121) and that *Pseudomonas aeruginosa* infection of macrophages induced

TLR-4/TRIF-dependent autophagy, which was attenuated by NLRC4/caspase-1-mediated cleavage of TRIF. Prevention of *in vivo* caspase-1-mediated cleavage of TRIF resulted in enhanced autophagy, reduced IL-1β production, and increased bacterial clearance (122). *L. monocytogenes* infection of macrophages was shown to induce TLR/IRAK1/IRAK4-dependent activation of NLRP3 (123, 124), which suggests that inflammasome–TLR interactions may have an important role in the bacterial recognition.

It has been also demonstrated that Clathrin-mediated endocytosis followed by TLR8- and TLR7-mediated recognition of HIV and HCV in monocytes and macrophages resulted in NLRP3 activation, which was independent of type I IFN production (125). Moreover, dendritic cells were shown to express higher levels of NLRP3 in the steady-state condition compared to macrophages and to secrete substantial amounts of mature IL-1β upon stimulation with TLR ligands independently of P2X7 signaling (126). On the other hand, chronic TLR stimulation by LPS has been shown to dampen NLRP3 activation through IL-10 induction (127), and TLR2/TLR4 engagement resulted in upregulation of plasminogen activator inhibitor type 2 (PAI-2), a serine protease inhibitor, which culminated in the stabilization of the autophagic protein Beclin 1 to promote autophagy, reduction of mitochondrial reactive oxygen species, NLRP3 protein level, and pro-IL-1β processing (128).

Although increasing evidence suggests that both TLRs and inflammasomes play an important role in promoting host–microbiota communication, studies regarding the interactions of these molecules in the context of the gut microbiota are still lacking and will further improve our knowledge on how commensal microorganisms and the immune system cooperate in the modulation of inflammatory and autoimmune diseases.

CONCLUDING REMARKS

The dynamic interactions that have been established between mammalian hosts and commensal microorganisms over millennia of coevolution resulted in the development of a well-structured immune system, which controls pathogenic infections while tolerates a highly diverse microbiota. Innate sensing of the gut microbiota through TLRs and inflammasomes contributes to intestinal homeostasis by stimulating the development/function of both regulatory and inflammatory cells and by promoting proliferation of IECs, epithelial integrity, mucus secretion, and containment of opportunistic infections. It has become clear that disturbances in the fine-tuning that governs host–microbiota interactions may lead to both local and systemic inflammatory and autoimmune diseases. In this review, we bring many examples of the dualistic role played by the gut microbiota in both promoting and regulating inflammation and autoimmunity. We believe that a better understanding of mechanisms involved in these complex interactions between host and commensals will further improve common prophylactic and therapeutic applications.

AUTHOR CONTRIBUTIONS

AI, CM, and RA wrote the manuscript. NC and RA reviewed the manuscript.

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Serum Cytokine Signature That Discriminates *Helicobacter pylori* Positive and Negative Juvenile Gastroduodenitis

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Gastroduodenitis caused by *H. pylori*, often acquired in early childhood, is found in about 50% of the adult population. Although *H. pylori* infections can remain asymptomatic, its virulence factors usually trigger epithelial vacuolization and degeneration, loss of microvilli, disintegration of cytoplasm, and leukocyte accumulation. It is believed that leukocyte infiltration is driven by cytokines produced locally in infected tissue. However, so far little is known about changes in serum cytokines in juvenile patients infected with *H. pylori*. Serum cytokine profiles were analyzed in 62 juvenile patients diagnosed with gastroduodenitis using the Bio-Plex multiplex assay. *H. pylori* infection was confirmed in 32 patients, while 30 patients were *H. pylori*-free. Cytokines CXCL5 and CXCL6, potent neutrophil chemoattractants, were upregulated in all patients diagnosed with gastroduodenitis. Serum levels of IL8, a prototype neutrophil attractant, remained unchanged in subjects with gastroduodenitis relative to controls. Therefore, our data suggest that CXCL5 and CXCL6 play a role in directing neutrophil trafficking into inflamed gastroduodenal tissue. In addition, the CCL25/GM-CSF ratio differed significantly between *H. pylori*-positive and -negative juveniles. Further, study is needed to evaluate the role of CCL25 and GM-CSF in the pathogenesis of the different etiologies of gastroduodenitis.

Keywords: *H. pylori*, virulence factors, cytokines, gastroduodenitis

INTRODUCTION

Helicobacter pylori (*H. pylori*) was identified in 1982 and suggested to be a causative agent for gastritis and stomach ulcers (Marshall and Warren, 1984). This helix-shaped gram-negative bacterium colonizes gastric mucosa and persists as a chronic infection (Marshall et al., 1985a; Morris and Nicholson, 1987). *H. pylori* is one of the most common gastrointestinal infections, being found in about 50% of the adult population (Sipponen et al., 1996; Kosunen et al., 1997). The majority of *H. pylori* infections remain asymptomatic, with only 15% of carriers developing symptoms (Atherton, 1998; Ernst and Gold, 2000). Infection, often acquired in early childhood,

has been shown to be associated with poor hygiene and impoverished living conditions (Malaty and Graham, 1994; Kivi et al., 2003; Konno et al., 2005; Dattoli et al., 2010). It is believed that *H. pylori* is transmitted via fecal-oral or oral-oral routes (Goh et al., 2011).

During gastric epithelium colonization, *H. pylori* establishes a persistent infection within the mucus layer without crossing the epithelial barrier (Noach et al., 1994). Infection is histologically characterized by surface epithelial degeneration, inflammation, and leukocyte infiltration into the gastric mucosa (Bodger and Crabtree, 1998; Peek et al., 2010).

H. pylori virulence factors are the main cause of tissue damage, and include vacuolating cytotoxin (vacA), cytotoxin associated gene A (cagA), and neutrophil-activating protein (HP-NAP; Atherton, 1998; Cellini and Donelli, 2000; Fu, 2014). Binding of *VacA* to gastric cells triggers epithelial vacuolization and degeneration, loss of microvilli, and cytoplasmic disintegration (Goodwin et al., 1986; Papini et al., 1994; Garner and Cover, 1996; Smoot et al., 1996). Animal studies have demonstrated that purified *vacA* toxin causes gastric epithelial damage with little effect on inflammatory leukocyte infiltration (Telford et al., 1994; Ghiara et al., 1995). In addition, cagA has been shown to be strongly associated with development of local inflammation and expression of pro-inflammatory cytokines (Peek et al., 1995). Moreover, it has been suggested that the cagA gene and nearby sequences code for proteins that act synergistically and promote production and secretion of pro-inflammatory cytokines (Tummuru et al., 1995; Censini et al., 1996), and that the virulence factor HP-NAP promotes neutrophil adhesion, chemotaxis, and activation (Satin et al., 2000). The combined effects of these virulence factors is inflammation of local tissue caused by damage to gastric epithelial cells, and activation of pro-inflammatory cytokine production.

Histologically, infiltration of gastric tissue by leukocytes is a hallmark of *H. pylori* infection. In tissue biopsies collected from patients infected with *H. pylori* both neutrophil infiltration (Kamada et al., 2006; Jaramillo-Rodríguez et al., 2011; Xu et al., 2012) and increased infiltration of CD4+ T helper lymphocytes in the lamina propria (D'Elios et al., 1997a) have been reported. Further, in gastric mucosa, it has been demonstrated that *H. pylori* infection activates predominantly Th1-type immune responses (D'Elios et al., 1997a), and immunohistochemically analyses of gastric biopsies have revealed an increased presence of CD8+ lymphocytes and macrophages (Bedoya et al., 2003). Animal models established that the early stage of infection is marked by neutrophil infiltration (Rossi et al., 2000). As infection progresses, a drop in neutrophil counts is followed by increased tissue infiltration with mononuclear leukocytes, mostly CD3+ lymphocytes. Initially scattered, lymphocyte infiltrates organize into small patches in the corpus and antrum of stomach (Rossi et al., 2000; Sepulveda and Patil, 2008). Later, the appearance of CD4+ and CD8+ lymphocytes in the periglandular area and beneath the basal lamina correlates with histological signs of gastric epithelial damage. Eventually, leukocyte infiltrates became organized in follicles containing CD21+, CD4+, and CD3+ lymphocytes (Rossi et al., 2000). Furthermore, the appearance of neutrophils at late stage leukocyte infiltration suggests active chronic gastritis. Together, these data indicate that initially *H. pylori* infection causes neutrophil infiltration of gastric mucosa, then as infection progresses neutrophils are replaced by lymphocytes. This leukocyte infiltration is the main cause of gastric epithelial damage in *H. pylori* infected tissue.

Exposure of gastric epithelium to *H. pylori* results in the production of a number of cytokines that stimulate migration of immune effector cells into inflamed tissue, including upregulation of IL8, CCL5, CCL3, IFN, IL10, IL12p40, and IL18 (Crabtree et al., 1994, 1995a,b; Yamaoka et al., 1996, 1998; Karttunen et al., 1997; Park et al., 2001; Dzierzanowska-Fangrat et al., 2008). Studies using animal models have demonstrated that the early stage of *H. pylori* infection is characterized by increased expression of IL1, IL8, IL6, and TNF-α in gastric mucosa (Harris et al., 2000; Rossi et al., 2000). Then, as the disease progresses, IL8 expression declines, while IL1, IL6, and TNF-α remain elevated (Rossi et al., 2000; Harris et al., 2000). It has been suggested that at late stages of the disease, there is a shift toward Th1 immunity, involving cytokines such as IFNγ and IL12 (D'Elios et al., 1997a; Haeberle et al., 1997; Pellicanò et al., 2007), and that persistent activation of the Th1 immune response is a cause of tissue damage in *H. pylori* infection (D'Elios et al., 1997b; Smythies et al., 2000). A combination of transcriptional analysis of tissue biopsies and histological findings has provided most of the information about cytokine activation of *H. pylori* infection. However, there is limited knowledge of serum cytokine expression in children infected with *H. pylori*, since the majority of data is from adult populations (Bayraktaroğlu et al., 2004; Mehmet et al., 2004; Abdollahi et al., 2011; Eskandari-Nasab et al., 2013). To date, for the most part, cytokines studies have been limited to the Th1 or pro-inflammatory class. This is unfortunate, as *H. pylori* infection often occurs during early childhood establishing a lifelong chronic infection. Understanding cytokine expression at the initial stages of infection will identify early markers, and improve disease diagnosis.

Here, we present data on cytokine activation in serum of children with gastroduodenitis. Sixty-two juveniles with gastroduodenitis were included in this study, with 30 having a diagnosis of *H. pylori* infection. Regardless of the presence or absence of *H. pylori* pathogenicity markers, there was upregulation of the potent neutrophil chemoattractants, CXCL5, and CXCL6. However, serum levels of IL8, a prototype neutrophil attractant, were not statistically different between diagnoses. Therefore, our data suggest that a novel set of chemokines, CXCL5, and CXCL6, play a role in neutrophil trafficking into inflamed gastroduodenal tissue. Further, an intriguing observation was that the CCL25/GM-CSF ratio differed significantly between *H. pylori* positive and negative children with gastroduodenitis.

MATERIALS AND METHODS

Subjects

Sixty-two patients (24 boys, 38 girls; age 14.0 ± 2.1) hospitalized in the Children's Republican Clinical Hospital (Kazan, Russia) with a diagnosis of gastritis and duodenitis were recruited for this study. Initial diagnosis of *H. pylori* infection in 30 of the patients

was based on clinical presentation and upper GI endoscopy. The presence of *H. pylori* was confirmed by urea breath test and PCR.

Biopsies were collected from each patient during upper GI tract endoscopy: two from antral part of the stomach along the major and minor curvatures, and 2–3 from the body of the stomach, along the major and minor curvatures. In addition, stomach biopsies were collected from three controls, who were found to be negative for any gastroduodenal pathology by diagnostic upper GI tract endoscopy. Biopsies were used for both PCR analyses and histological studies.

Serum samples were collected from all 62 juveniles with gastritis and duodenitis, as well as from 20 age- and sex-matched controls. All controls were negative for symptoms of upper GI tract infection or gastritis. Serum samples were stored at −80°C. The Ethics Committee of Kazan State Medical University approved this study (N6, 06.25.2012) and informed consent was obtained from the legal guardian of each study subject, in accordance with the Declaration of Helsinki and the article 20, Federal Law "Protection of Health Right of Citizens of Russian Federation" (N323-Ф3, 11.21.2011).

Urea Breath Test

Breath ID Hp (Exalenz, USA) was used to confirm *H. pylori* infection. This breath test measures the presence of ^{13}C labeled CO_2 in the patient's breath after ingestion of a solution containing ^{13}C labeled urea. After 10 min, exhaled air is collected and tested for the presence of $^{13}CO_2$, which indicates *H. pylori* infection.

PCR Detection of *H. pylori*

DNA was extracted from biopsy tissue using the Helicopol Kit (Lytech, Moscow). *H. pylori* positive biopsies were analyzed by PCR for a pathogenicity marker profile using the Helicopol II Kit (Lytech, Moscow). Briefly, 2 μl total DNA was mixed with 4 μl 10x PCR buffer, 2 μl 25 mM $MgCl_2$, 0.5 μl (100 pmol) each of primers, 40.7 μl distilled water, and 0.3 μl (2.5 U) Taq DNA polymerase. The reaction mixture was then subjected to 35 cycles, each consisting of 30 s at 94°C, 30 s at 50°C, and 2 min at 72°C. PCR products were analyzed on a 1% agarose gel.

Cytokine Analysis

Serum cytokine levels were analyzed using a Bio-Plex Pro Human Chemokine Panel (40-Plex; Bio-Rad, Hercules, CA), a multiplex magnetic bead-based antibody detection kit.

Immunohistochemical Analysis

After initial analysis, there were surplus clinical diagnostic biopsy specimens from 21 *H. pylori* positive and 13 negative cases. These were used for immmunohistochemical analysis. Punch biopsies were fixed in 4% paraformaldehyde for 4 h at 4°C, and then cryoprotected with 30% sucrose in PBS. Immunohistochemical staining was performed on 5 μm thick sections. Slides were deparaffinized with xylene and rehydrated through a graded alcohol series. Tissue morphology was evaluated by light microscopy using hematoxylin-eosin staining. Additionally, Alcian blue (pH 2.5) and periodic acid Schiff (PAS) staining were performed to detect the presence of sialomucins.

Statistical Analysis

Statistical analysis was performed using the STATISTICA 7.0 Software Package (StatSoft, Tulsa, OK) and the IBM SPSS Statistics 20 Software Package (IBM Corp, Armonk, NY, U.S.). Data are presented as the median (5–95% range) for continuous variables. Differences between independent study groups were tested by non-parametric methods. To address type 1 error, Kruskal–Wallis ANOVA by Ranks test for multiple independent samples was followed by the multiple comparison (all pairs) non-parametric *post-hoc* Steel-Dwass test Nonparametric multiple comparisons were made [recalculated and confirmed] by the Steel-Dwass all pairs test using JMP® 13.0.0 Software (SAS Institute Inc, USA). Differences were considered significant at $P < 0.05$. Jonckheere's non-parametric trend test was performed to compare three group medians when they were arranged in order. Cytokine profiles between subject groups were differentiated using forward stepwise discriminant function analysis.

RESULTS

Patients

Sixty-two patients (24 boys and 38 girls) were admitted to the Children's Republican Clinical Hospital (Kazan, Russia) with a diagnosis of gastritis and duodenitis. The main symptoms were pain in the epigastric area, vomiting, nausea, headache, and fatigue. Biopsy samples collected from the antrum of the stomach were analyzed for the presence of *H. pylori* antigens. Thirty samples were positive for *H. pylori* and 32 samples negative. The surplus biopsy tissue from 21 positive and 13 negative juveniles (of the 62 enrolled in the study) was used for histological studies.

Three *H. pylori* positive biopsies had massive leukocyte infiltration of the lamina propria (**Figure 1**) with moderate incomplete metaplasia of the epithelium (**Figure 2**). Moderate lymphocyte infiltration of the lamina propria was found in 11 positive biopsies, with four having moderate epithelial metaplasia. Finally, four had moderate lymphocyte infiltration of the lamina propria with no sign of metaplasia. Based on histological evaluation, diagnosis of chronic atrophic gastritis was established in 9 (42.9%) of the *H. pylori* positive biopsies.

Histological analysis of the 13 *H. pylori* negative biopsies revealed epithelial metaplasia in eight biopsies, with lymphocyte infiltration in five cases. Two biopsies had lymphocyte infiltration and metaplasia. The remaining two biopsies had no sign of lymphocyte infiltration or metaplasia. Diagnosis of chronic atrophic gastritis was established in 6 (46.2%) of the *H. pylori* negative biopsies. The gastritis activity was graded in all biopsies according to the Histological Division of the Sydney System and "Up-Dated Sydney System" (Tytgat, 1991; Dixon et al., 1997; **Table 1**). This classification determines the activity grade based on the presence of polymorphonuclear leucocytes in combination with mononuclear inflammatory infiltrate, intestinal metaplasia, atrophy, and detection of *H. pylori* organisms. *H. pylori* positive biopsies were characterized by a higher grade of chronic inflammation compared to *H. pylori*-free biopsies (**Table 1**).

All patients were examined by gastroduodenoscopy. Hyperemia and edema were detected in the gastric mucosa

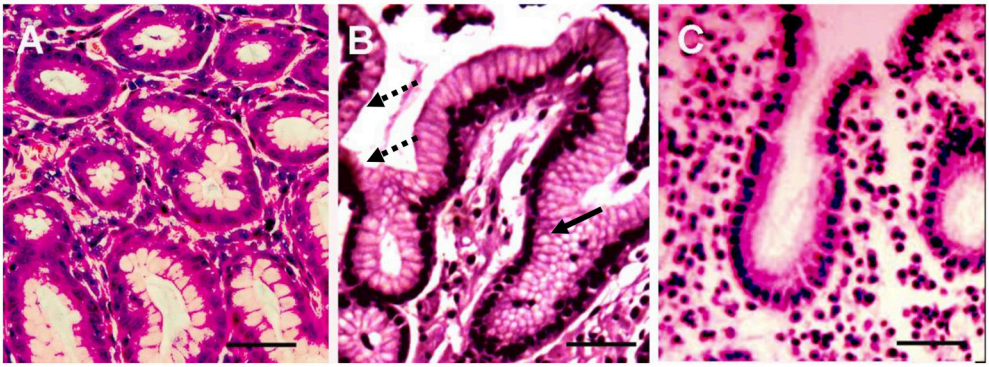

FIGURE 1 | Histological sections of three representative biopsies. Gastric biopsy sections (3–5 μm) of control **(A)**, and *H. pylori* positive **(B)** and *H. pylori* negative **(C)** gastroduodenitis cases were deparaffinized and stained with hematoxylin and eosin (H&E). The gastric epithelium phenotype of the *H. pylori* positive juvenile **(B)** resembles the phenotype of colonic epithelium, characterized by multiple intracytoplasmic mucin droplets of varying sizes and shapes (solid arrow), and the absence of a brush border (dashed arrow). H&E; x100; Bar represents 20 μm.

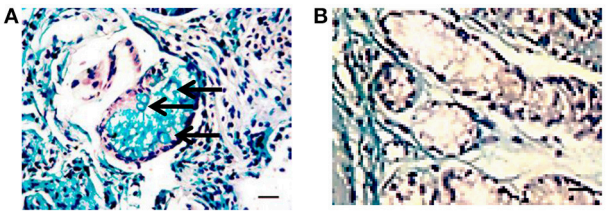

FIGURE 2 | Histochemistry of gastric epithelium metaplasia in a *H. pylori* positive biopsy. Gastric biopsy sections (3–5 μm) were deparaffinized and stained with Alcian blue (pH 2.5) and PAS followed by H&E staining. Presence of sialomucins (stained blue, solid arrow) demonstrates incomplete metaplasia (Filipe et al., 1994). **(A)** *H. pylori* positive gastric biopsy; **(B)** *H. pylori* negative gastric biopsy (Bar represents 20 μm).

of some, but not all, subjects. No visible changes were observed in the esophagus. Erosion with fibrin deposits, hyperemia, and edema were detected in 3 (14%) of *H. pylori* positive subjects. Five (16.1%) *H. pylori* positive cases were characterized by duodenal bulbar deformity with multiple scars, hyperemia and edema, with no deformation, or scarring observed in *H. pylori* negative stomachs. Two (6.4%) *H. pylori* positive stomachs had multiple ulcers, while all *H. pylori* negative patients were all ulcer-free. Overall, *H. pylori* positive stomachs were characterized by pronounced histological abnormalities compared to *H. pylori* negative stomachs.

Cytokine Profile

A total of 40 cytokines were analyzed in the serum of all subjects (**Table 2**). Children with gastroduodenitis had similar profiles, with the cytokine activation profile in *H. pylori* positive serum closely resembling *H. pylori* negative. For example, significant upregulation of CXCL2, CCL1, IL2, CCL7, CCL22, CXCL16, and CXCL12 was detected in both groups. Interestingly, the serum level of a major chemoattractant for neutrophils, IL8, did not differ significantly between patients with gastroduodenitis and controls. However, serum levels of CXCL5 and CXCL6, chemoattractant factors for neutrophils, were significantly upregulated in sera from all *H. pylori* positive juveniles, and not linked to the presence of neutrophil infiltration in biopsied tissue. Although serum levels of TNF-α were significantly higher in both *H. pylori* positive and negative sera compared to controls (**Table 2**), concentrations of this cytokine were within the normal range for this age (Mózes et al., 2011).

Significantly increased levels of CCL15, CCL20, and MIF were detected in both *H. pylori* positive and negative serum compared controls. However, only MIF levels differed significantly between the two groups. In contrast, no changes in serum levels of CCL2, CCL3, CCL8, CCL11, CCL17, CCL19, CCL21, CCL23, CCL24, CCL26, CCL27, CXCL9, CX3CL1, IL6, IL8, IL10, and IFN-γ were found either between the two groups of patients with gastroduodenitis, or between juveniles with gastroduodenitis and the control group.

We compared cytokine activation in *H. pylori* positive patients with different histological presentations. The histological data was used to divide the patients into four groups; group one was characterized by severe lymphocyte and neutrophil infiltration of the lamina propria with moderate epithelial metaplasia, group two had moderate lymphocyte infiltration of the lamina propria and moderate epithelial metaplasia, group three had mild infiltration of lamina propria and mild epithelial metaplasia, and group four had no lymphocyte infiltration and no epithelial metaplasia. The pattern of cytokine upregulation was similar in all groups and was characterized by increased serum levels of chemoattractants for lymphocytes, monocytes, natural killer cells and dendritic cells such as CCL1, CCL22, CCL15, CXCL16, and CXCL12.

Next, forward stepwise discriminant function analysis was utilized to identify cytokines that differed between control and *H. pylori* positive and negative sera. Fourteen cytokines were selected to generate a classification matrix [model summary: Wilks' lambda = 0.15498; $F_{(28, 102)} = 5.6106$, $p < 0.0001$, IL4, IL8, CCL3, CCL1, CCL7, IFNγ, CXCL12, CCL2, CXCL10,

TABLE 1 | Histological examination of the gastric biopsies from *H. pylori* positive and negative gastroduodenitis cases.

Histological findings	*H. pylori* positive, abs. (%), $n = 21$	*H. pylori* negative, abs. (%), $n = 13$	Control abs. (%) $n = 3$
No visible lymphocyte infiltration or few inflammatory cells	9 (42.9%)	12 (92.3%)	3 (100%)
Moderate leukocyte infiltration of the lamina propria (Grade I)	9 (42.9%)	1 (7.7%)	–
Severe leukocyte infiltration of the lamina propria (Grade II)	3 (14.2%)	0	–
Metaplasia (+)	12 (57.1%)	8 (61.5%)	–
Metaplasia (–)	9 (42.9%)	5 (38.5%)	3 (100%)

CCL23, MIF, TNF-α, CXCL1, and CXCL9]. Discriminant analysis revealed greater differences between patients with gastroduodenitis (both *H. pylori* positive and negative) and controls (Squared Mahalanobis distances 34.32) than between *H. pylori* positive and negative patients (Squared Mahalanobis distances 2.56). Furthermore, the 14 cytokine-based classification matrix yielded 100% correct predictions for controls (predicted classifications vs. observed classifications in the classification matrix), with lower percentages of correct predictions for cases that were *H. pylori* positive (80%) and *H. pylori* negative (76.66%).

In Crohn's disease altered expression of GM-CSF and CCL25 has been suggested to play a role in the pathogenesis of inflammatory gastrointestinal disease (Samson et al., 2011). Therefore, we sought to determine whether these two cytokines were involved in the pathogenesis of *H. pylori*-related gastroduodenitis. Serum levels of GM-CSF were significantly upregulated in *H. pylori* negative subjects, while in *H. pylori* positive subjects, GM-CSF levels were similar to controls (**Table 2**), and significantly lower ($^*P < 0.05$; **Table 2**) than *H. pylori*-free serum. In addition to increased GM-CSF levels, *H. pylori* negative serum was characterized by significantly lower levels of CCL25 (**Table 2**), suggesting bidirectional activation of these cytokines in negative serum. To further analyze the activation pattern of these cytokines in *H. pylori* negative and positive serum, we compared the CCL25/GM-CSF ratio in three independent study groups using Kruskal–Wallis ANOVA by Ranks test (P-level = 0.0083), followed by *post-hoc* Jonckheere's non-parametric trend test for multiple comparisons (P-level = 0.006; **Figure 3**). The CCL25/GM-CSF ratio differed significantly between each group. *H. pylori* positive and negative juveniles were positioned on either side of controls (**Figure 3**), suggesting that the CCL25/GM-CSFratio reflects an essential difference in gastroduodenitis pathogenesis with *H. pylori* positive and negative stomach having distinct forms.

DISCUSSION

H. pylori infection is often acquired early in childhood (McCallion et al., 1996; Suerbaum and Michetti, 2002; Tkachenko et al., 2007). This bacterium colonizes gastric mucosal epithelium, establishes a chronic infection (Marshall et al., 1985b; Morris and Nicholson, 1987), and once established, releases numerous virulence factors causing apoptosis and vacuolization of the gastric epithelium, and functional disruption of the gastric epithelial barrier (Goodwin et al., 1986; Papini et al., 1994; Garner and Cover, 1996; Smoot et al., 1996).

Currently, little is known about systemic activation of cytokines in children infected with *H. pylori*. The serum cytokine profile of infected children suggests a strong activation of chemoattractants for mononuclear leukocytes (**Table 2**). For example, in *H. pylori* positive gastroduodenitis we demonstrate increased serum levels of chemoattractants for mononuclear lymphocytes, such as CXCL10, CCL22, and CXCL16 (Taub et al., 1996; Andrew et al., 1998; Huang et al., 2008). This observation is supported by histological examination of biopsies, where increased leukocyte infiltration was detected (**Table 1** and **Figure 1**). Interestingly, there were no differences in the serum cytokine profiles of patients with distinct histological presentations suggesting that tissue pathology is determined by *in situ* cytokine activation, which is not reflected in circulating cytokine levels. Serum levels of the prototype neutrophil chemoattractant IL8 (Gessler et al., 2004), remained unchanged in juveniles with gastroduodenitis. Bayraktaroglu et al described the same phenomenon in adults where IL-8 serum levels in *H. pylori* positive cases did not differ from controls (Bayraktaroğlu et al., 2004). However, increased levels of IL8 transcripts in tissue have been documented in *H. pylori* patients (Yamada et al., 2013; Nagashima et al., 2015), suggesting that upregulation of IL-8 may be a local characteristic of inflammation of gastrointestinal tissue. The molecular mechanisms regulating systemic neutrophil migration into inflamed tissue remains to be determined. Here, we demonstrate upregulation of CXCL5 and CXCL6, potent chemoattractants for neutrophils (Territo et al., 1989; Chertov et al., 1996; Mei et al., 2012) in serum from juveniles with gastroduodenitis. CXCL5, constitutively expressed by enterocytes, coordinates with CXCR2 the transmigration of neutrophils (Mei et al., 2012). Increased numbers of CXCL6 positive mucosal cells have been observed in Crohn's disease biopsies (Yamada et al., 2013), where, upon activation, upregulation of CXCL6 is more sustained than IL8 (Wuyts et al., 2003). It has been suggested that CXCL6 could play a role in supporting chronic inflammation by facilitating neutrophil migration at a late stage of infection (Wuyts et al., 2003). In this study, we present novel data on the upregulation of CXCL5 and CXL6 in serum during gastroduodenitis. Serum levels of these cytokines did not differ between *H. pylori* positive and negative children with gastroduodenitis, suggesting that upregulation of CXCL5 and CXCL6 is a tissue-specific response to inflammation, and not driven by a specific pathogen.

TABLE 2 | Serum cytokine profile of children diagnosed with *H. pylori* positive and negative gastroduodenitis.

Analyte	*H. pylori* positive	*H. pylori* negative	control
	Median (pg/ml; 5–95% range)	Median (pg/ml; 5–95% range)	Median (pg/ml; 5–95% range)
IL-1β	11.10 (3.61–22.30); $P < 0.05$	8.93 (1.57–20.82); $P < 0.05$	2.50 (1.45–4.93)
IL2	38.24 (18.35–60.99); $P < 0.05$	32.13 (6.30–55.06)	17.98 (4.67–34.56)
IL4	64.28 (19.01–87.12); $P < 0.05$	58.32 (30.52–116.86); $P < 0.05$	8.40 (3.30–18.00)
IL6	28.30 (11.60–51.29)	25.84 (6.30–64.58)	16.22 (10.25–76.07)
IL8	29.00 (9.17–48.30)	25.59 (8.90–54.76)	8.32 (3.81–56.00)
IL10	142.41 (12.51–250.46)	119.02 (43.21–214.17)	77.95 (57.01–210.31)
IL16	2835.29 (91.60–11326.32); $P < 0.05$	2762.31 (381.46–6959.23); $P < 0.05$	678.23 (402.53–2713.07)
CCL1	203.26 (55.32–276.00); $P < 0.05$	178.84 (86.83–261.92); $P < 0.05$	46.00 (24.62–120.80)
CCL2	68.41 (3.23–164.97)	67.02 (12.29–100.79)	43.23 (34.30–345.63)
CCL3	24.12 (5.40–36.22)	21.51 (10.66–65.00)	28.99 (2.06–87.70)
CCL7	347.20 (21.11–595.61); $P < 0.05$	309.99 (95.47–550.32); $P < 0.05$	118.14 (61.00–448.84)
CCL8	87.86 (11.20–333.22)	115.22 (17.55–284.38)	78.49 (6.51–141.19)
CCL11	95.50 (47.20–156.00)	89.40 (63.60–135.80)	99.36 (57.30–167.20)
CCL13	210.14 (13.51–499.87); $P < 0.05$	251.50 (19.57–733.77); $P < 0.05$	34.27 (3.05–210.04)
CCL15	1654.79 (34.23–41566.08); $P < 0.05$	13334.57 (211.26–43075.98); $P < 0.05$	72.94 (23.65–242.02)
CCL17	518.65 (29.93–2962.22)	476.64 (95.91–1426.65)	243.07 (23.54–1341.02)
CCL19	1270.66 (47.13–4730.73)	1075.69 (194.00–2762.13)	642.28 (120.30–3283.56)
CCL20	35.41 (3.56–181.76); $P < 0.05$	45.20 (2.33–135.31); $P < 0.05$	4.34 (2.33–8.02)
CCL21	153.20 (134.20–195.40)	198.30 (156.20–225.30)	167.30 (136.20–201.20)
CCL22	3337.42 (116.17–8142.66); $P < 0.05$	3532.51 (515.56–5486.09); $P < 0.05$	378.38 (5.78–1858.06)
CCL23	570.75 (23.60–1450.51)	763.31 (46.20–1901.47)	252.75 (66.61–731.94)
CCL24	236.20 (156.50–328.80)	296.40 (178.30–301.69)	294.00 (166.00–315.00)
CCL25	1094.47 (60.90–2606.17)	351.70 (95.91–1426.65); $P < 0.05$	501.52 (29.40–2019.70)
CCL26	36.37 (26.20–78.67)	46.37 (29.30–66.29)	27.20 (21.27–78.40)
CCL27	96.67 (84.50–126.30)	113.34 (82.50–146.20)	85.60 (76.45–124.30)
CXCL1	877.52 (106.00–1495.04); $P < 0.05$	813.00 (340.69–2495.34); $P < 0.05$	327.00 (216.83–694.48)
CXCL2	924.71 (34.76–6357.27); $P < 0.05$	1506.60 (149.96–4479.89); $P < 0.05$	26.52 (5.36–55.10)
CXCL5	548.17 (126.00–2913.34); $P < 0.05$	758.31 (154.00–3379.31); $P < 0.05$	167.00 (123.00–209.00)
CXCL6	151.67 (34.00–479.22); $P < 0.05$	134.32 (64.54–711.35); $P < 0.05$	34.00 (34.00–108.23)
CXCL9	973.81 (58.21–2921.36)	876.86 (148.47–5175.09)	313.88 (114.60–1303.63)
CXCL10	568.92 (85.11–2082.65); $P < 0.05$	686.86 (89.18–3958.80); $P < 0.05$	87.00 (50.01–99.84)
CXCL11	135.81 (6.18–457.00); $P < 0.05$	126.97 (19.55–949.43); $P = 0.000004$	3.70 (1.78–12.26)
CXCL12	4423.42 (163.20–7225.97); $P < 0.05$	3659.66 (875.50–6827.47); $P < 0.05$	107.70 (89.40–1365.15)
CXCL16	1103.74 (9.53–2096.30); $P < 0.05$	1291.65 (128.47–1931.20); $P < 0.05$	171.11 (8.88–442.83)
CXCL13	86.49 (10.00–218.93)	97.56 (7.02–177.21)	11.05 (3.79–208.33)
CX3CL1	120.30 (67.10–176.50)	127.90 (66.80–150.30)	100.70 (68.30–167.20)
GM-CSF	8.30 (2.80–456.83)	33.59 (12.88–230.11); $P < 0.05$, $^{*}P < 0.05$	13.3 (10.20–15.30)
INF-γ	145.72 (15.26–243.31)	132.90 (36.60–245.16)	83.00 (40.25–164.07)
MIF	375.98 (78.00–46690.84); $P < 0.05$	6641.92 (525.81–89114.28); $P < 0.05$, $^{*}P < 0.05$	23.67 (9.28–207.56)
TNF-α	74.57 (2.56–131.86); $P < 0.05$	62.12 (24.66–104.25); $P < 0.05$	10.23 (3.45–36.97)

P—significance between gastroduodenitis group and healthy control, Steel-Dwass test.

*P—significance gastroduodenitis groups, Steel-Dwass test.

CXCL6 is a neutrophil chemoattracting factor produced by endothelial cells and macrophages exposed to IL1β or LPS (Wuyts et al., 2003). Additionally, transcriptional activation of both CXCL6 and CXCL5 has been demonstrated in cells stimulated with IL17 (Ruddy et al., 2004; Numasaki et al., 2005). Here, we show upregulation of IL17 in *H. pylori* positive juveniles suggesting that IL17 activation of epithelial cells and local macrophages causes upregulation of CXCL5 and CXCL6, which in turn promotes neutrophil chemotaxis into gastric tissue. It should be noted that CXCL6 synergy with MCP1 facilitates neutrophil chemotaxis (Gijsbers et al., 2005). Therefore, neutrophil chemotaxis may result from a combined action of several of cytokines.

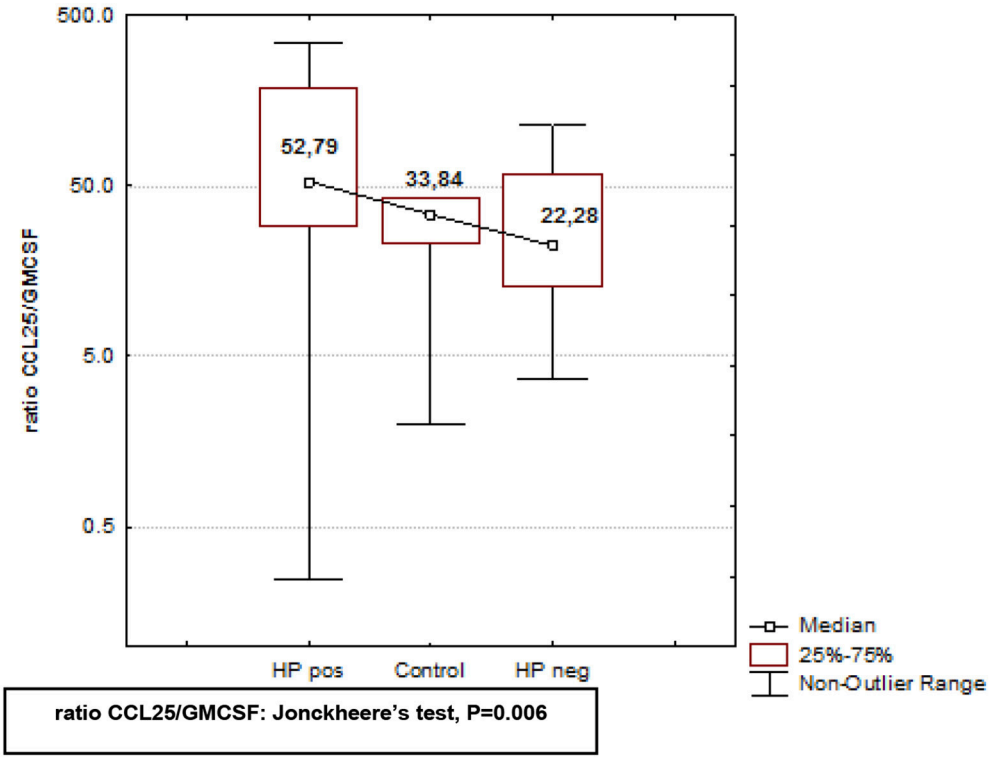

FIGURE 3 | Analysis of CCL25/GMCSF ratio in *H. pylori* positive and negative subjects, and healthy controls. CCL25/GMCSF ratio in serum of *H. pylori* positive, *H. pylori* negative, and healthy controls was analyzed using using Kruskal–Wallis ANOVA by Ranks test, followed by the *post-hoc* non-parametric Jonckheere's-test for ordered medians. CCL25/GM-CSF ratio differed significantly between *H. pylori* positive and *H. pylori* negative subjects, and healthy controls ($P = 0.006$).

The most intriguing observation made in this study was that GM-CSF and CCL25 may play a role in the pathogenesis of gastroduodenitis, and that changes in serum levels of GM-CSF and CCL25 may reflect the host's reaction to disease. Analysis of the CCL25/GM-CSF ratio indicates that *H. pylori* positive and negative gastroduodenitis are unrelated clinical entities. Our data support the observation made by Samson et al. suggesting a role for GM-CSF and CCL25 in the pathogenesis of inflammatory gastrointestinal disease (Samson et al., 2011). These authors demonstrated that CD patients with high serum level of GM-CSF neutralizing antibodies had increased number of iliac epithelial cells expressing CCL25. Local upregulation of CCL25 has been shown to facilitate iliac inflammation by stimulating CCR9-driven T lymphocyte migration. Our data provide further evidence for a role of both CCL25 and GM-CSF in the pathogenesis of inflammatory gastrointestinal diseases. Although our analysis revealed no correlation between the CCL25/GM-CSF ratio, histological presentation, and localization of gastrointestinal inflammation, we believe further study should be conducted to fully understand the function of CCL5 and GM-CSF in the pathogenesis of inflammatory gastrointestinal disease.

We have demonstrated upregulation of serum CXCL5 and CXCL6 in subjects diagnosed with gastroduodenitis, whereas serum levels of the neutrophil attractant, IL8, did not differ from controls. Therefore, we suggest that neutrophil accumulation in tissue in children with gastroduodenitis is directed by a distinct set of chemokines including CXCL5 and CXCL6. Additionally, we present the first evidence for a potential role for CCL25 and GM-CSF in the pathogenesis of gastroduodenitis, and that the CCL25/GM-CSF ratio can be utilized to for the discrimination of gastroduodenitis caused by *H. pylori* from gastroduodenitis due to other causes. Further study, using animal model for *H. pylori* gastroduodenitis is needed to determine the roles of CCL25 and GM-CSF in the pathogenesis of gastroduodenitis.

AUTHOR CONTRIBUTIONS

SK, AR, VL, EM—cytokine profile analysis, SA, AK, DS, RF—clinical examination of patients, FK—morphology, YD and GC—biostatistics.

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An *in silico* approach reveals associations between genetic and epigenetic factors within regulatory elements in B cells from primary Sjögren's syndrome patients

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Recent advances in genetics have highlighted several regions and candidate genes associated with primary Sjögren's syndrome (SS), a systemic autoimmune epithelitis that combines exocrine gland dysfunctions, and focal lymphocytic infiltrations. In addition to genetic factors, it is now clear that epigenetic deregulations are present during SS and restricted to specific cell type subsets, such as lymphocytes and salivary gland epithelial cells. In this study, 72 single nucleotide polymorphisms (SNPs) associated with 43 SS gene risk factors were selected from publicly available and peer reviewed literature for further *in silico* analysis. SS risk variant location was tested revealing a broad distribution in coding sequences (5.6%), intronic sequences (55.6%), upstream/downstream genic regions (30.5%), and intergenic regions (8.3%). Moreover, a significant enrichment of regulatory motifs (promoter, enhancer, insulator, DNAse peak, and expression quantitative trait loci) characterizes SS risk variants (94.4%). Next, screening SNPs in high linkage disequilibrium ($r^2 \geq 0.8$ in Caucasians) revealed 645 new variants including 5 SNPs with missense mutations, and indicated an enrichment of transcriptionally active motifs according to the cell type (B cells > monocytes > T cells ≫ A549). Finally, we looked at SS risk variants for histone markers in B cells (GM12878), monocytes ($CD14^+$) and epithelial cells (A548). Active histone markers were associated with SS risk variants at both promoters and enhancers in B cells, and within enhancers in monocytes. In conclusion and based on the obtained *in silico* results that need further confirmation, associations were observed between SS genetic risk factors and epigenetic factors and these associations predominate in B cells, such as those observed at the FAM167A–BLK locus.

Keywords: Sjögren's syndrome, genetics, epigenetics, polymorphism, *in silico*, histone modifications, B cells

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Introduction

Primary Sjögren's syndrome (SS) is a systemic autoimmune epithelitis affecting exocrine glands, such as salivary and lacrimal glands (1). The clinical manifestations of SS include dry mouth (xerostomia), dry eyes (keratoconjunctivitis sicca), systemic features, and patients have a 20- to 40-fold increased risk of developing lymphoma (2–4). Histological examination shows focal and peri-epithelial T and B cell infiltration plus macrophages in exocrine glands and parenchymal organs, such as kidney, lung, and liver (5). SS is characterized by the presence of circulating autoantibodies (Ab) against the sicca syndrome (SS)A/Ro and SSB/La ribonucleoprotein particles (6).

It is estimated that there are over 120 million single nucleotide polymorphisms (SNPs) in the human genome (NCBI dbSNP database, Build 143) and, among them, hundreds are disease risk variants for autoimmune diseases (AID) with the particularity that they are for the vast majority excluded from protein-coding regions (exon) and present within regulatory areas (7, 8). Regulatory SNPs control genes through an effect on (i) the transcriptional machinery when present within a gene regulatory region [promoter, enhancer, insulator (a gene regulatory element that blocks interaction between enhancers and promoters), and expression quantitative trait loci (eQTL)], (ii) the spliceosomal complex formation that controls intron excision, (iii) the activation of mRNA non-sense-mediated decay (NMD), and (iv) the control of messenger RNA stability through microRNA (3′-UTR). In SS, the list of genetic variations is growing with the particularity that the odds ratio (OR) is usually modest ($OR < 1.5$) with the exception of the HLA genes that have a significant OR (usually >2) (9). The associated risk genes analysis supports immunopathological pathways in SS, such as antigen presentation, cytokine signaling, and the NF-κB pathway (10). The characterization of regulatory SNPs in SS remains to be established.

In SS, several arguments support a role for epigenetic deregulation in disease initiation and progression (11, 12). The first clue was that two drugs, procainamide and hydralazine, induced SS in humans by blocking DNA methylation (13). Moreover, defects in DNA methylation characterize T cells, B cells, and salivary gland epithelial cells from SS patients (14–16), and such defects were associated with the expression of genes usually repressed by DNA methylation, such as transposons and miRNAs in salivary glands from SS patients (17, 18). Last, but not least, histone epigenetic markers and ribonucleoprotein post-translational modifications are immunogenic leading to autoAb production (14).

Accordingly, the aim of this work was to test the association between genetic and epigenetic determinants in SS. In the following, we pursue a two-staged analysis. First, we characterized a large panel of SS risk variants to reveal that they are predominantly present within regulatory elements. Second, we further explored the striking associations of those regulatory elements with cellular specificity and particularly in immune cells.

Materials and Methods

SS Genetic Risk Factors

Data mining based on peer reviewed literature information (PubMed) and publicly available databases (centralgwas.org) served in the compilation of a list of 43 gene risk factors and their reported variants ($n = 72$) in SS (Table S1 in Supplementary Material) (19–49). The number of SS patients and controls were also reported as well as the OR average (95%), when available. The gene list used in this study was manually updated further to include gene function, SNP number (dbSNP database), and genomic location according to the human genome reference GRCh38. Genetic variants and their observed associations with clinical and functional phenotype were submitted to The National Center for Biotechnology Information (NCBI) ClinVar database[1]. The gene list was tested with the FatiGO web interface AmiGO2[2] for functional enrichment.

Functional/Regulatory Genome Annotation Data

The variant effect predictor (VEP) tool[3] was used to determine the location of the variants (exon, intron, 5′/3′-UTR, Up/Downstream genic sequence, and intergenic section) and their consequences [missense, non-coding transcript, splice donor variant, and target of non-sense-mediated mRNA decay (NMD)].

Assessment of the SNPs functional relevance was further completed by requesting Regulome DB V1.1[4], and HaploRegV2[5], data bases (50, 51) for promoter [RNA polymerase II (Pol II) binding], enhancer (H3K27Ac, H3K36me3, and/or H3K27me3 binding in the absence of Pol II binding), insulator [CCCCTC-binding factor (CTCF)], transcription factor (TF) binding, DNase peak, and eQTL.

Linkage Disequilibrium

Following SNP selection, the HaploRegV2 web portal was used to identify SNPs in linkage disequilibrium (LD, $R^2 \geq 0.80$) in Europeans from the 1000 genome project using a maximum distance between variants of 200 kb in order to cover the enhancer elements (51).

Statistical Analysis

Pearson's Chi-squared test with Yate's continuity correction, when appropriate, was used to evaluate the significance of differences between the regulatory motifs and the histone chromatin immunoprecipitation (ChIP) experiments. A probability (P) of <0.05 was considered significant.

Results

Autoimmune-Related Genes Associated with SS

A list of 43 SS-associated gene risk factors corresponding to 72 SNPs, referred to as SS risk variants, was extracted from the scientific literature (**Figure 1**). Among the risk factors, half (36/72) were associated with another AID (systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis, inflammatory bowel disease, autoimmune thyroiditis disease, insulin-dependent diabetes, primary biliary cirrhosis, autoimmune hepatitis), allergy, infections, and cancer, including B/T cell lymphomas. This partial

[1] http://www.ncbi.nlm.nih.gov/clinvar
[2] http://amigo2.geneontology.org
[3] http://www.ensembl.org
[4] http://regulome.stanford.edu
[5] http://www.broadinstitute.org

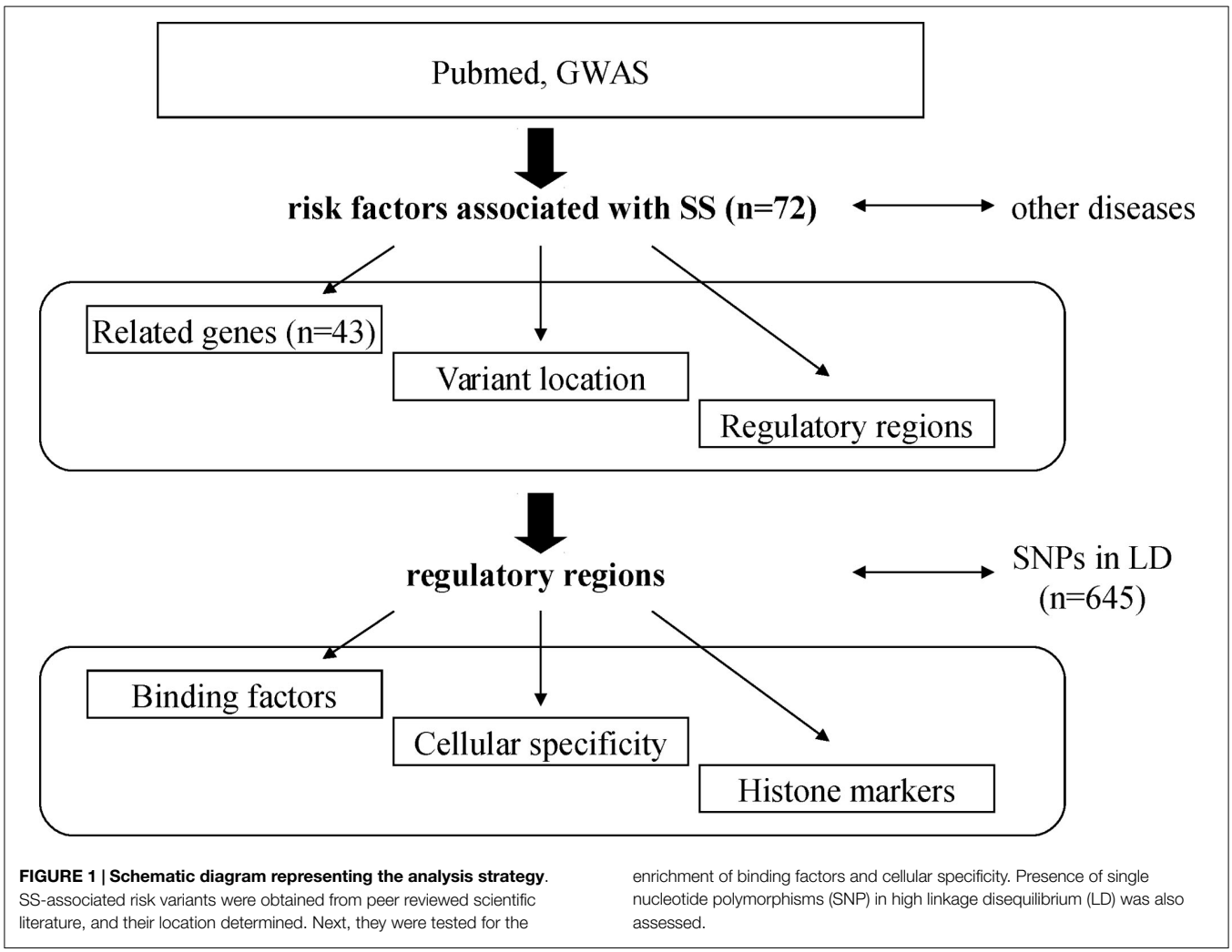

FIGURE 1 | Schematic diagram representing the analysis strategy. SS-associated risk variants were obtained from peer reviewed scientific literature, and their location determined. Next, they were tested for the enrichment of binding factors and cellular specificity. Presence of single nucleotide polymorphisms (SNP) in high linkage disequilibrium (LD) was also assessed.

overlap suggests that both common and distinct genetic traits are present in SS and equally distributed.

Variant Location

Next, we used the VEP predictor tool in order to test the location of the different variants (**Figure 2**). Applied to the 72 SS risk variants, the VEP tools identified 4/72 (5.6%) exonic variants with missense mutations (IL17F, rs763780; MBL2, rs1800450; PTPN22, rs2476601; and TNFA1P3, rs2230926), and 40/72 (55.6%) intronic variants including an alternative splice donor variant (IRF5, rs2004640) and 4 variants that were predicted as targets of non-sense-mediated mRNA decay (NMD: ICA1, rs17143355; SLC25A40, rs10276819; STAT1, rs13005843; and TNIP1, rs6579837). Moreover, two 5′-UTR variants (CD14, rs2569190; and NCR3, rs11575837), one 3′-UTR variant (IL10, rs3024498), 12/72 (16.7%) upstream genic variants, 7/72 (9.7%) downstream genic variants, and 6/72 (8.3%) intergenic variants (>10 kb) were also observed.

Regulatory Regions and DNA Binding Molecules

We then used a combination of three tools based on information from the ENCODE program (VEP) and from both the ENCODE and the Roadmap Epigenome programs (RegulomDB and HaploReg v2) to determine whether SS risk variants are likely to be within promoters, enhancers, or insulators. These regulatory motifs were defined according to the available ChIP results from multi-cell analysis showing 21/72 (29.2%) promoters, 41/72 (56.9%) enhancers, and 5/72 (6.9%) insulators. Of particular note, within the four SNPs with missense mutations, one promoter and two insulators were detected (**Figure 3**). Moreover, 34/72 (47.2%) DNase hypersensitive regions (DNase peak) and 12/72 (16.7%) eQTL were recovered. Looking specifically at promoters and enhancers, data from ChIP experiments revealed that NF-κB ($n = 5$), STATs ($n = 3$), and EGR-1 ($n = 3$) were predominant in promoters, and NF-κB ($n = 3$) in enhancers. For the remaining 5/72 (6.9%) SNPs, no regulatory functions were assigned which is significantly lower than the expected rate of 56.2% ($P < 10^{-6}$) (50).

Genes in High Linkage Disequilibrium

In order to improve the analysis, we used the HaploReg v2 tool to include 645 new SNPs that were identified to be in high LD with the 72 annotated SNPs (**Table 1**). This tool identifies 34 new genes, including one microRNA (Mir4752), five SNPs with

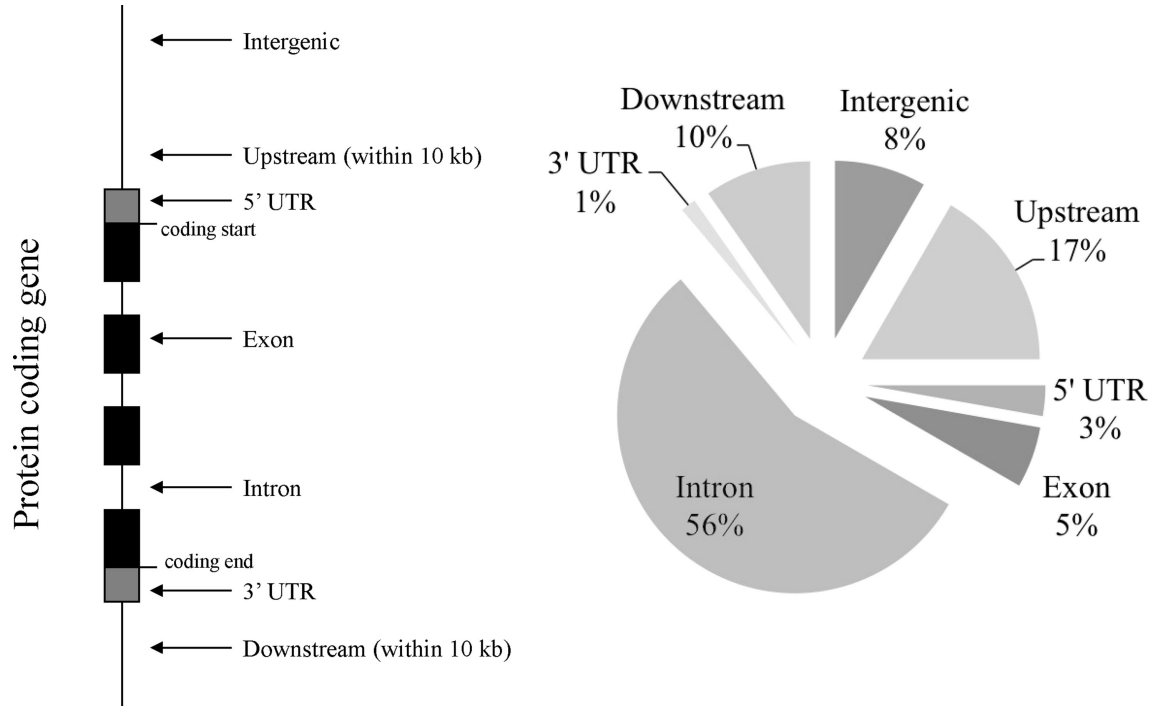

FIGURE 2 | Occurrences of SS risk variants according to the protein-coding gene location.

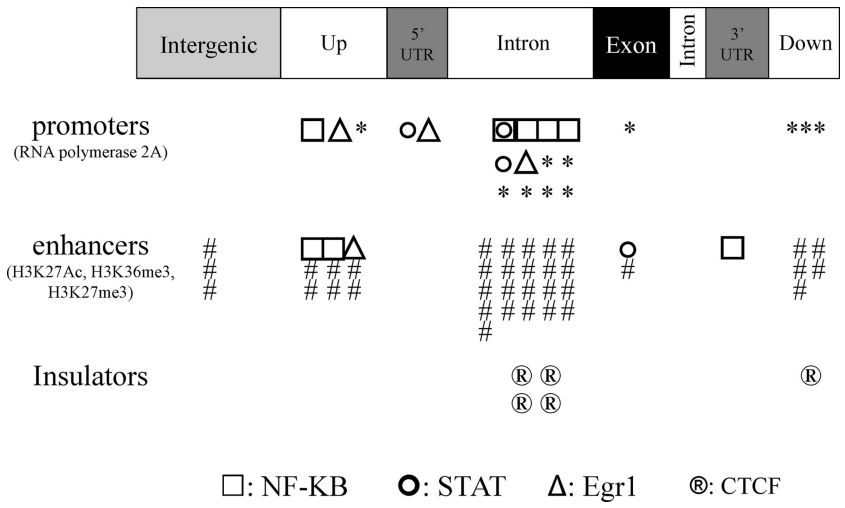

FIGURE 3 | Regulatory SS risk variants were subdivided into three groups: promoters (polymerase II binding), enhancers (H3k27Ac, H3k36me3, and/or H3k7me3 binding), and insulators (CTCF binding). Regulatory SS risk variants were further located according to the protein-coding gene, and the predominant transcription factor associated with each SS risk variant is indicated (NF-κB, STATs, and Egr1).

missense mutations (rs1041981 in LTA; rs78957773 in MCCD1, rs2230539 in PKN1, and rs52817781 plus rs2233290 in TNIP1), and indicated an overall 15.8-fold enrichment of enhancers in human embryonic cells (H1; $P = 0.0001$) and 4.9-fold enrichment of enhancers in the GM12878 EBV-transformed lymphoblastoid B cell line ($P = 0.0014$). A significant enrichment of transcriptionally active sites, identified by DNAse enrichment analysis, was observed in the SNP set with a 21-fold increase in GM12878 lymphoblastoid B cells ($P < 10^{-6}$), 10.8-fold increase in $CD14^+$ monocytes ($P = 0.00002$), 9.2-fold increase in $CD20^+$ B cells ($P = 0.001$), 8.2- to 8.5-fold increase in T cells ($P < 0.005$), and 5.7-fold increase in the A549 epithelial cell line ($P = 0.005$). Moreover, gene ontology biological process analysis (AmiGO2) identified "regulation of immune response" ($P = 2.16 \times 10^{-18}$ and 9.2×10^{-17}), "positive regulation of cytokine production" ($P = 1.56 \times 10^{-11}$ and 1.22×10^{-13}), "response to molecule of

TABLE 1 | Gene risk factors associated with primary Sjögren's syndrome (SS).

SS gene risk factors extracted from the literature
BAK1, BCL2, BLK, C4A, CCL2, CD14, CD40, CD40LG, CHRM3, CXCR5, EBF1, FAM167A, GTF2I, HLA DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA, ICA1, IKBKE, IL10, IL12A, **IL17F**, IL21, IRF5, LILRA3, **LTA***, **MBL2**, NCR3, NFKB1, OR2B11, **PKN1***, **PTPN22,** SLC25A40, STAT1, STAT4, TNF, **TNFA1P3**, TNFSF13B, **TNIP1***, TNPO3, TNFSF4, Trim21
New genes in high linkage disequilibrium
ABCB1, AC053545.3, ADAD1, AF213884.2, APBB3, ATP6V1G2, ATP6V1G2-DDX39B, BTNL2, C6orf10, CCL11, CCL7, CTD-2049J23.2, DBF4, GGNBP1, HCG23, HLA-DPA1, IL2, KIAA1109, **MCCD1***, Metazoa_SRP, MIR4752, NDUFA2, NFKBIL1, PTGER1, RP11-10J5.1, RP11-356I2.2, RP11-356I2.4, RP3-527F8.2, RP5-998H6.2, RSBN1, RUNDC3B, SLC25A40, snoU13, XXbac-BPG254F23.7

Bold missense mutations.
**Missense mutations detected by linkage disequilibrium.*

bacterial origin" ($P = 4.9 \times 10^{-7}$ and 6.2×10^{-9}), and "cellular response to interferon" ($P = 1.82 \times 10^{-7}$ and 1.86×10^{-7}) as the most significant functions associated with the SS gene risk factors tested alone or associated with the genes revealed by the LD analysis, respectively.

Cell Type-Specific Analysis Revealed Activated Enhancer and Promoter Histone Markers at SS Risk Variants in B Cells

To further explore cell type specific activation in promoters and enhancers at SS risk variants and according to the critical role played in the disease by epithelial cells, lymphocytes, and macrophages, we selected from the 18 ENCODE available cells: the human lung adenocarcinoma cell line A549 for epithelial cells, the GM12878 lymphoblastoid cells for B cells, and the peripheral blood $CD14^+$ monocytes for macrophages. For these three cell types, we mapped SS risk variants to markers of active promoters (H3K4me2, H3K4me3, and H3K9Ac), and to markers of active enhancers (H3K36me3 and H3K4me1) (52). In addition, H3K27Ac was selected as a marker of activity, and H3K27me3 as an inactive marker of enhancers.

As shown in **Figure 4A** and with regards to the 21 promoter SS risk variants, the three active promoter markers (H3K4me2, H3K4me3, and H3K9Ac) were significantly enriched in B cells (GM12878) in contrast to the epithelial cells (A549) and monocytes ($0.01 < P < 0.0006$, Chi square with Yate's correction). The active marker H3K27Ac was enriched in B cells and monocytes in contrast to epithelial cells ($P = 0.0001$ and $P = 0.02$, respectively).

The same analysis was performed with the 41 enhancer SS risk variants (**Figure 4B**) revealing an enrichment of the enhancer active marker H3K36me3 in both B cells and monocytes in contrast to A459 cells ($P = 0.02$ and $P = 0.005$, respectively). The active marker H3K27Ac was enriched in B cells ($P = 0.0001$), and, although not significant, there is a trend for a monocyte enrichment in contrast to epithelial cells. In summary, these findings highlight the critical role of epigenetic factors in B cells to control both promoter and enhancer SS risk variants, and in monocytes to control enhancer SS risk variants.

FAM167A–BLK Locus

In order to validate our observations, and based on three reports, including the genome wide association study (GWAS) performed by Lessard et al., in 395 patients with SS and 1975 controls from European origins (31, 35, 41), the FAM167A–BLK locus (Chr 8:11421463-11564604) was selected to position the 8 FAM167A–BLK SS risk variants plus two 5′-UTR variants selected from the LD analysis and previously identified as lupus risk variants (53). These two SNPs are in high LD with 4/8 SS risk variants [rs922483 is in high LD with rs2736340 ($r^2 = 0.81$), rs13277113 ($r^2 = 0.83$), and rs2736345 ($r^2 = 0.96$); and rs2250788 is in high LD with rs2254546 ($r^2 = 0.98$)]. As shown in **Figure 4C**, the 10 selected SNPs were positioned in the FAM167A–BLK locus revealing three groups. The first group contains an isolated SNP (rs12549796) that was present in an intronic part of the FAM167A gene. A second group ($n = 7$) was present in the vicinity of the BLK promoter and exon 1, and a third group ($n = 2$) was present ~35 kb downstream BLK promoter.

Next, as revealed by querying the Ensembl database using H3K27Ac to mark active promoters and enhancers, SNPs were positioned within 9/10 H3K27Ac active motifs in B cells (GM12878), which is in contrast to 2/10 H3K27Ac active motifs in monocytes, and none in epithelial cells. Such associations between genetic and epigenetic factors within regulatory elements in B cells for the FAM167A–BLK locus were further reinforced by using the RegulomeDB tool that summarizes results from the ENCODE and Epigenetic Roadmap programs. As indicated **Table 2**, the RegulomeDB tool supports that SS risk factors at FAM167A–BLK locus would predominantly affect B cells (lymphoblastoid and naive B cells) and, to a lesser extent, monocytes, T cells (naïve, TH2, and Treg), mesenchymal stem cells, and fibroblasts.

Discussion

Primary SS is an autoimmune disease with a genetic basis in which at least 40 gene risk factors may be involved, including BLK, IRF5, STAT4, and the HLA locus. However, these genetic risk factors alone cannot explain all of the disease risk factors and, in particular, environmental risk factors (e.g., viruses, hormones . . .) that are likely to play a critical role in the process of the disease. Given the complexity of the disease, epigenetic analyses are conducted to provide new insights into the disease as DNA methylation patterns, chromatin structures, and microRNA are influenced both by the genetic machinery and by environmental factors (13, 54, 55). The primary role of the epigenome is to regulate, in a cell-specific manner, cellular development, and differentiation and such effects vary between individuals with age as revealed by testing identical twins (56), or between smokers and non-smokers (57). Furthermore, genetic variants and, in particular, non-coding and regulatory SNPs can influence cell type specific regions marked by accessible regions, thus opening new perspectives to better characterize disease risk factors and cell types contributing to the diseases which was the aim of the present *in silico* analysis.

Applied to SS, such strategy was fruitful in suggesting the existence of associations between genetic and epigenetic alterations in the setting of the disease. Indeed, a cell-specific overlap

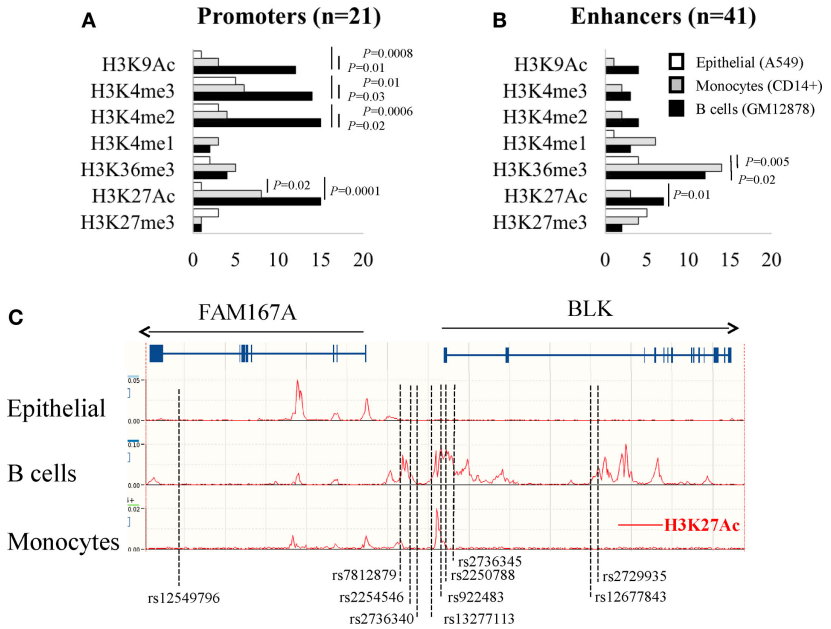

FIGURE 4 | Analysis of histone modifications in the promoters (A) and enhancers (B) of SS risk variants within A549 epithelial cells, B cell lymphobastoid GM12878 cells, and CD14$^+$ monocytes. (C) In the lymphoblastoid GM12878 B cell line, SS genetic variants and the active histone markers H3k27Ac are co-localized in and around the FAM167A–BLK locus (http://www.ensembl.org).

TABLE 2 | Summary of the cell type enrichment markers for FAM167-BLK variants according to the RegulomeDB prediction web tool.

SNP	Promoter (histone markers)	Enhancer (histone markers)	Open chromatin (DNase)	eQTL	Pol II (ChIP-Seq)
rs12549796	No	Mesenchymal stem cells	Fibroblast	No	No
rs7812879	No	B lymphoblastoid, B cells, monocytes	No	B lymphoblastoid	No
rs2254546	No	B cells	No	B lymphoblastoid	No
rs2736340	No	B cells	No	B lymphoblastoid, monocytes	No
rs13277113	B lymphoblastoid	B cells, monocytes	B lymphoblastoid, B cells	B lymphoblastoid	No
rs922483	B lymphoblastoid, B cells, T cells, monocytes	Fibroblast	B lymphoblastoid, B cells, TH2, Treg	B lymphoblastoid	B lymphoblastoid
rs2250788	B lymphoblastoid, B cells, T cells, monocytes	Fibroblast	B lymphoblastoid, B cells	No	B lymphoblastoid
rs2736345	B lymphoblastoid, B cells, T cells	Fibroblast, Mesanchymal stem cells	B lymphoblastoid, B cells, TH2	B lymphoblastoid	B lymphoblastoid
rs12677843	No	No	No	No	No
rs2729935	No	B lymphoblastoid, B cells	No	No	No

exists between identified SS risk variants and the regulatory switches found by the ENCODE program, thus suggesting that DNA–protein binding and gene transcription are affected by the SNPs. Remarkably, almost all SS risk variants tested (94.4%) had *in silico* evidence of regulatory functions including the 3/4 missense SNPs and the 37/40 intronic SNPs. In addition and according to our *in silico* observations that need further confirmation, it could be postulated that SS risk variants control DNA-protein binding leading to the regulation of cell-specific promoters (Pol II, NF-κB, STATs), enhancers (NF-κB), and insulators (CTCF). These results also suggest that there is an effect on some common pathways (NF-κB, STATs) previously described to be affected in SS (10).

The genetic and epigenetic fine mapping of autoimmune risk factors was recently performed in 21 AID with the notable exception of SS (7). In line with our observations, it was observed that autoimmune risk variants were mostly non-coding (90%) and map predominantly to H3K27Ac positive immune-cell enhancers (60%) and promoters (8%). Next, a T cell signature was observed in nearly all of the AID tested except in lupus and primary billiary cirrhosis (two AID frequently associated with SS) that present a B cell signature, and type I diabetes with pancreatic islets. Finally, it was reported that autoimmune risk factors were enriched within binding sites for immune-related TFs, such as Pu-1 and NF-κB. As a consequence, the physiopathology of AID needs to be updated according to the recent progress in epigenetics (54).

Some limitations are inherent in this type of study. First, cells used in the ENCODE program are predominantly cell lines that are different from primary cells, such as the lymphoblastoid GM12878 B cell line, that results from EBV transformation of peripheral blood mononuclear cell using phytohemagglutinin as a mitogen. New results using primary cells, which are available from the Epigenome Roadmap program further supports similarities between lymphoblastoid GM12878 B cells and purified human $CD20^+$ B cells as we observed for the FAM167A–BLK locus when using the RegulomeDB tool. Second, although the ENCODE program is an extensive resource; the program is limited to certain cell types and DNA binding elements that limit the interpretation. Third, many SNPs are in tight genetic linkage and, as a consequence, genetic risk variants may not be causal, but rather reveal the presence of a linked SNP that is functionally relevant to the pathogenesis. Such a situation may be suspected for different SNPs tested from our selection since the LD analysis has revealed new missense mutations as well as new gene risk factors that need to be tested, such as chemokines (CCL7 and CCL11), cytokines (IL2) and the miRNA4752. Two SNPs in CCL11 have been associated with germinal center-like structure formation in SS patients (47), and CCL11 (Eotaxin) circulating levels were reduced in SS patients (58).

While the function of the protein encoded by FAM167A is unknown, the tyrosine kinase BLK controls B cell development and is activated after B cell receptor engagement. The FAM167A–BLK locus is associated with several AID, such as SS, lupus, rheumatoid arthritis, scleroderma, and vasculitis. Among them, two risk alleles (rs132771113 and rs9222483) are known to control BLK transcription during B cell development (53, 59). Moreover, by integrating epigenetic fine mapping, we further observed that all BLK-associated SS risk variants, including the two previously described, were all present within epigenetic marks in B cells. Altogether, this example illustrates the value of integrating epigenetic resources for investigating the complex mechanisms by which non-coding risk variants could modulate gene expression.

Last but not least, the B cell subset identified from our *in silico* study deserves several comments. First, B cell qualitative abnormalities have been reported in SS with important perturbations in peripheral blood B cell profiling and B cell migration within exocrine glands (5, 60). Second, the association between the incidences of B cells in salivary gland epithelial cells has been addressed as well as the formation of ectopic germinal centers and transformation to B cell lymphoma (61). Third, non-HLA genetic associations in SS are predominantly related to B cell genes (BTK, CD40, EBF-1 . . .) as we observed in our selection. Fourth, a recent study reported DNA methylation changes in B cells and such changes predominate within loci containing SS risk factors (16). Altogether, these observations provide rationale for targeting B cells in SS along with the observations that depleting B cells with Rituximab or targeting BAFF with Belimumab are both effective (62, 63).

In conclusion, we have tested, as a proof of concept, a novel approach that integrates both epigenetic information and results from genomic analysis to further enhance the value of the genetic risk factors highlighted in complex diseases, such as SS. Future work needs to be done in order to confirm experimentally the cellular specificity and the functional role of the characterized regulatory SNPs. Another consequence is that such approach could be used to select and/or propose future therapeutic drugs in SS as epigenetic mechanisms are reversible.

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Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00437

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Dysbiosis May Trigger Autoimmune Diseases via Inappropriate Post-Translational Modification of Host Proteins

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The gut ecosystem with myriads of microorganisms and the high concentration of immune system cells can be considered as a separate organ on its own. The balanced interaction between the host and microbial cells has been shaped during the long co-evolutionary process. In dysbiotic conditions, however, this balance is compromised and results in abnormal interaction between the host and microbiota. It is hypothesize here that the changed spectrum of microbial enzymes involved in post-translational modification of proteins (PTMP) may contribute to the aberrant modification of host proteins thus generating autoimmune responses by the host, resulting in autoimmune diseases.

Keywords: microbiome, dysbiosis, intestine, post-translational modification, autoimmune disease

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INTRODUCTION

Intricate host–microbe symbiotic relationships in the human gut have evolved during the long-term coevolution between the two. It resulted in fine-tuned inter kingdom molecular adaptations that benefit both sides (Donia and Fischbach, 2015). In particular, the commensal microbiota benefits from the continuous food supply, exogenous and endogenous, and constant physico-chemical conditions. Advantages for the host organism include metabolic, structural, protective, and other beneficial functions exerted by the commensal microbiota. The importance of commensal microbiota for the proper development and functioning of the host immune system is also well-recognized (Paun and Danska, 2015).

Our knowledge on the topic expands continuously. The complex microbiota of our gastrointestinal tract consists of at least 1000 bacterial species, the majority of which belong to the Firmicutes and Bacteroidetes phyla (Qin et al., 2010). The microbiota composition and function is very dynamic and multiple environmental factors affect its quantity, quality and functionality. Age, mode of delivery, infant feeding practices, the use of drugs (antibiotics), diet, industrial food additives, epidemiology, climate, eco-catastrophes, migrations, and many more are the influential factors (Lerner, 2011; Chassaing et al., 2015; Lerner and Matthias, 2015a,b,c).

It is not our intention to cover all the aforementioned factors that may affect host-microbe interaction. Here, we will provide an update on the dysbiotic situations, where the normal composition of the commensal is compromised, which may result in pathologies such as autoimmune diseases. More specifically, we will be focusing on the microbial enzymatic machinery that is involved in post-translational modification of proteins (PTMP). We hypothesize that the enzymes produced by the dysbiotic microbial community may process luminal proteins differently

than that of the normal community. The abnormal PTMP may produce neo-epitopes that are autoimmunogenic and may induce systemic autoimmune responses resulting in autoimmune diseases.

THE ROLE OF INTESTINAL DYSBIOSIS IN HUMAN AUTOIMMUNE DISEASES

Presently, it is apparent that the microbiota and its products have a profound effect on the development and maintenance of the immune system. Germ-free animals, for example, have an impaired immune system that can be functionally restored after the inoculation of commensal bacteria (MacPherson et al., 2001, 2002; Mazmanian et al., 2005). The extent of dependency of the immune system on commensals may even suggest the commensalocentric view. At the same time, not all commensals are alike. The dysbiotic populations, with no identifiable pathogens, can still confer the susceptibility to immune-mediated diseases (Paun and Danska, 2015). Our focus here on autoimmune diseases and several mechanisms of the microbial involvement in the promotion of autoimmunity have been suggested (Chervonsky, 2013). The first one is the molecular mimicry, where microbial peptides are identical, or similar enough to self-peptides. Second, it could be bystander activation during infection, with the induction of costimulation and cytokine production by APCs, which, at the same time, may presents self-antigens. The third suggested mechanism is the "amplification of autoimmunity by cytokines" elicited by microbial activation of professional APCs and the innate lymphoid cells to produce proinflammatory cytokines by T cells. And the fourth suggestion is the involvement of the whole human intestinal system, including dysbiotic community, exogenous enzymes produced by the dysbiotic populations, and the corresponding PTMP activity that generates neo-epitopes.

The multiple animal models of human autoimmune diseases (AD) suggest the direct involvement of commensal microbiota in disease development. Under the germ-free conditions no disease is developing in the animal models of IBD, rheumatoid arthritis and multiple sclerosis, supporting the notion of "no bugs, no disease," while in some others they are only attenuated (Wu and Wu, 2012). In some models of the human ADs, causality is strengthened by the reintroduction of specific microorganisms restoring the disease severity.

Some members of the gut microbiota have been linked to ADs. Changing a single bacterial species and/or the entire commensal community can alter the outcome of a specific AD due to the imbalance of pathological/protective immune responses (Wu and Wu, 2012). The summary of specific microbial species in relation to defined animal models of ADs and their functions, in relation to disease progression, is shown in **Table 1**.

ENZYMES FROM DYSBIOTIC POPULATIONS CAPABLE OF PTMP

Endogenous and microbial enzymes have the capacity of intestinal enzymatic neo-antigen generation by PTMP. The corresponding modifications taking place in the intestine include peptides crosslinking, de/amination/deamidation, de/phosphorylation, a/deacetylation, de/tyrosination, de/glutamylation, de/glycylation, ubiquitination, palmitoylation, glycosylation, galactosylation, arginylation, methylation, citrullination, sumoylation, and carbamylation. For example, human endogenous intestinal enzymes and microbial transglutaminases (tTg, mTg) induce multiple neo-epitopes on the Tg-peptide docked complex or citrullination by peptidylarginine deiminase resulting in the formation of autoantibodies in celiac disease and rheumatoid arthritis, respectively. A similar phenomenon is observed in other ADs. Bacterial species, their corresponding enzymes capable of PTM of host proteins and potential involvement in disease are listed in **Table 2**.

THE ROLE OF POST-TRANSLATIONAL MODIFICATION IN AUTOIMMUNITY INDUCTION

Bacteria possess an amazing capacity for adaptation and survival strategies, including differential expression of transcriptome and proteome, variations in growth physiology, and in developmental behavior. PTMP contribute substantially to this adaptability and bacterial cell cycle regulation (Grageasse et al., 2015). On the other hand, the microbial PTMP has a paramount significance to the host. Their enzymatic apparatus is capable to transform naïve/self or non-self-peptides to autoimmunogenic ones. Several examples are worth mentioning.

A well-described PTMP is the citrullination, where an arginine residue in a protein is converted to a citrulline residue. The host enzyme is activated during apoptosis, autophagy, and NETosis processes, which are well-known as being implicated in autoimmunity (Valesini et al., 2015). In fact, some anti-citrullinated protein antibodies may serve as good diagnostic markers for autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Felty's syndrome (Muller and Radic, 2015). The citrullinated proteins have also been detected in polymyositis and IBD. Some bacteria such as *Porphyromonas gingivalis* possess the corresponding enzymes, peptidylarginine deiminases, which may citrullinate human proteins (Wegner et al., 2010). The authors suggested that *P. gingivalis*-mediated citrullination of bacterial and host proteins may lead to the generation of antigens driving the autoimmune response in RA. Experimental verification of the specificity and activity of peptidylarginine deiminase from *P. gingivalis* revealed that it is primarily cell surface associated, heat stable, and display the optimal activity under alkaline conditions characteristic for the inflamed environment (Abdullah et al., 2013). Moreover, the enzyme has very broad substrate specificity and modifies arginine residues in all positions of all proteins tested. Thus, its presence in inflamed tissue may foster autoimmune reactions by altering host epitopes. In experimental models of periodontal disease and arthritis it has been found that peptidylarginine deiminase from *P. gingivalis* is a key contributor to the pathogenesis of these diseases (Gully et al.,

TABLE 1 | Autoimmune disease and associated dysbiotic characteristics*.

Autoimmune disease	Dybiosic characteristics	Microbial species	Action
Inflammatory bowel disease	Reduction of *Firmicutes*+*Bacteroides*, overgrowth of *Proteobacteria*	*Proteus mirabilis, Klebsiella pneumoniae* *Segmented filamentous bacteria* *B. fragilis* *B. thetaiotaomicron*	Induce Induce Attenuate Attenuate
Rheumatoid arthritis	Increased diversity, Increase in *Porphyromonas, Prevotella*, *Leptotricha,* and *Lactobacillus* species	*Porphyromonas gingivalis, Prevotella nigrescens,* *Segmented filamentous bacteria* *Lactobacillus bifidus,* *Clostridium*	Induce Induce Induce Induce
Type 1 diabetes	Decreased diversity, Increased *Bacteroidetes,* Decreased *Bifidobacteria,* and butyrate-producing bacteria	*Bacteroides* *Segmented filamentous bacteria*	Attenuate Attenuate
Celiac disease	Increased diversity	*Lachnoanaerobaculum, Prevotella, Actinomycetes* *Lachnoanaerobaculum umeaense*	Induce Induce
Multiple sclerosis	Decreased *Clostridia* clusters XIVa and IV and *Bacteroidetes*	*Segmented filamentous bacteria* *Bacteroides fragilis, Lactobacillus paracasei, Lactobacillus plantarum*	Induce Attenuate

**Adapted from references (Wu and Wu, 2012; Lerner and Matthias, 2015b; Paun and Danska, 2015).*

TABLE 2 | Bacterial enzymes capable of post-translational modification of host proteins and potential involvement in disease.

Bacterium	PTMP	Relation to disease
Porphyromonas gingivalis	Peptidylarginine deiminase	RA, SLE, Felty's syndrome, periodontal disease (Muller and Radic, 2015)
Bacteroides fragilis	Ubiquitination	Inflammatory bowel and autoimmune diseases (Patrick et al., 2011)
Shigella flexneri	Demyristoylation	Affects cellular growth, signal transduction, autophagasome maturation, and organelle function (Burnaevskiy et al., 2013)
Shigella flexneri	Deamidation	Inhibits acute inflammatory responses (Sanada et al., 2012)
Shigella flexneri	Protein kinase	Prevents phospho-IκBα degradation and NF-κB activation to establish infection (Kim et al., 2005)
Gram-negative bacteria	Kinases, phosphatases, phospholyases, and serine/threonine acetylases	Host phosphoproteome modulation to establish infection (Grishin et al., 2015)
Escherichia coli, Salmonella, Shigella, Chlamydia, and Legionella	Ligases and deubiquitinases	Modulation of host ubiquitin pathways to establish infection (Zhou and Zhu, 2015)
Streptococcus pyogenes, Enterococcus faecalis, Listeria monocytogenes, Streptococcus pneumoniae, Pseudomonas aeruginosa, Capnocytophaga canimorsus, Treponema denticola,	Glycosidases	Alteration of host glycobiome for immunomodulation, adherence, and nutrition to establish infection (Sjögren and Collin, 2014)
Legionella, Chlamydia, Bacillus	Histone methylases	Remodeling the host epigenetic machinery (Rolando et al., 2015)
Short chain fatty acids-producing commensals	Inhibition of histone deacetylases	Anti-proliferative and anti-inflammatory effects (Schilderink et al., 2013)
Bacteria at mucosal surfaces	Proteases	Generation of host damage-associated molecular patterns (Sofat et al., 2015)

2014). In human subjects, there is a strong association between the presence of *P. gingivalis* peptidylarginine deiminase and diseases such as RA and periodontitis (Laugisch et al., 2015; Shimada et al., 2015). In both diseases, the microbial enzyme convert arginine to a citrulline residue, thus creating a neo-antigen.

More recently, an additional PTMP activity, lysine acetylation, has been detected in *P. gingivalis* in addition to its citrullination capacity (Butler et al., 2015). A unique homolog of the eukaryotic ubiquitin has been found in the predominant bacterium of the human gut, *Bacteroides fragilis* (Patrick et al., 2011). In eukaryotes post-translational regulation by ubiquitination plays an important role in regulation of intracellular proteolysis and modification of protein function. The ubiquitination process is also central for immune surveillance and response to invading pathogens. Its presence in a predominant human gut bacterium may have important implications for our understanding of AD development.

One of the factors contributing to autoimmune progression is the cellular environment that may change PTMP (Cañas et al., 2015). PTMP of histones alter the chromatin architecture thus generating "open" and "closed" states, and these structural changes can modulate gene expression under specific conditions. While methylation and acetylation are the best-characterized histone PTMP, citrullination by the protein arginine deiminases represents another important player in this process (Slade et al., 2014). Juxtaposition of citrullinated histones with infectious pathogens and complement and immune complexes may compromise the tolerance to nuclear autoantigens and promote autoimmunity (Muller and Radic, 2015). Like a double-edged sword, histones can be post-translationally modified also by Tg cross-linking, a well-described PTMP (Lerner and Matthias, 2015c). Likewise, microbial transglutaminase, a member of the Tg family, is known for its pivotal function in bacterial survival (Lerner and Matthias, 2015c). Bacterial glyosidases are involved in PTMP and many key proteins of the immune system are glycosylated (Sjögren and Collin, 2014). The glycosylation sites of IgE, IgM, IgD, IgE, IgA, and IgG are functionally important, and they are responsible for the well-documented association between alterations of the serum glycome and autoimmunity. The altered glycan theory of autoimmunity has been recently suggested (Maverakis et al., 2015). It implies that each AD has a unique glycan signature characterized by the site-specific relative abundances of individual glycan structures on immune cells and extracellular proteins. This especially concerns the site-specific glycosylation patterns of different immunoglobulin classes and subclasses (Maverakis et al., 2015).

A well-characterized example of PTMP is the tTg in celiac disease that will be discussed in the next section.

WHERE PTMP DOES TAKES PLACE IN THE GUT?

This topic still needs to be revealed in greater details, but there are some hints for at least two ADs. In celiac disease, the autoantigen is tTg, capable of deamidating, or transamidating gliadin (Reif and Lerner, 2004; Lerner et al., 2015a). This PTMP occurs below the epithelium, resulting in neo-epitopes of gliadin docked on the tTg, inducing anti-tTg, or anti neo-epitope tTg autoantibodies. These are the well-known serological markers of celiac disease (Lerner, 2014; Lerner et al., 2015b). More recently, a family member of tTg, the microbial Tg that is heavily used in the food industry, has been shown as a potent inducer of specific antibodies in celiac disease patients (Lerner and Matthias, 2015d). Interestingly, the same food additive has been suggested as a new environmental trigger and potential inducer of celiac disease (Lerner and Matthias, 2015a,c).

A number of PTMPs are relevant to IBD. These pathways include phosphorylation, neddylation hydroxylation, and cleavage of cytokine precursor forms by the inflammasome. The interplay of these rapid response mechanisms enables rapid adaptation to incoming inflammatory signals. Cytokine induced barrier breakdown allows for bacterial translocation to the basal aspect of intestinal epithelial cells. Bacterial antigens and endogenous danger signals are recognized by the adaptive and innate immune system, triggering a variety of reactions including apoptosis, increased cytokine release, loss of tight junctional proteins, and barrier breakdown (Ehrentraut and Colgan, 2012).

In rheumatoid arthritis, citrullination is a major post-translational modification of arginine, which converts naïve peptides into the immunogenic neo-epitopes. This PTMP constitutes the basis for the specific prediction of disease activity due to the production of anti-citrullinated protein antibodies (Lerner and Matthias, 2015b). It has been suggested that infectious agents that release toxins such as lipopolysaccharides at mucosal surfaces may trigger the inflammatory response with a potential to cause citrullination of various proteins such as fibronectin, fibrinogen, and collagen (Sofat et al., 2015).

Theoretically, it can be assumed that PTMP may take place in the lumen, on the intestinal, or buccal mucosal surfaces, in the interepithelial spaces, or below the epithelium. There are more questions, however, than definitive answers.

THE HYPOTHESIS

We hypothesize here that the PTM enzymes of dysbiotic gut microbiota behave like a Trojan horse. They are essential for the microbial growth and survival in the gut, but are detrimental to the human host. This enzymatic machinery is capable of PTMP, turning naïve peptides to immunogenic ones by generating, or exposing neo-epitopes, thus compromising tolerance and inducing autoimmunity. Peptides crosslinking, de/amination/deamidation, de/phosphorylation, a/deacetylation, de/tyrosination, de/glutamylation, de/glycylation, ubiquitination, palmitoylation, glycosylation, galactosylation, arginylation, methylation, citrullination, sumoylation and carbamylation are some examples for PTMP taking place in the intestine. The corresponding microbial enzymes that encounter the closely related host substrates under the optimal local conditions, act as post-translational modifiers of the host's peptides in the initiation, progression and maintenance of human systemic ADs.

SUMMARY

There are a number of factors, genetic and environmental, that have been identified as conducive to dysbiotic conditions in humans. We hypothesize here that the spectrum and activities of enzymes, which are normally involved in PTMP, become

biased in the dysbiotic microbial community. The examples are given demonstrating how these activities may affect post-translational modification of host proteins thus generating new aberrant epitopes. These epitopes may generate host autoimmune responses and trigger autoimmune diseases. Much less is known, however, how to convert the "pathobiota" back to the "normobiota" to restore the balanced host-microbe interaction with a normal PTMP pattern. Answering this question will be the basis for the development of efficient therapeutic strategies to prevent autoimmune diseases.

AUTHOR CONTRIBUTIONS

AL and RA designed, performed literature search and wrote the manuscript; TM designed, constructed table, edited and wrote the manuscript.

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REVIEW
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Update on Pyrin Functions and Mechanisms of Familial Mediterranean Fever

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Mutations in the *MEFV* gene, which encodes the protein named pyrin (also called marenostrin or TRIM20), are associated with the autoinflammatory disease familial Mediterranean fever (FMF). Recent genetic and immunologic studies uncovered novel functions of pyrin and raised several new questions in relation to FMF pathogenesis. The disease is clinically heterogeneous reflecting the complexity and multiplicity of pyrin functions. The main functions uncovered so far include its involvement in innate immune response such as the inflammasome assemblage and, as a part of the inflammasome, sensing intracellular danger signals, activation of mediators of inflammation, and resolution of inflammation by the autophagy of regulators of innate immunity. Based on these functions, the FMF-associated versions of pyrin confer a heightened sensitivity to a variety of intracellular danger signals and postpone the resolution of innate immune responses. It remains to be demonstrated, however, what kind of selective advantage the heterozygous carriage conferred in the past to be positively selected and maintained in populations from the Mediterranean basin.

Keywords: familial Mediterranean fever, autoinflammation, pyrin, innate immunity, cytoskeleton

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INTRODUCTION

Autoinflammatory diseases are a group of genetically determined multisystem disorders caused primarily by the dysfunctions in innate immunity. These rare disorders are characterized by recurrent episodes of generalized inflammation and fever in the absence of infectious or autoimmune causes (McDermott et al., 1999; Brydges and Kastner, 2006). Familial Mediterranean fever (FMF; MIM 294100) is one of the most common and best characterized hereditary autoinflammatory syndromes (Ting et al., 2006; Masters et al., 2009). FMF is an ethnically restricted disease, which predominantly affects people of Mediterranean descent, mainly Armenians, Turks, Arabs and Jews. The carrier rate in these populations is estimated to be as high as 1:5 to 1:7 (Daniels et al., 1995; Gershoni-Baruch et al., 2001; Yilmaz et al., 2001; Sarkisian et al., 2008). Other Mediterranean populations are less affected by the disease, although the number of cases is still substantial (Touitou, 2001; La Regina et al., 2003).

GENETICS OF FMF

Familial Mediterranean fever has long being considered as an autosomal recessive disease caused by mutations in the *MEFV* gene, which is composed of 10 exons and encodes a 781 amino

acids protein called pyrin or marenostrin or TRIM20 (The French FMF Consortium, 1997; The International FMF Consortium, 1997). According to the INFEVERS database, more than 60 FMF-associated mutations have been identified so far, with the majority of them being extremely rare mutations (http://fmf.igh.cnrs.fr/infevers/). The FMF-associated mutations are predominantly located within exon 10 of the gene, and they primarily result in amino acid substitutions. The phenotypic variability of the disease is thought to be partially associated with particular mutations and allelic heterogeneity (Dewalle et al., 1998; Shohat et al., 1999). The most frequent mutations causing FMF are the five missense mutations: in exon 10 these are M694V, M694I, V726A, and M680I and in exon 2 – E148Q (Touitou, 2001). Among these five mutations, M694V is associated with a more severe form of the disease (Shinar et al., 2000), and E148Q mutation – with a milder form of the disease (Touitou, 2001).

Despite being long considered as an autosomal recessive disorder, there are some cases with the involvement of only one *MEFV* heterozygous mutation and even without any apparent *MEFV* mutation (Booty et al., 2009; Marek-Yagel et al., 2009; Ben-Zvi et al., 2015). Although the genetic defect causing the disease is well-known, a broad spectrum of *MEFV*-associated phenotypes suggest additional genes or immune factors, which may modulate the innate immune response in FMF. A growing number of studies confirm this hypothesis. It has been suggested that major histocompatibility complex class (HLA) I chain-related gene A has a modifier effect on the disease phenotype (Turkcapar et al., 2007). More recently, differential effects of HLA class I and class II alleles on FMF such as subsets of clinical forms and response to colchicines treatment have been demonstrated for the Japanese population (Yasunami et al., 2015).

The high carrier frequency in affected populations suggests the selective advantage conferred by the heterozygous state such as protection against presumptive pathogen(s). Comparative analyses of amino acid substitutions in the ret finger protein (rfp) domain of pyrin among primates and diseased people have demonstrated that some human mutations actually represent the recapitulation to the ancestral amino acid states and these exist as wild type in other species (Schaner et al., 2001). Inspection of the dN/dS ratios performed by the authors revealed the signature of episodic positive selection. Another study of population genetics of FMF involving the sequence analysis of a larger *MEFV* region, from exon 5 to the 3′-UTR, essentially supported the hypothesis of heterozygote advantage/overdominance for *MEFV* mutations (Fumagalli et al., 2009). The meta-analysis of population genetics in FMF suggests that the mutations are not uniformly distributed in various populations and differ in phenotypes, neutrality and other population genetics characteristics (Papadopoulos et al., 2008). The authors identified Jews as the candidate population for founder effects in *MEFV* mutations due to genetic isolation and genetic drift.

Positive selection to maintain the high frequency of the heterozygotes should be sufficiently strong to overcome the negative effects such as the increased morbidity and mortality rates among the homozygotes and compounded heterozygotes (Twig et al., 2014). There have been suggestions that the mutated forms of the protein may confer an increased protection against tuberculosis (Cattan, 2003) or brucellosis (Ross, 2007) but none had direct evidence provided. Other potential effects of the mutation carrier state are thought may be associated with the protection against asthma (Brenner-Ullman et al., 1994), atopy (Sackesen et al., 2004), or allergy (Kalyoncu et al., 2006). But it is not clear then why this protection mechanism against allergic diseases had not emerged worldwide but remained restricted to the existing geographical and ethnical boundaries.

PYRIN: STRUCTURE AND PROTEIN–PROTEIN INTERACTION

MEFV encodes the protein called pyrin (also known as marenostrin or TRIM20), which is supposed to play a key role in apoptotic and inflammatory signaling pathways. The protein belongs to the large family of proteins sharing a conserved domain structure with the tripartite motif (TRIM) consisting of an N-terminal RING domain, B-box domain(s) and a C-terminal coiled-coil domain (Weinert et al., 2015). In TRIM20 the RING domain with the ubiquitin ligase activity is replaced by the PYD domain, which belongs to the death domain superfamily (Martinon et al., 2001; Gumucio et al., 2002; Kohl and Gruütter, 2004). It also carries an additional ∼200-amino acid C-terminal B30.2/rfp/PRY/SPRY domain. The majority of the disease-associated mutations are located in the C-terminal B30.2 domain.

During the last decade the functions of pyrin have been the subject of intensive research, and advances in our understanding of its functionality are the result of identification of its interaction with different proteins and oligomers. In particular, the PYD domain, via cognate pyrin domain association, interacts with an adapter protein denoted apoptosis-associated speck-like protein with a caspase recruitment domain (ASC; Richards et al., 2001), and thus participates in the regulation of apoptosis, inflammation, and IL-1β processing (Gumucio et al., 2002). The B30.2/rfp/PRY/SPRY domain-mediated protein interactions indicate the role of this domain as an adaptor module to assemble macromolecular complexes (Perfetto et al., 2013). As a part of the pyrin inflammasome, pyrin also acts as a pattern recognition receptor sensing pathogen modification and inactivation of Rho GTPases (Xu et al., 2014). Paradoxically, pyrin also acts as a component directing the inflammasome components, NLRP1, NLRP3, and pro-caspase 1, to selective autophagic degradation (Kimura et al., 2015). On its own, the dimerization of B30.2 domains by means of the CHS domain appears to be crucial for the recognition of higher order oligomers (Weinert et al., 2015).

REGULATION OF INNATE IMMUNITY BY PYRIN

Since the positional cloning and characterization of the *MEFV* gene in 1997 by two consortia (The French FMF Consortium, 1997; The International FMF Consortium, 1997), numerous

hypotheses have been proposed explaining the potential role of the protein encoded in regulation of innate immune responses. Through N-terminal PYD domain pyrin modulates caspase-1 and IL-1β activation exerting proinflammatory (Yu et al., 2006, 2007; Seshadri et al., 2007; Gavrilin et al., 2012) or anti-inflammatory (Chae et al., 2006; Papin et al., 2007; Hesker et al., 2012) regulatory effects, depending on the experimental system employed. In a number of studies the NALP3 inflammasome complex has been implicated in the pathogenesis of FMF (Papin et al., 2007; Omenetti et al., 2014). More recently, homozygous knock-in mice harboring the mouse pyrin protein fused to the human B30.2 domain containing FMF-associated mutations has been shown to secrete the large amounts of IL-1β in a NLRP3-independent manner (Chae et al., 2011) suggesting the formation of an inflammasome that does not include NLRP3. The pro-inflammatory feature of the pyrin function and the formation of the pyrin inflammasome has been confirmed in several later studies (Gavrilin et al., 2012; Mankan et al., 2012; Xu et al., 2014), which suggested that FMF-associated mutations are gain-of-function for pyrin and pyrin itself promotes ASC oligomerization and forms a caspase-1-activating complex. To add even more to the complexity of functions performed by pyrin, a recent study has established that it is involved in the specific autophagic degradation of cytoplasmic regulators of innate immunity thus contributing to the resolution of inflammatory response (Kimura et al., 2015).

PYRIN AND DANGER SIGNALS

Initially, the similarity of pyrin with other known transcriptional factors has led to speculations that pyrin may itself be a nuclear factor (The International FMF Consortium, 1997; Centola et al., 2000). It has been shown that the protein product of an alternatively spliced mRNA is able to translocate to the nucleus (Papin et al., 2000). The subsequent studies, however, have demonstrated the cytosolic localization of the full-length pyrin. The first evidence for the interaction of the N-terminal part of pyrin with microtubules and co-localization of pyrin with actin has been obtained by Mansfield and co-workers (Mansfield et al., 2001) suggesting that pyrin is a part of unique cytoskeleton signaling pathway. Subsequently, Waite et al. (2009) confirmed this observation by demonstrating the interaction of pyrin, ASC and actin as well as by identifying new interactions of pyrin with the adaptor protein PSTPIP1, which regulates the cytoskeleton and cell migration. Demonstration of the interaction between the B-box/coiled-coil domains of pyrin with PSTPIP1, the protein that is mutated in PAPA syndrome (Pyogenic Arthritis, Pyoderma gangrenosum, and Acne), confirmed the important role played by pyrin in cytoskeletal signaling pathways (Shoham et al., 2003; Waite et al., 2009). Mutations in PSTPIP1 increase its affinity to pyrin thus suggesting a molecular link between FMF and PAPA syndrome (Shoham et al., 2003).

In a more recent study by Xu et al. (2014) pyrin has been shown to activate caspase-1 in response to Rho GTPases modifying toxins from a number of pathogenic bacteria. They suggested that pyrin recognizes downstream Rho modifications, most likely involving the actin cytoskeleton modifications (Xu et al., 2014). Subsequent studies by Kim et al. (2015) confirmed the link between the aberrant actin depolymerization and inflammasome formation. Moreover, the authors suggested that IL-18 but not IL-1β is implicated in the development of autoinflammatory disease. However, it was shown that pyrin inflammasome is triggered by the lack of actin polymerization using inactivation mutation of the actin-depolymerizing cofactor Wdr1 in mice raised in a gnotobiotic facility (Kim et al., 2015). Thus, the direct participation of pyrin in bacterial sensing has been questioned in this study. Moreover, another interpretation of results by Xu et al. (2014) is that pyrin responds to the modification and inactivation of the host's proteins by bacterial toxins rather than directly detecting a microbial product (Foley, 2014). In the light of recent findings implicating Rho GTPase in the direct regulation of the pyrin inflammasome the role of the host protein modification as a danger signal is much more prevalent than that of bacterial toxins (Park et al., 2015). Similarly, pertussis toxin with the abolished ADP-ribosyltransferase activity is unable to induce pyrin-dependent inflammasome to cleave pro-IL-1β into the active form (Dumas et al., 2014). Thus the bacterial toxins or components are not the primary danger signals for pyrin inflammasome, there must be modifications of the host proteins in order to be perceived as danger signals.

The relationship between pyrin and cytoskeleton can be additionally confirmed by the therapeutic efficiency of colchicine, an alkaloid with the antimitotic activity, which is recommended and widely used as the first line therapy for the treatment of FMF (Zemer et al., 1986). Moreover, responsiveness to colchicine still has a diagnostic power in FMF. Colchicine exerts various anti-inflammatory effects mostly related to microtubule disruption and depolymerization in a dose-dependent manner (Sackett and Varma, 1993). In a recent study by Taskiran and others the re-organization of actin cytoskeleton in THP-1 cells by colchicine has been described (Taskiran et al., 2012). The mechanisms of its action in FMF have not been completely elucidated and remain under active investigation. It is generally accepted that the therapeutic action of colchicine in FMF is mainly due to the effects on leukocyte migration, signal transduction, and gene expression (Dinarello et al., 1976; Ben-Chetrit et al., 2006; Chae et al., 2008).

Pyrin is primarily expressed in cells of myeloid lineage, mainly polymorphonuclear neutrophils, eosinophils, and monocytes (Centola et al., 2000). Seemingly unprovoked trafficking of these cells to the serosal/synovial membranes is a key event in acute inflammatory attacks of FMF (Ben-Chetrit and Levy, 1998). The regulatory mechanisms implicated in leukocyte migration depend on Ca^{2+} influx and signaling events that result in changes in cytoskeletal organization, such as assembly and disassembly of F-actin, which provide forces necessary for cell migration (Vicente-Manzanares and Sánchez-Madrid, 2004). The regulation of these activities has recently been shown to involve the Rho family of small GTPases, including Rho, Rac, and Cdc42 (Endlich et al., 2001; Provenzano and Keely, 2011). The

Rho family of GTPases are the key regulators of a variety of cellular activities including motility, proliferation, apoptosis, and, particularly, of actin cytoskeleton rearrangements (Spiering and Hodgson, 2011). It was shown that Rho activation is sufficient to promote migration of monocytes across endothelial cells (Honing et al., 2004). In our experiments, we have observed a phenomenon of heightened sensitivity of neutrophils from FMF patients toward *in vitro* conditions in the inductor-free media (Manukyan et al., 2013a,b). This suggests that the host-derived stress signals could be responsible for the activation of these cells. Similar results with the use of monocytes have been obtained later by another group (Sugiyama et al., 2014). Experiments *in vitro* unavoidably impose mechanical forces on cells that may affect cytoskeletal structure and modulate cellular behavior (Wang et al., 1993; Matthews et al., 2006). Thus, the routine experimental procedures involving external mechanical forces applied to cells may lead to the generation of danger signals that are sensed by the pyrin inflammasome. Recent investigations showing the pyrin inflammasome activation in response to actin modifications (Xu et al., 2014; Kim et al., 2015), together with our results suggesting the excessive activation of neutrophils from FMF patients in *ex vivo* experiments (Manukyan et al., 2013a,b), warrants further studies of pyrin-cytoskeleton interactions, especially in the case of FMF.

CLINICAL ASPECTS

Clinically FMF divided into two phenotypes, types 1 and 2 (Shohat and Halpern, 2011). Type 1 is characterized by recurrent episodes of fever and polyserositis. Fever is the main symptom of the disease which is present in 95% of acute inflammation episodes, with the body temperature reaching usually above 38°C (Sohar et al., 1967). Fever is accompanied by sterile peritonitis, synovitis, pleuritis, and rarely by pericarditis and erysipelas-like skin lesions (Shohat and Halpern, 2011). A typical attack lasts 12 h to 2–3 days and resolves spontaneously. The frequency, duration, intensity, and symptoms experienced during attacks are highly variable between patients. During attack-free period patients are asymptomatic. About 50% of the patients are reported to have prodromal symptoms (Lidar et al., 2006). The first clinical presentation of type 2 FMF is amyloidosis, in the absence of other clinical symptoms (Shohat and Halpern, 2011).

The manifestation of the disease is diverse, ranging from asymptomatic to the potentially life-threatening states. The main and potentially lethal complication of the disease is secondary (AA) amyloidosis, which usually affects the kidneys. Amyloidosis is the result of tissue deposition of amyloid, which is a proteolytic cleavage product of the acute phase reactant serum amyloid A (SAA; Sohar et al., 1967). Overproduction of SAA leads to the extracellular accumulation of fibrillar protein and the development of amyloidosis (van der Hilst et al., 2005). Presently the administration of colchicine (1–3 mg/day) is the preferred treatment for FMF allowing extended periods of remission as well as preventing amyloidosis in the majority of FMF patients (Pras, 1998). Since 5–10% of the patients are colchicine resistant or intolerant to the drug, the efforts toward developing alternative treatment were undertaken. As an alternative approach, specific anti-cytokine therapies, such as IL-1 receptor and tumor necrosis factor antagonists were tested with a limited number of patients (Nakamura et al., 2007; Ben-Zvi and Livneh, 2014). Although, genotyping of the *MEFV* mutations is the preferred method in FMF diagnostics, it is confirmatory to the clinical diagnosis. Presently, there are no definitive biochemical markers that could serve as diagnostic ones. Usually FMF attacks are accompanied by a non-specific increase in acute phase reactants such as C-reactive protein, fibrinogen, and serum amyloid A as well as by the increased white blood cell count with low-grade neutrophilia. The diagnosis of FMF remains clinical, since mutations have reduced penetrance and cannot always be identified on both alleles (Tunca et al., 2002). Clinical manifestations of FMF may overlap with the phenotypes of autoinflammatory disorders and, to a certain degree, with autoimmune diseases, which may potentially complicate diagnosis of FMF. One distinguishing feature of autoinflammatory syndroms is responsiveness to IL-1β blocking therapy, although there are exceptions (Bodar et al., 2005; Arostegui et al., 2007; Kuijk et al., 2007; Gattorno et al., 2008).

Several studies have demonstrated association of *MEFV* mutations with different inflammatory pathologies, such as systemic onset juvenile idiopathic arthritis (Uslu et al., 2010), inflammatory bowel disease (Akyuz et al., 2013), Behçet's disease (Atagunduz et al., 2003), ulcerative colitis (Giaglis et al., 2006), Fibromyalgia syndrome (Feng et al., 2009), rheumatoid arthritis (Rabinovich et al., 2005), ankylosing spondylitis (Akkoc et al., 2010), and others. Other pathologies, which are associated with mutations in the *MEFV* gene, are two vasculitis types, Henoch-Schonlein Purpura (Gershoni-Baruch et al., 2003; Tunca et al., 2005) and polyarteritis nodosa (Tunca et al., 2005; Aksu and Keser, 2011). Clinical manifestations of these vasculitis types, which accompany FMF, might be considered as a clinical manifestation of the latter. The diseases listed represent multi-factorial immunological disorders with a marked involvement of the inflammatory component. A growing number of similar cases, which are reported to be associated with the *MEFV* gene,

TABLE 1 | Structural and functional roles of pyrin in cellular processes.

Role	Reference
Inflammasome assembly	Chae et al., 2011; Perfetto et al., 2013; Vajjhala et al., 2014
Sensing intracellular danger signals by the inflammasome (aberrant actin depolymerisation, protein modification by bacterial toxins)	Dumas et al., 2014; Xu et al., 2014; Kim et al., 2015
Activation of mediators of inflammation by the inflammasome (IL-1β, IL-18)	Chae et al., 2011; Kim et al., 2015
Pyrin-cytoskeloton interactions	Mansfield et al., 2001; Shoham et al., 2003; Waite et al., 2009
Apoptosis	Martinon et al., 2001; Richards et al., 2001; Gumucio et al., 2002
Autophagy of innate immunity regulators	Mandell et al., 2014; Kimura et al., 2015

suggest that pyrin is a key regulatory element of the innate immune responses and can affect inflammatory processes during these disorders.

CONCLUDING REMARKS

Understanding the role of pyrin in innate immunity has progressed rapidly in recent years, uncovering its numerous functions in the cell from the formation of several supramolecular structures and inflammasome assembly, to sensing various intracellular danger signals, to mounting the innate immune responses, and to the resolution of inflammation (**Table 1**). The evidences for positive selection of mutations in the *MEFV* gene are given in several works cited in this review, and they have a strong support from the formal analytical approaches of population genetics. What remains unclear, however, why the mutations have been selected and maintained in the Mediterranean populations and what are the mechanistic explanations for the advantage at the biochemical level? In particular, we know well about the negative effects of the homozygous or compounded heterozygous mutant allele combination, which result in a higher morbidity/mortality rate. At the cellular level this genetics presumably results in the impaired assembly of pyrin inflammasomes, in the launch of excessive and extended inflammatory responses, and in a less efficient resolution of inflammation. Given the multiple functions of pyrin and the expected pleiotropic effects of mutations in the *MEFV* gene, the significance and contribution of each of these functions are difficult to ascertain in terms of clinical presentation in pathology or the heterozygote advantage at the phenotypic level. All we know at the clinical level is that the homozygous or compounded heterozygous state results in the enhanced and extended inflammatory response to some of the innocuous factors that are tolerated well and handled efficiently by the normal immune system. The diseased state is certainly disadvantageous (although it cannot be excluded that certain conditions in the past have been conducive to select such traits) but we don't know about the possible advantages conferred by the heterozygote state. Investigation of this previously ignored group could potentially reveal the characteristics and traits that were responsible for the selective advantage and maintenance of these mutations in the Mediterranean populations.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Multiplex analysis of serum cytokines in humans with hantavirus pulmonary syndrome

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Hantavirus pulmonary syndrome (HPS) is an acute zoonotic disease transmitted primarily through inhalation of virus-contaminated aerosols. Hantavirus infection of endothelial cells leads to increased vascular permeability without a visible cytopathic effect. For this reason, it has been suggested that the pathogenesis of HPS is indirect with immune responses, such as cytokine production, playing a dominant role. In order to investigate their potential contribution to HPS pathogenesis, we analyzed the serum of hantavirus-infected subjects and healthy controls for 68 different cytokines, chemokines, angiogenic, and growth factors. Our analysis identified differential expression of cytokines that promote tissue migration of mononuclear cells including T lymphocytes, natural killer cells, and dendritic cells. Additionally, we observed a significant upregulation of cytokines known to regulate leukocyte migration and subsequent repair of lung tissue, as well as cytokines known to increase endothelial monolayer permeability and facilitate leukocyte transendothelial migration. Conversely, we observed a downregulation of cytokines associated with platelet numbers and function, consistent with the thrombocytopenia observed in subjects with HPS. This study corroborates clinical findings and extends our current knowledge regarding immunological and laboratory findings in subjects with HPS.

Keywords: hantavirus pulmonary syndrome, serum, cytokines, chemokines, growth factors, immune response, hantaviruses

Introduction

Hantavirus pulmonary syndrome (HPS) is a severe life threatening disease caused by members of the genus *Hantavirus*. In the United States, these members include Sin Nombre virus, Bayou virus, Black Creek Canal virus, and New York virus, while South American members include Andes virus and Laguna Negra virus (1–5). Although HPS was first diagnosed as a clinical entity in 1993 in response to the four corners outbreak (6), retrospective studies have identified hantavirus-associated fatalities as early as 1978 (7). HPS cases have been reported in 34 states with the majority occurring in the Southwestern states; however, several have been reported in the Northwestern and Midwestern states. Through April 2014, the Center for Disease Control and Prevention has confirmed 639 total cases of HPS in the U.S., with the majority occurring in New Mexico (94 cases), Colorado (81 cases), and Arizona (72 cases) (8). Although the prevalence of HPS is low in the U.S., 36% of all reported HPS cases have resulted in death, underscoring the potential impact to public health.

Clinically, HPS manifests with fatigue, fever, muscle pain, headache, dizziness, nausea, and vomiting (9). Soon after onset, individuals present with bilateral diffuse interstitial edema resembling acute respiratory distress syndrome (10). Rapidly progressing pulmonary edema, myocardial depression, and hypovolemia are the leading cause of death (11). There is no specific treatment for HPS; therefore, medical care is mainly supportive with early diagnosis resulting in more successful outcomes.

Hantaviruses do not produce a visible cytopathic effect; consequently, it is believed that cytokines produced by infected cells either directly or indirectly lead to a compromised endothelial monolayer, which in turn, leads to vascular leakage. Indeed, increased numbers of cytokine-producing cells have been observed in lung and spleen tissue of HPS cases (12). We as well as others have demonstrated that endothelial cells produce the chemokines, CCL5 and CXCL10, when infected with Sin Nombre virus (13, 14). These cytokines are strong chemoattractants for mononuclear leukocytes including monocytes, lymphocytes, and natural killer (NK) cells (15, 16). Expression of these chemokines may explain the postmortem observation of monocytic interstitial pneumonia in fatal HPS cases; however, it remains to be determined whether these chemokines are expressed during active HPS. In contrast to CCL5 and CXCL10 and atypical of most viral infections, *in vitro* culture studies show that only a slight upregulation of type I interferon (IFN) is observed when endothelial cells are infected with hantaviruses. These data are also consistent with clinical observations that suggest that a robust IFN-α response is not characteristic of hantavirus infection (17, 18).

Although limited data exist regarding cytokine expression in subjects with HPS, a study by Borges et al. evaluated the concentrations of 11 serum analytes by ELISA. A cytokine profile was reported that defined the differential expression of a selected number of Th1 and Th2 cytokines (19). Specifically, they observed significantly elevated levels of IL-6, IFN-γ, sIL-2R, TNF-α, and decreased IL-10 when compared to controls, suggesting that activation of Th1 and Th2-type immune responses are involved. While ELISA is commonly used for such studies, it has limitations such as the necessity of a large sample volume and this issue is compounded when one wishes to analyze multiple analytes. High-throughput multiplex analysis by Luminex xMAP technology allows the simultaneous detection and quantitation of many analytes and uses a small amount of serum or plasma. In the present study, we utilized Luminex xMAP technology to conduct a comprehensive evaluation of 68 different cytokines, chemokines, angiogenic, and growth factors (hereafter referred to collectively as cytokines) in subjects with HPS, including 38 cytokines previously not investigated in association with this disease. Changes in 40 cytokines were detected in the serum of subjects with HPS when compared to healthy controls; 25 cytokines were significantly upregulated while15 were downregulated. A subset of these cytokines known to influence the migration of mononuclear effectors was upregulated, as were cytokines known to play a role in lung microbial defense and tissue repair. Another subset of cytokines associated with thrombocyte counts and function was downregulated. This study corroborates clinical findings and extends our current knowledge by providing a more comprehensive basis for the immune responses and morphology observed in laboratory and histological findings in subjects with HPS.

Materials and Methods

Subjects

Twelve clinical diagnostic serum specimens collected from 2008 to 2012 by the Nevada State Health Laboratory (NSHL) and with a confirmed diagnosis of HPS were utilized in this study. The NSHL serves as a regional reference laboratory and routinely screens subjects suspected of having HPS, by the presence of anti-hantavirus antibodies. These deidentified diagnostic specimens were deemed to be exempt from IRB approval by the University of Nevada (UNR), Research Integrity Office (Reference #616225-1) as meeting the exemption criteria defined by the Department of Health and Human Services under Human Subject Research Code 45 CFR 46.102(f). Information of each HPS case was limited to diagnosis, gender, and antibody titer range. Forty-two serum samples from healthy individuals collected under informed consent were used as controls (Human subjects protocol # B12-031). Control subjects were chosen to be consistent with published demographics of typical HPS cases regarding age and gender (male to female ratio of 54–46%, respectively, and mean age of 39.4 years) (20).

HPS Screening

Serum anti-hantavirus antibody titers were evaluated by ELISA, according to the methods described by Feldmann et al. (21). Serum dilutions (1:100–1:6400) were tested for the presence of anti-hantavirus IgG and IgM using recombinant nucleocapsid protein supplied by the United States Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA). Subjects with antibody titers greater than twofold above that of negative controls were considered positive.

Multiplex Analysis

The levels of serum cytokines were analyzed using Bio-Plex (Bio-Rad, Hercules, CA, USA) multiplex magnetic bead-based antibody detection kits following the manufacturer's instructions. The Bio-Plex Pro Human Chemokine Panel (40-Plex); Bio-Plex Pro Human Th17 Cytokine Panel; Bio-Plex Pro Human Cytokine 27-plex Panel; and Bio-Plex Human Cytokine 21-plex Panel were used for analysis of a total of 68 analytes. Fifty microliters of serum from each respective case and control was analyzed using a Luminex 200 analyzer with MasterPlex CT control software and MasterPlex QT analysis software (MiraiBio, San Bruno, CA, USA). Standard curves for each analyte were generated using standards provided by manufacturer. Serum samples from HPS cases were heat inactivated and tested for the presence of infectious virus prior to Luminex analysis. The effect of heat inactivation on cytokine stability was evaluated and those that could not be normalized were excluded from analysis.

Statistical Analysis

Mann–Whitney non-parametric analysis was utilized to identify differences in medians between HPS cases and controls. In addition, we performed classification analysis using the tree-based

ensemble machine learning algorithm Random Forest (RF) (22). For this analysis, 500 random trees were built using six predictors for each node, and auto-bootstrap out-of-bag sampling was used for testing the model as previously described (23).

Results

Anti-Hantavirus Titer in HPS Serum

Twelve serum samples from subjects suspected of having hantavirus infection were tested for the presence of anti-hantavirus IgG and IgM antibodies. Antibody titers twofold greater than those of the control samples were considered diagnostic for hantavirus infection (**Table 1**). Previous reports suggest that anti-hantavirus IgM and IgG change with disease progression (24, 25). As reported by MacNeil and coworkers, early stage HPS is characterized by high IgM titers that peak within 11–14 days after onset whereas cases with early stage HPS often have no SNV-specific IgG titer (24). In contrast to IgM titers, median IgG titers typically displayed an increasing trend for a longer interval after the onset of disease. In light of the deidentified nature of our HPS cases, we used antibody titers to assess the stage of their illness. Six of our cases had high serum titer of IgM while IgG levels were low or undetectable, indicative of early stage disease. For the remaining six cases, high serum titers were observed for both IgG and IgM, consistent with late onset HPS.

Differential Expression of Serum Cytokine in HPS Cases

A total of 68 serum cytokines were measured for HPS cases and controls (**Tables 2–4**). To the best of our knowledge, 38 of these cytokines were previously uninvestigated in the context of HPS (indicated by an asterisk in **Tables 2–4**). A significant increase in the serum levels of 25 of 68 (36.7%) cytokines were observed for the HPS cases when compared to healthy controls (**Table 2**). The greatest difference was observed for IL-6, CXCL10, CX3CL1, MIF, and MIG, all of which were upregulated fivefold over those of controls ($p < 0.001$). In contrast, 15 of 68 (22.1%) cytokines were downregulated in HPS cases when compared to controls (**Table 3**), the greatest differences were observed for CXCL12, CCL21, CCL22, CCL27, and sCD40L ($p < 0.001$). Additionally, the majority of downregulated cytokines belonged to the homeostatic and inflammatory chemokine family. Of the 68 cytokines investigated, 28 (41.2%) were not statistically different when comparing cases and controls (**Table 4**).

Analysis of Serum Cytokines in Early vs. Late Stage HPS

In order to investigate the possibility that differential expression of cytokines occurs between subjects with early and late stage HPS, we compared these two subgroups with each other and to healthy controls. Surprisingly, we observed only five cytokines to be differentially expressed between the two subgroups of HPS

TABLE 1 | Antibody titer in serum from HPS cases.

Subject	IgM titer	IgG titer	Stage
1	>6400	<400	Early
2	>6400	<400	Early
3	>6400	<400	Early
4	>400	Negative	Early
5	>400	Negative	Early
6	>400	Negative	Early
7	<6400	>6400	Late
8	<6400	>6400	Late
9	<6400	>6400	Late
10	<6400	>6400	Late
11	<6400	>6400	Late
12	<6400	>6400	Late

TABLE 2 | Cytokines upregulated in HPS cases compared to healthy controls.

Analyte	Case (pg/mL), $n = 12$	Control (pg/mL), $n = 41$	p Value
Upregulated In HPS serum			
IL-1α	537.7 ± 95.0	179.12 ± 15.7	0.0001
IL-2RA	455.3 ± 84.6	177.3 ± 7.3	0.0001
IL-2	11.7 ± 3.6	4.7 ± 0.8	0.005
IL-3	415.1 ± 86.4	140.5 ± 11.4	0.0001
IL-6	87.9 ± 22.7	10.8 ± 2.0	0.0001
IL-10	49.2 ± 31.5	15.7 ± 1.1	0.05
IL-12(p40)	927.3 ± 175.1	280.7 ± 22.9	0.0001
IL-17A*	23.3 ± 6.8	7.5 ± 0.2	0.0001
IL-17F*	74.3 ± 19.3	17.9 ± 4.8	0.0001
IL-18*	1651.6 ± 495.1	803.6 ± 66.7	0.006
IL-22*	42.1 ± 12.1	22.7 ± 0.5	0.004
CCL23*	705.9 ± 102.5	375.7 ± 37.6	0.0004
CXCL10	2834.2 ± 913.5	197.8 ± 18.8	0.0001
CX3CL1*	1456.6 ± 321.2	241.3 ± 13.2	0.0001
GM-CSF	55.3 ± 9.7	14.2 ± 2.5	0.0001
M-CSF	4811.7 ± 167.7	415.1 ± 26.5	0.0001
VEGF	179.2 ± 122.7	48.8 ± 6.1	0.05
MIF*	4779.9 ± 2229	540.6 ± 70.5	0.001
CXCL9*	2702.7 ± 891	355.0 ± 93.0	0.0001
TNFβ	227.9 ± 26	147.9 ± 12.9	0.007
IFNα	191.9 ± 26.2	123.6 ± 9.8	0.005
LIF*	346.7 ± 40.9	216.9 ± 10.7	0.0001
b-NGF*	122.0 ± 13.8	98.3 ± 3.9	0.03
SCF*	1180.8 ± 233.9	469.3 ± 30.9	0.0001
TRAIL*	391.9 ± 82.4	266.7 ± 14.8	0.02

TABLE 3 | Cytokines downregulated in HPS cases compared to healthy controls.

Analyte	HPS (pg/mL), $n = 12$	Control (pg/mL), $n = 41$	p Value
Downregulated in HPS serum			
CCL1*	41.7 ± 0.3	43.3 ± 0.4	0.03
CCL5	1210.5 ± 230	5520.3 ± 670	0.001
CCL11	18.5 ± 0.9	47.1 ± 2.6	0.0001
CCL13*	37.1 ± 10.2	135.0 ± 14.1	0.0005
CCL17*	70.4 ± 31.5	241.4 ± 22.3	0.0004
CCL19*	156.2 ± 58.9	418.5 ± 38.1	0.001
CCL21*	979 ± 193	3504.6 ± 119	0.0001
CCL22*	276.2 ± 101	1112.8 ± 60.4	0.0001
CCL24*	356.7 ± 93.8	597.8 ± 49.5	0.02
CCL26*	16.4 ± 2.5	27.6 ± 1.9	0.005
CCL27*	319.8 ± 65.7	1411.4 ± 79.9	0.0001
CXCL6*	25.7 ± 44	48.2 ± 2.3	0.0002
CXCL12*	166.7 ± 32.7	2367.3 ± 104.3	0.0001
CXCL16*	183.4 ± 44.0	618.3 ± 27.9	0.0001
sCD40L	89.3 ± 54.4	2014.2 ± 128	0.0001

TABLE 4 | No significant difference in cytokine expression between HPS and healthy controls.

Analyte	HPS, *N* = 12 (pg/mL)	Healthy control, *n* = 41 (pg/mL)	*p* Value
IL-1	4.59 ± 0.1	4.8 ± 0.2	0.52
IL-1RA	93.7 ± 50.7	50.4 ± 7.4	0.15
IL-1β	2.7 ± 0.1	7.9 ± 1.9	0.1
IL-4	64.9 ± 5.2	78.4 ± 5.2	0.18
IL-5*	6.3 ± 0.6	5.9 ± 0.1	0.27
IL-7	5.3 ± 2.1	5.5 ± 0.5	0.9
IL-9*	9.9 ± 1.7	19.7 ± 11.9	0.66
IL-13	8.7 ± 0.5	8.9 ± 0.34	0.59
IL-15	9.7 ± 4.1	5.7 ± 0.07	0.07
L-16*	252.2 ± 53.4	317.2 ± 40.9	0.4
IL-21	30.7 ± 8.7	30.1 ± 6.0	0.96
IL-23	102.6 ± 25.3	95.1 ± 17.4	0.83
IL-25*	1.6 ± 0.3	2.4 ± 0.3	0.2
IL-31*	18.5 ± 3.2	21.4 ± 2.2	0.49
IL-33*	402.6 ± 125.7	723.3 ± 100.0	0.11
CCL3	18.3 ± 3.7	43.9 ± 9.5	0.15
CCL7*	196.9 ± 27	169.1 ± 23.1	0.55
CCL8	75.8 ± 11.3	95.8 ± 6.8	0.15
CXCL1*	215.9 ± 34.9	232.4 ± 13.4	0.6
CXCL2*	236.8 ± 37.7	302.1 ± 25.3	0.2
CXCL5*	1085.8 ± 230.1	798.1 ± 90.6	0.43
CXCL11*	23.5 ± 5.2	41.3 ± 10	0.35
FGF*	14.8 ± 1.3	20.7 ± 2.7	0.24
GCSF	26.2 ± 13	26.4 ± 3.3	0.98
HGF*	973.7 ± 284.6	869.9 ± 82.4	0.64
IFNγ	20.1 ± 5.0	15.4 ± 1.6	0.24
DCGF-β*	6605.8 ± 1808	4692.7 ± 353.1	0.1
PDGF	889.9 ± 302	1095.5 ± 62.1	0.29

cases (**Table 5**). Of these, median IL-33 and CXCL6 levels were greater in the early stage subjects whereas median CCL23, CXCL1, and TNF-β were greater in the late stage subjects. As expected, differences in cytokine expression between subgroups and controls were consistent with differences observed between total HPS cases and controls (data not shown).

Classification of Cytokines by Importance

Given the complex interactions of cytokines with immune and non-immune cells, clarification of how distinct cytokines contribute to a pathological situation is often difficult to resolve. In order to provide insight into this issue, we implemented the machine logic algorithm RF to analyze our data set and potentially identify the most important cytokines that define this disease. For our analysis, 500 random decision trees were constructed with six predictors at each node, and auto-bootstrap out-of-bag sampling was implemented to test the accuracy of model. This model accurately identified HPS cases with 100% specificity and 73.81% sensitivity (**Table 6**). The 10 most significant cytokines for delineating HPS in decreasing order of importance are: M-CSF, CXCL16, sCD40, CXCL12, CCL22, IL-1a, CCL21, IL-12p40, CCL17, and IL-1b.

Discussion

The microvascular endothelium is principal target of hantavirus infection in humans and its infection in lung tissue results in significant pathology (26). Infection of endothelial cells leads to increased vascular permeability without an observable cytopathic effect; therefore, the pathogenesis of HPS is likely indirect with immune responses, such as cytokine production, playing an important role. The cytokines that we observed to be upregulated in the serum of HPS cases are involved in a number of antiviral defense mechanisms including proliferation, maturation, and activation of leukocytes, as well as survival of leukocytes, and regulation of endothelial monolayer permeability (**Table 2**). High levels of IL-1α, IL-6, MIF, and TNF-β suggest a strong proinflammatory milieu in the serum of HPS cases, thus promoting both inflammation and activation of immune responses. We also observed stem cell proliferation factors to be upregulated, potentially promoting the proliferation and differentiation of subsets of immune effector cells. For example, proliferation of myeloid progenitors is strongly supported by IL-3, GM-CSF, and M-CSF. Increased serum concentrations of GM-CSF and M-CSF also suggest proliferation of monocytes and granulocytes (neutrophils, eosinophils, and basophils). Upregulation of the pluripotent factor, SCF, was also observed in association with HPS, suggesting increased proliferation of T lymphocytes, NK cells, and dendritic cells.

We observed a subset of 15 serum cytokines to be downregulated in our HPS cases (**Table 4**). Twelve of these cytokines are involved in chemotaxis of lymphocytes, such as B cells, T cells, and NK cells, to sites of infection. Some of these cytokines, including CCL22, CXCL12, and CCL17, are associated with activation of Th2-type immunity and are potent recruiters of Th2 cells to the lungs, as well as activators of pre-B cells (27–29). A number of cytokines identified as differentially expressed in the present study are consistent with putative immune responses of lung tissue. For example, we observed the upregulation of serum IL-17F, CXCL16, and IL-22, which are involved in the regulation of leukocyte migration into lung tissue, as well as lung tissue repair (30–33). Upregulation of IL-17F has also been observed in the lung tissue of asthmatic cases and its level positively correlated with disease severity (30, 34, 35). Overexpression of IL-17F promotes neutrophil infiltration and increased airways sensitivity and thus has a significant impact on lung function (35). IL-22 is considered a key cytokine for mucosal tissue repair (36) and by activating antimicrobial responses in lung epithelial cells; it has been shown to be critical for host defense as well. Also, IL-22 promotes lung epithelial cell proliferation (37) and therefore, based on our analyses, the cytokine profile observed in our HPS cases is consistent with a pulmonary antimicrobial response and subsequent mononuclear cell migration into the lung.

The serum cytokine profile observed in our HPS subjects also suggests a mobilization of mononuclear immune effector cells (**Table 2**). IL-12(p40) is an autocrine chemoattractant released by activated macrophages and promotes Th1-type immunity (38, 39). Additionally, serum levels for several potent T lymphocyte and NK chemoattractants were upregulated, including CXCL10, MIG, and CCL23 (15, 16, 40, 41). MIF and VEGF, which are regulators of mononuclear cell transendothelial migration, were upregulated as well. Migration of leukocytes can also be facilitated by the upregulation of adhesion molecules on the surface of endothelial cells in response to VEGF, IL-1α, and IL-6 (42, 43).

TABLE 5 | Serum cytokine profile during early and late stages of HPS.

Analyte	HPS early (pg/mL)	HPS late (pg/nL)	Control (pg/mL)	*p* Value*	*p* Value**	*p* Value***
IL-1α	316.3 ± 60.4	545 ± 98.1	179.12 ± 15.7	0.006	0.0001	
IL-2RA	345.5 ± 92.5	454.9 ± 44.2	177.3 ± 7.3	0.001	0.0001	
IL-2	11.9 ± 5.5	10.1 ± 4.6	4.7 ± 0.8		0.02	
IL-3	269.3 ± 39.8	331.6 ± 87.2	140.5 ± 11.4	0.0004	0.0002	
IL-6	50.4 ± 30.5	109.9 ± 25.9	10.8 ± 2.0	0.003	0.0001	
IL-10	34.6 ± 12.4	14.6 ± 0.5	15.7 ± 1.1		0.02	
IL-12(p40)	613.9 ± 77.3	815.1 ± 196.3	280.7 ± 22.9	0.0001	0.0001	
IL-15	13.8 ± 7.5	5.6 ± 0.4	5.7 ± 4.1	0.008		
IL-17A	20.1 ± 6.2	24.6 ± 10.5	7.5 ± 0.2	0.0001	0.001	
IL-17F	61.0 ± 24.8	52.7 ± 10.7	17.9 ± 4.8	0.009	0.03	
IL-22	32.2 ± 12.5	55.8 ± 24.4	22.7 ± 0.5	0.0005		
IL-33	651.5 ± 179.2	74.5 ± 19.3	723.3 ± 100			0.03
CCL5	1339.8 ± 409.3	1168.7 ± 182.5	5520.3 ± 670	0.02	0.05	
CCL11	20.0 ± 1.3	17.5 ± 0.6	47.1 ± 2.6	0.0002	0.0009	
CCL17	124.1 ± 51.5	17.7 ± 5.6	241.4 ± 22.3		0.003	
CCL19	250.1 ± 64.4	126.3 ± 32.9	319.6 ± 38.1		0.02	
CCL21	732.9 ± 136.8	1185.5 ± 303.1	3504.6 ± 119	2.8E-11	0.0001	
CCL22	458.6 ± 159.4	108.5 ± 39.9	1112.8 ± 60.4	0.0004	0.0001	
CCL23	489.8 ± 112.4	990.7 ± 122.7	375.7 ± 37.6		0.0001	0.02
CCL24	229.5 ± 53.3	608.2 ± 180.2	597.8 ± 49.5		0.007	
CCL26	18.5 ± 4.3	13.9 ± 2.6	27.6 ± 1.9		0.03	
CCL27	306.4 ± 91.9	391.2 ± 97.1	1411.4 ± 79.9	0.0001	0.0003	
CXCL1	470.0 ± 50.8	954.3 ± 176.8	232.4 ± 13.4		0.03	0.01
CXCL5	2005.6 ± 1072.8	225.0 ± 49.9	708.1 ± 90.6	0.02		
CXCL6	32.9 ± 8.3	21.3 ± 10.9	48.2 ± 2.3		0.04	0.003
CXCL10	2785.2 ± 146.2	3843.1 ± 1266	197.8 ± 18.8	0.0001	0.0001	
CXCL12	191.1 ± 47.2	181.0 ± 42.6	2367.3 ± 104.3	0.0001	0.0001	
CXCL16	201.5 ± 73.8	175.2 ± 29.9	618.1 ± 27.9	0.0001	0.0001	
CX3CL1	1020.4 ± 440.6	1710.3 ± 309.1	241.3 ± 13.2	0.0001	0.0001	
GM-CSF	67.3 ± 9.7	43.6 ± 16.6	14.2 ± 2.5	0.0001	0.005	
DCGF-β	4611 ± 1036.1	11215.6 ± 3269	4692.7 ± 353.1		0.0002	
LIF	253.3 ± 39.1	386.8 ± 50.4	216.9 ± 10.7		0.0001	
M-CSF	1721.7 ± 475.2	3563.8 ± 1221.1	415.1 ± 26.5	0.0001	0.0001	
MIG	2924.6 ± 1596.1	3152.6 ± 2746.3	355.0 ± 93.0	0.0003	0.0001	
MIF	1977.3 ± 540.6	666.2 ± 200.9	540.6 ± 70.5	0.008	0.0001	
sCD40L	157.8 ± 85.1	15.7 ± 4.3	2014.2 ± 128	0.0001	0.0001	
SCF	798.8 ± 207.6	1390.8 ± 443.1	469.3 ± 30.9	0.006	0.0001	
TNFβ	167 ± 16.4	226.9 ± 16.1	147.9 ± 12.9			0.04
VEGF	286.5 ± 48.8	101.4 ± 28.5	48.86.1	0.01	0.02	

**p value early phase to control; **p value late phase to control; ***p value early to late phase.*

MIF and VEGF promote expression of the adhesion molecules, E-selectin, ICAM-1, and VCAM-1, and increase vascular permeability (44, 45). Additionally, VEGF can decrease tight junctions between endothelial cells enabling transmigration of immune effector cells (42, 46). The observed increased serum levels of CXCL1, which may lead to release of VEGF-A from hantavirus-activated endothelial cells, further suggests that upregulation of VEGF plays a role in HPS (47, 48).

Cytokines including CXCL10, MIF, MIG, IL-12(p40), IL-17A, and CCL23 are known to promote proliferation and migration of mononuclear immune cells, such as T lymphocytes, NK cells, monocytes, and dendritic cells (15, 49–51). Consequently, our data support the previous observations of others whereby mononuclear cell and immunoblasts are the principal cellular infiltrate in the lungs of HPS cases (12). Nevertheless, the observed cytokine expression also is consistent with the activation and migration of neutrophils. Previous studies suggest that the cytokines, IL-17F, VEGF, CXCL1, GM-CSF, and IL-22, promote neutrophil migration and lung tissue repair (52–54). These data corroborate a previous report by Mori et al., who observed low-level neutrophil infiltration in the lungs of HPS case (12). Interestingly, serum level of CXCL8, the prototype neutrophil chemoattractant, was not significantly elevated in the HPS cases in our study; however, it was identified as one of the top 10 cytokines by our RF analysis, suggesting its expression, or lack thereof, plays an important role in HPS pathology. Our data further suggest that a Th17 shift occurs in HPS (55). In the presence of IL-23, non-Th17 cells can produce IL-17 (56); however, we observed no differential expression of serum IL-23 in HPS cases. Therefore, it is likely that activated Th17 lymphocytes were the source of IL-17 in the serum of our HPS cases.

Expression of IL-17 and IL-22 in HPS suggests a developing antimicrobial state in the lung. It has been reported that IL-17 and IL-22 activate β-defensins and the S100 family of proteins (52, 57). *In vivo* studies using knockout mice have demonstrated that IL-17 and IL-22 are crucial for bacterial defense in the lung (58, 59). Furthermore, it has been reported that IL-17R signaling is mandatory for the establishment of an antibacterial response

TABLE 6 | Random forest analysis of serum cytokines in HPS vs. controls.

Variable	Score (%)	Changes in HPS serum	Variable	Score (%)	Changes in HPS serum
M-CSF	100.0000	Upregulated	IL-17F	19.4733	Upregulated
CXCL16	98.7888	Downregulated	CCL3	19.1369	Unchanged
sCD40L	96.8968	Downregulated	CCL1	18.8358	Downregulated
CXCL12	85.5322	Downregulated	CXCL11	18.5085	Unchanged
CCL22	78.4301	Downregulated	DCGFB	17.9953	Unchanged
IL-1α	74.0061	Upregulated	IL-4	17.7238	Unchanged
CCL21	70.3732	Downregulated	IL-25	17.4077	Unchanged
IL-12(p40)	62.9938	Upregulated	IL-33	16.6915	Unchanged
CCL17	62.8689	Downregulated	CCL7	16.5016	Unchanged
IL-1β	61.4314	Unchanged	TRAIL	15.7741	Upregulated
CCL5	61.0088	Downregulated	IL-9	14.3617	Unchanged
IL-3	58.5351	Upregulated	IL-18	12.0276	Upregulated
CCL13	58.0210	Downregulated	IL-7	10.5668	Unchanged
CXCL9	52.4830	Upregulated	IL-22	10.2808	Upregulated
CXCL10	50.3664	Upregulated	CXCL2	9.3174	Unchanged
CCL11	48.5759	Downregulated	MIF	8.8718	Upregulated
CCL27	46.5450	Downregulated	IL-16	8.8434	Unchanged
CXCL5	46.0115	Unchanged	b-NGF	7.9004	Upregulated
CX3CL1	45.6097	Upregulated	IL-31	7.0565	Unchanged
GM-CSF	43.1944	Upregulated	IL-10	6.3552	Upregulated
IFNα	41.2777	Upregulated	CCL8	6.1227	Unchanged
LIF	40.7034	Upregulated	IL-17A	5.3501	Upregulated
CCL24	39.4832	Downregulated	INFG	4.9877	Unchanged
IL-2RA	38.9993	Upregulated	GCSF	4.4335	Upregulated
PDGF	36.2886	Unchanged	IL-1	4.1642	Unchanged
CCL19	34.6337	Downregulated	FGF	4.0244	Unchanged
IL-6	31.5406	Upregulated	HGF	3.9312	Unchanged
CXCL6	30.4604	Downregulated	IL-1RA	3.5669	Unchanged
IL-15	25.5225	Unchanged	CXCL1	3.3770	Unchanged
TNFβ	24.6500	Upregulated	IL-5	2.6487	Unchanged
SCF	24.2768	Upregulated	IL-23	2.2930	Unchanged
IL-2	22.9123	Upregulated	VEGF	0.9825	Upregulated
CCL26	21.2675	Downregulated	IL-13	0.0038	Unchanged
CCL23	20.7866	Upregulated			

to *M. pneumoniae*, systemic fungal infection, *B. fragilis*, and *E. coli* (60–63). Consistent with this statement, a protective role for IL-22 was recently reported for experimental influenza A virus infection (64).

We also observed a subset of cytokines involved in the regulation of platelet counts and function to be downregulated in the serum of our HPS subjects, including sCD40L, CCL5, CCL22, and CXCL12 (**Table 3**). Consistent with our observations and the pathophysiology of HPS, CXCL12 and CCL22 act on platelets to rapidly stimulate their adhesion (65), and CCL5 and sCD40L are released by activated platelets (66–68). Wenzel and coworkers reported that serum levels of sCD40L closely correlate with platelets counts and that they are increased upon thrombocyte transfusion (69). Viallard et al. also reported a correlation between thrombocyte counts and serum sCD40L, implying that it may be used as a surrogate marker for platelet counts (66). Decreased thrombocyte counts are also well documented in association with HPS (2, 70) and our observation of downregulated sCD40L presents a potential biomarker for the thrombocytopenia. Notwithstanding, decreased serum CCL22 might also reflect the development of the thrombocytopenia observed in HPS cases. It has been shown that CCL22 is capable of aggregating platelets in the presence of low concentrations of thrombin or adenosine diphosphate (ADP), and can rapidly stimulate platelets adhesion (65). It is noteworthy that endothelial cells do not produce this cytokine; dendritic cells are the main source of CCL22 (71). Therefore, the thrombocyte aggregation and depletion observed in HPS may be the result of cytokine-driven immune responses.

Serum levels of CCL21 and CCL27 were also downregulated in the serum of our HPS subjects. These cytokines have tissue-specific activity; for example, CCL21 orchestrates dendritic cell and T cell trafficking to the lymph nodes (72–74) and CCL27 regulates migration of immune effector cells to the skin (75). Taken together, these findings suggest that the cytokines expressed during HPS promote lung tissue infiltration while reducing leukocyte trafficking to other organs and tissues.

In order to investigate the contribution of each respective cytokine to the disease process, we conducted classification analysis by RF. Of the 10 most important cytokines identified by this analysis, 3 were significantly upregulated, as determined by Mann–Whitney analysis; however, we also observed 6 to be downregulated. This observation underscores the importance of cytokine inhibition in the disease process and further suggests that depressed serum cytokine expression may be an important biomarker for monitoring disease progression.

Overall, the majority of downregulated serum cytokines were associated with Th2-type immune activation; these included CCL21, CCL17, CCL13, and CCL11. Furthermore, the cytokines

significantly upregulated in HPS cases were those promoting Th1-type immunity; these included CXCL9, CXCL10, and IL-12(p40). The cytokines M-CSF, CXCL12, IL-3, LIF, GM-CSF, CCL24, which facilitate activation, differentiation, and bone marrow mobilization of myeloid progenitors, were also identified by RF analysis to differentiate HPS cases from controls. RF analysis further identified chemokines associated with platelet aggregation as important in differentiating cases from controls. Interestingly, sCD40L and CXCL12 were ranked, respectively, as the third and fourth most import cytokine in our RF analysis. Chemokines, such as sCD40L, CXCL12, and CCL17, which are stored in platelet granules, are released upon platelet aggregation, a process that is critical in HPS pathology (2, 76–78). Accordingly, nadir platelet counts in HPS may explain low serum CXCL1, CCL17, and sCD40. Taken together, RF analysis supports the supposition that HPS pathogenesis may be characterized by Th1-type immune responses and thrombocytopenia.

In summary, our data suggest that HPS is characterized by a serum cytokine profile that is consistent with putative immune responses in lung tissue. Strong activation of mononuclear immune effectors including T lymphocytes, NK cells, and dendritic cells is also suggested by this cytokine profile. Additionally, our data imply that decreased counts and increased aggregation of thrombocytes in HPS might be explained in part by the immune response to viral infection. Lastly, to the best of our knowledge, our data provide the first evidence of Th17 lymphocyte activation in association with HPS. The data presented in this study are suggestive of putative *in vivo* immune mechanisms and may identify the role of these cytokines in HPS pathophysiology; however, future studies using animal models would be necessary to definitively confirm their involvement.

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REVIEW
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Hantaviral Proteins: Structure, Functions, and Role in Hantavirus Infection

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Hantaviruses are the members of the family Bunyaviridae that are naturally maintained in the populations of small mammals, mostly rodents. Most of these viruses can easily infect humans through contact with aerosols or dust generated by contaminated animal waste products. Depending on the particular Hantavirus involved, human infection could result in either hemorrhagic fever with renal syndrome or in Hantavirus cardiopulmonary syndrome. In the past few years, clinical cases of the Hantavirus caused diseases have been on the rise. Understanding structure of the Hantavirus genome and the functions of the key viral proteins are critical for the therapeutic agents' research. This paper gives a brief overview of the current knowledge on the structure and properties of the Hantavirus nucleoprotein and the glycoproteins.

Keywords: Hantavirus, nucleocapsid protein, glycoprotein, reassortment, MxA protein

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INTRODUCTION

Hantaviruses comprise genus *Hantavirus* within family Bunyaviridae (Elliott, 1990). Humans become infected by either inhaling virus contaminated aerosols or having contact with the urine or droppings of infected animals (Jonsson et al., 2010). In humans hantaviruses cause either hemorrhagic fever with renal syndrome (HFRS) or Hantavirus cardiopulmonary syndrome (HCPS). Generally, each distinct Hantavirus is maintained in nature in the populations of the particular small mammal (rodent or insectivore) host species. Murinae-associated hantaviruses cause HFRS, while Sigmodontinae associated hantaviruses usually cause HCPS. Most of the Arvicolinae-borne hantaviruses (Prospect Hill virus and Tula virus being the most prominent ones) seem to be non-pathogenic for humans (Plyusnin et al., 1994; Schmaljohn and Hjelle, 1997). In accord with the geographic distribution of the virus specific natural hosts, HFRS is mainly diagnosed in Europe and Asia, with murine-borne Hantaan virus (HTNV), Dobrava-Belgrade virus (DOBV), and Seoul virus, as well as arvicoline-borne Puumala virus (PUUV), serving as the main causative agents. HCPS is endemic in the Americas and is caused by a variety of the Sigmodontinae-borne New World Hantaviruses, with Andes virus (ANDV) and Sin Nombre virus (SNV) being the most prominent sources of human infections. Mortality rates vary from 0.3 to 10% for HFRS and between 30 and 40% for HCPS (Jonsson et al., 2010; Macneil et al., 2011; Krautkramer et al., 2013). HFRS clinical symptoms include fever, renal dysfunction, haemorrhagic manifestations, and shock. HCPS is characterized by fever, myalgia, headache, and gastrointestinal symptoms, followed by non-cardiogenic pulmonary oedema, and shock. A summary of the geographic distribution and host affiliation of the most prominent hantaviruses and the diseases they cause is given in **Table 1**.

TABLE 1 | Representative hantaviruses and their rodent hosts.

Rodent host subfamily	Rodent host species	Virus species	Disease	Geographic distribution
Murinae	*Apodemus agrarius*	Hantaan	HFRS	Russia, China, and Korea
Murinae	*A. flavicollis*	Dobrava-Belgrade	HFRS	Balkans and Europe
Murinae	*Rattus norvegicus*	Seoul	HFRS	Worldwide
Sigmodontinae	*Peromyscus maniculatus*	Sin Nombre	HCPS	Western Canada and USA
Sigmodontinae	*P. maniculatus*	Monongahela	HCPS	Eastern Canada and USA
Sigmodontinae	*P. leucopus*	New York	HCPS	USA
Sigmodontinae	*P. leucopus*	Blue River	HCPS	USA
Sigmodontinae	*Sigmodon hispidus*	Black Creek Canal	HCPS	USA
Sigmodontinae	*Oryzomys palustris*	Bayou	HCPS	USA
Sigmodontinae	*S. hispidus*	Muleshoe	HCPS	USA
Sigmodontinae	*S. alstoni*	Cano Delgadito	Not known	Venezuela
Sigmodontinae	*Oligoryzomys longicaudatus*	Andes	HCPS	Argentina, Chile, and Uruguay
Sigmodontinae	*O. longicaudatus*	Oran	HCPS	Argentina
Sigmodontinae	*O. Flavescens*	Lechiguanas	HCPS	Argentina and Uruguay
Sigmodontinae	*Calomys laucha*	Laguna Negra	HCPS	Paraguay and Bolivia
Arvicolinae	*Clethrionomys glareolus*	Puumala	HFRS	Russia, Europe, and Asia
Arvicolinae	*Microtus pennsylvanicus*	Prospect Hill	Not known	North America
Arvicolinae	*M. ochrogaster*	Blood Land Lake	Not known	North America
Arvicolinae	*M. arvalis*	Tula	Not known	Europe, Russia, and Asia
Arvicolinae	*M. californicus*	Isla Vista	Not known	USA and Mexico

HANTAVIRUS GENOME STRUCTURE AND LIFE CYCLE

Hantavirus virions have spherical shape with size varying between 80 and 120 nm. Hantavirus genome is comprised of three segments of single stranded negative sense RNA. Based on their size, these three segments are named small (S), medium (M), and large (L). L segment encodes viral polymerase, while M and S segments encode the precursor (GPC) for two viral surface glycoproteins (G1 and G2, or alternatively called Gn and Gc), and the nucleocapsid (N) protein, respectively. Each virion generally contains equimolar amounts of genomic RNA, with a single molecule of the viral RNA-dependent RNA polymerase (RdRp) being attached to each segment of viral RNA. All viral RNA segments are coated with the molecules of the N protein forming ribonucleoproteins (RNPs; Elliott, 1990). These are enclosed by an envelope consisting of a lipid bilayer, with G1 and G2 surface glycoproteins embedded into it (Elliott, 1990).

Hantavirus virion attachment to the host cell via cellular receptors is followed by endocytosis. RNPs are released to the cytoplasm from the late endosome following pH-mediated membrane fusion. Transcription and translation take place either at the site of RNPs release or at the endoplasmic reticulum–Golgi intermediate compartment (ERGIC). In case of the latter, the RNPs are transported to ERGIC. The viral polymerase, RdRp, possesses transcriptase, replicase and endonuclease functions; thus, it carries out both virus transcription (**Figure 1A**) and replication (**Figure 1B**). To initiate transcription, RdRp cleaves cellular mRNA forming capped primers. Recently, cellular endonucleases have also been suggested to participate in capped primer formation by cleaving cellular mRNAs which cap-structures are protected from degradation by the specifically bound viral N protein (Mir et al., 2008). These capped primers initiate transcription of viral mRNAs. S segment derived mRNA serves as a template for the N protein, and for some particular hantaviruses also it produces a non-structural NSs protein (**Figure 1C**). M segment derived mRNA produces GPC on the ER membrane-bound ribosomes. G1 and G2 glycoproteins are transported from the ER to the Golgi complex or to the plasma membrane where assembly takes place (**Figure 2**). Old World hantaviruses assemble at the Golgi while New World hantaviruses assemble at the plasma membrane (Elliott, 1990; Ravkov et al., 1998; Spiropoulou, 2013) (**Figure 2**). After assembly, the newly formed envelope contains spike-like projections (Elliott, 1990; Welsch et al., 2007; Hepojoki et al., 2012) formed by the tetramers of the viral surface glycoproteins, which apparently play an important role in both virus assembly and cell entry (Welsch et al., 2007; Lyles, 2013; Yamauchi and Helenius, 2013). Newly assembled virions are released through exocytosis.

STRUCTURE AND PROPERTIES OF HANTAVIRUS GLYCOPROTEINS

Hantavirus surface glycoproteins G1 and G2 are coded by the M segment and are expressed as a polyprotein precursor, GPC, which is cleaved by a cellular protease during translocation to ER yielding mature G1 and G2 glycoproteins (**Figure 1C**) (Pensiero and Hay, 1992; Plyusnin et al., 1996). Cryo-electron microscopy and cryo-electron tomography studies have shown that G1 and G2 proteins form square-shaped surface spikes protruding from the viral membrane, with each spike complex made of four G1 and four G2 subunits (Huiskonen et al., 2010; Battisti et al., 2011). It has been shown that these G1/G2

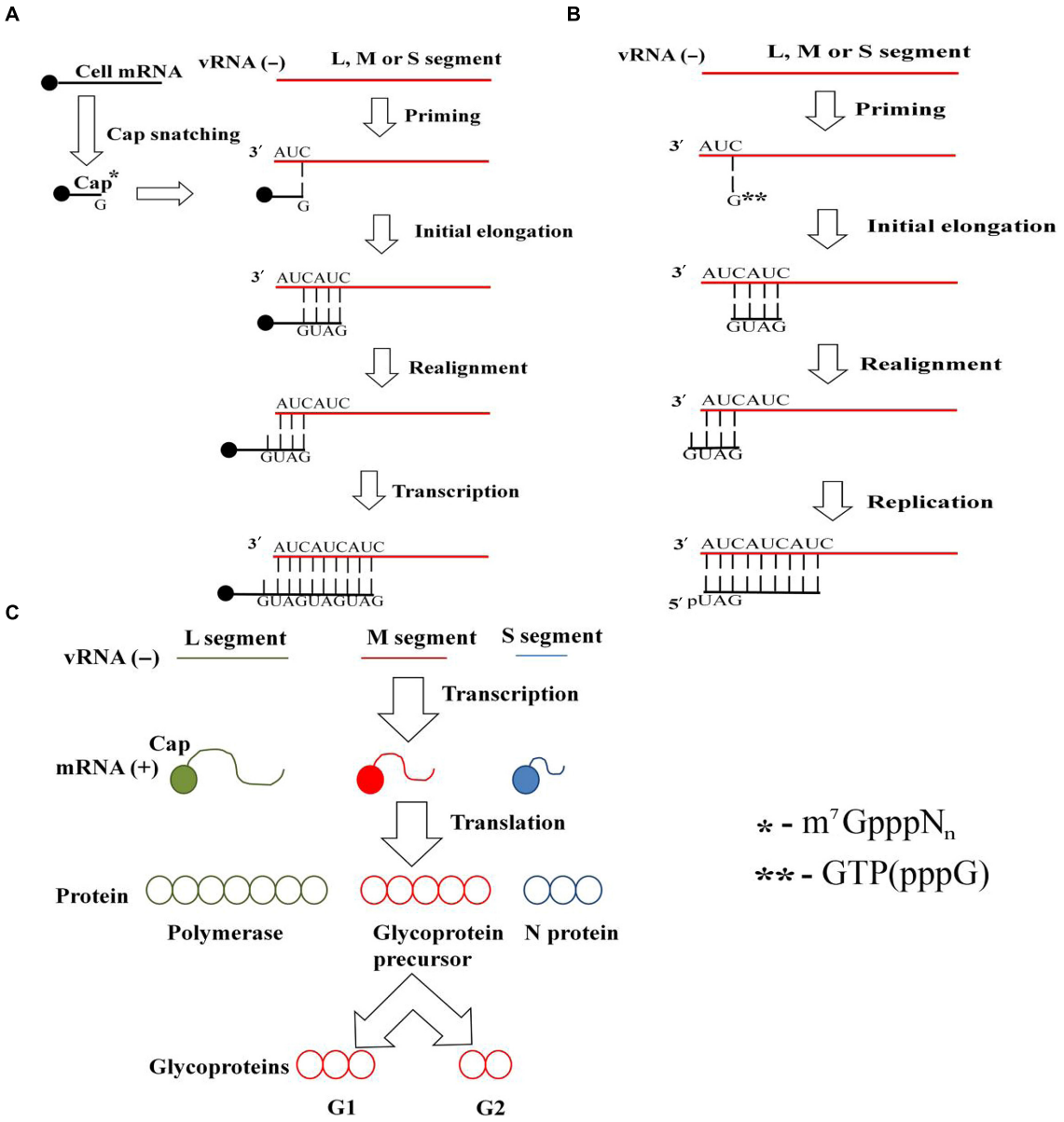

FIGURE 1 | Hantavirus transcription, replication and translation. (A) Hantavirus transcription. Transcription occurs through a prime and realign mechanism. Cellular mRNA is cleaved by either Hantavirus RNA-dependent RNA polymerase (RdRp) or cellular endonucleases in a process called cap snatching, thus forming a capped primer (m^7GpppN_n). It is this capped primer that initiates transcription by aligning its guanidine to the 3′ cytosine of the vRNA. After synthesis of several nucleotides, the nascent RNA slips back and realigns. Final elongation then takes place, producing an extra copy of viral mRNA. **(B)** Replication of Hantavirus RNA. Replication takes place in cytoplasm of the infected cell, using prime and realign mechanism. RdRp attached to the 3′ end of vRNA aligns guanidine triphosphate (pppG) residue to the first cytosine of the virus RNA and synthesizes the first three nucleotides of the new cRNA strand. The nascent RNA slips back and realigns after successive addition of bases. Then, final elongation takes place, resulting in production of the full length cRNA. In turn, this positive strand anti-genomic cRNA serves as a template for producing a large amount of the new strands of vRNA. **(C)** Hantavirus transcription and translation. Negative sense viral RNA serves as a template for the viral RdRp, which initiates transcription by cap-snatching mechanism and generates viral mRNA. Viral mRNAs are translated producing N protein, glycoprotein precursor (which is cleaved to form G1 and G2 glycoproteins), and RdRp from the small (S), medium (M), and large (L) segment-originated mRNA, respectively.

glycoprotein heterodimers may interact with specific cellular surface proteins, β_3-integrins, facilitating cellular entry of HCPS-causing hantaviruses (Gavrilovskaya et al., 1998).

Both G1 and G2 glycoproteins are built in a similar way, each containing a large globular domain, a hydrophobic transmembrane sequence and a small C-terminal cytoplasmic tail. Since no matrix proteins exist connecting Hantavirus nucleoproteins and envelope proteins, it is suggested that there is a direct interaction between N protein and cytoplasmic tails of G1 and G2. Nuclear magnetic resonance spectroscopy has

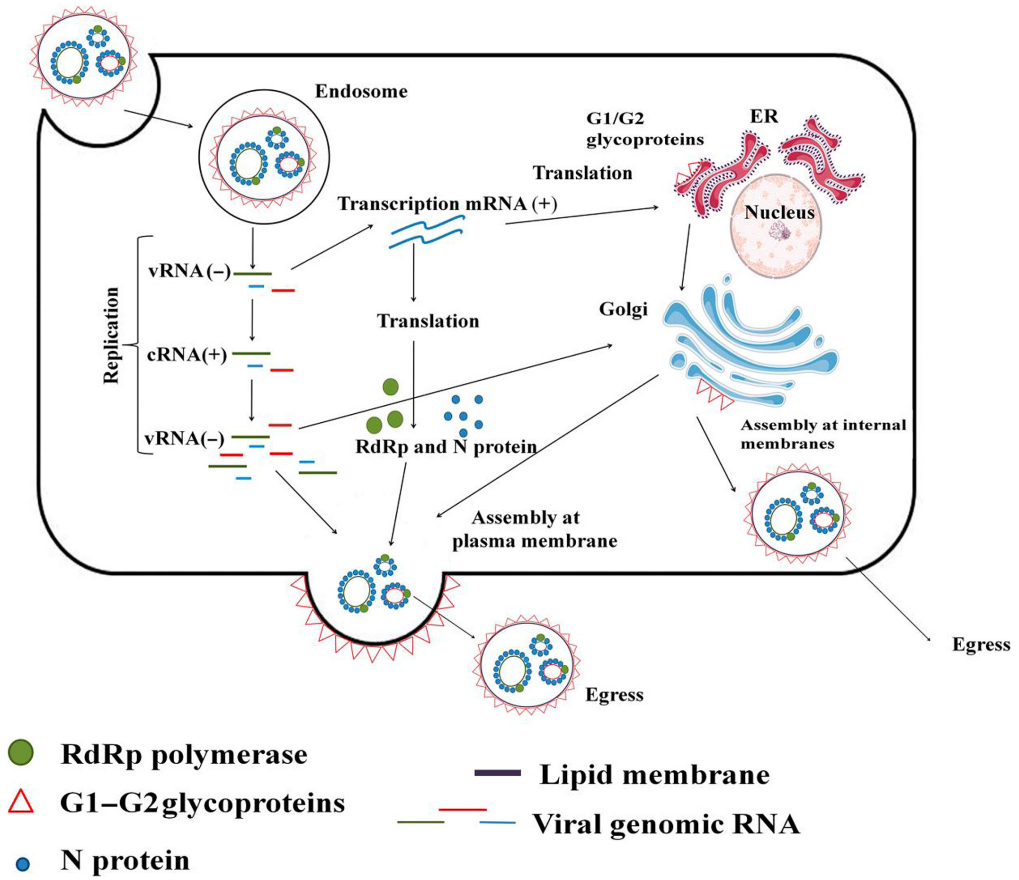

FIGURE 2 | Hantavirus life cycle. Virion binds to the cell surface membrane receptor and enters the cell via endocytosis. Once inside the cell, RNPs are released from the late endosome via pH-mediated membrane fusion. Virion-supplied RdRp is driving initial mRNA transcription which takes place in cytoplasm. Viral genomic (minus sense) RNA serves as a template for generation of mRNA utilized for protein synthesis. When sufficient amounts of the viral proteins are produced, RdRp switches to the replication mode synthesizing full length anti-genomic (plus sense) RNA, which in turn serves as a template for producing a large amount of the new full length minus sense viral RNA molecules. Newly synthesized vRNA becomes encapsidated with N protein forming ribonucleoprotein and transported into perinuclear membrane system, from where they will be transported to Golgi for initiation of virion formation. Egress takes place at the plasma membrane.

shown that a part of the G1 tail (residues 543–599) has a double ββα-fold zinc finger made up of a highly conserved motif that has high similarity between ANDV and Prospect Hill viruses (PHV) (Estrada et al., 2009, 2011). It has been suggested that these zinc fingers play a role in virus assembly (Estrada and De Guzman, 2011).

GLYCOPROTEIN INTERACTION AND TRAFFICKING

Maturation of Bunyavirus glycoproteins takes place in the Golgi complex (Pettersson and Melin, 1996). During maturation, G1 and G2 glycoproteins are N-glycosylated. Three glycosylation sites are located on G1 and only one on G2 glycoprotein. Both G1 and G2 glycoproteins are sensitive to endoglycosidases H and F. It has been reported that G1 and G2 targeting to Golgi depends on conformational interaction between these two glycoproteins (Shi and Elliott, 2002; Deyde et al., 2005). Furthermore, it appears that G1 glycoprotein plays an important role in facilitating Golgi trafficking of both glycoproteins. For example, when G1 glycoprotein is expressed individually, it becomes partially localized to Golgi, while the majority of this protein is localized in the endoplasmic reticulum (Deyde et al., 2005). However, when G2 is individually expressed, it becomes exclusively localized in the endoplasmic reticulum (Ruusala et al., 1992; Spiropoulou et al., 2003). It is important to note that glycoproteins from different hantaviruses are capable to interact resulting in proper targeting to the Golgi complex (Deyde et al., 2005).

GLYCOPROTEIN-INDUCED VIRULENCE

Viral infection ignites innate immune responses aimed to reduce viral replication. Type I interferons (IFNs) play a pivotal role in providing direct antiviral protection as well as activating natural killer (NK) cells, the key effector cells of the innate immune response. On the other hand, in order to survive viruses develop mechanisms to prevent elimination by inhibiting pathways

activating type I IFN transcription (Finlay and McFadden, 2006; Yoneyama and Fujita, 2007). It has been shown that expression of the G1 protein cytoplasmic tail of pathogenic hantaviruses (Alff et al., 2006) inhibits the induction of IFN-β. This ability distinguishes pathogenic hantaviruses from non-pathogenic ones, as the latter are incapable of inhibiting IFN-β induction (Alff et al., 2006). The G1 cytoplasmic tail of the pathogenic hantaviruses has been shown to inhibit IFN-β transcription by binding to TRAF3 (Alff et al., 2008) and preventing RIG-I/TBK1-directed IRF3 phosphorylation (Mackow et al., 2014; Matthys et al., 2014). TRAF3 is an E3 ubiquitin ligase that forms a TBK1–TRAF3 complex, which is crucial for IRF3 phosphorylation. Phosphorylation of IRF3 is vital in IFNβ induction.

HANTAVIRUS REASSORTMENT AND COMPATIBILITY OF THE HANTAVIRUS PROTEINS

Reassortment, i.e., exchange of the genome segments between different virus strains, plays an essential role in maintaining segmented viruses and can produce new strains with novel characteristics and improved survivability. The evolution of the Rift Valley Fever virus (RVFV) presents an example of how novel reassortants can be naturally generated in endemic areas (Sall et al., 1997). Rapid virus evolution caused by such reassortment may bring about global outbreaks (Webster and Laver, 1971; Young and Palese, 1979). Also, ability to generate reassortants may put people living in the endemic areas at the risk of generating uncontrolled chimeric viruses by using live attenuated vaccines (Sall et al., 1997). Initially, genetic reassortment has been shown among members of arthropod-borne Bunyaviridae (Beaty et al., 1985; Chandler et al., 1991). Later on, genetic reassortment between different Hantavirus strains in nature has been documented as well (Henderson et al., 1995; Li et al., 1995). Li et al. (1995) proposed that such reassortment could lead to the emergence of new Hantavirus strains with novel epidemiological characteristics.

Reassortment is most likely to occur between genetically different strains of the same Hantavirus, or between closely related hantaviruses circulating within closely related rodent host species. Despite being rather rare, reassortment among hantaviruses that are distantly related is also possible in nature. It is well-known that Hantavirus infection could "spill over" to the non-specific host when different animal species share the same ecological niche. This could potentially result in dual infection of a single animal. Replication of two different hantaviruses in the same host organism may produce reassortants with new characteristics (Henderson et al., 1995; Rodriguez et al., 1998).

In vitro studies have proven that genetic reassortants can be developed between distantly related hantaviruses (Rodriguez et al., 1998; Kang et al., 2002). In details, the ability of two distantly related hantaviruses to develop reassortants *in vitro* have been investigated by Rizvanov et al. (2004). The authors have shown that ANDV and SNV (**Figure 3**) can generate reassortants with novel infectivity characteristics which differ

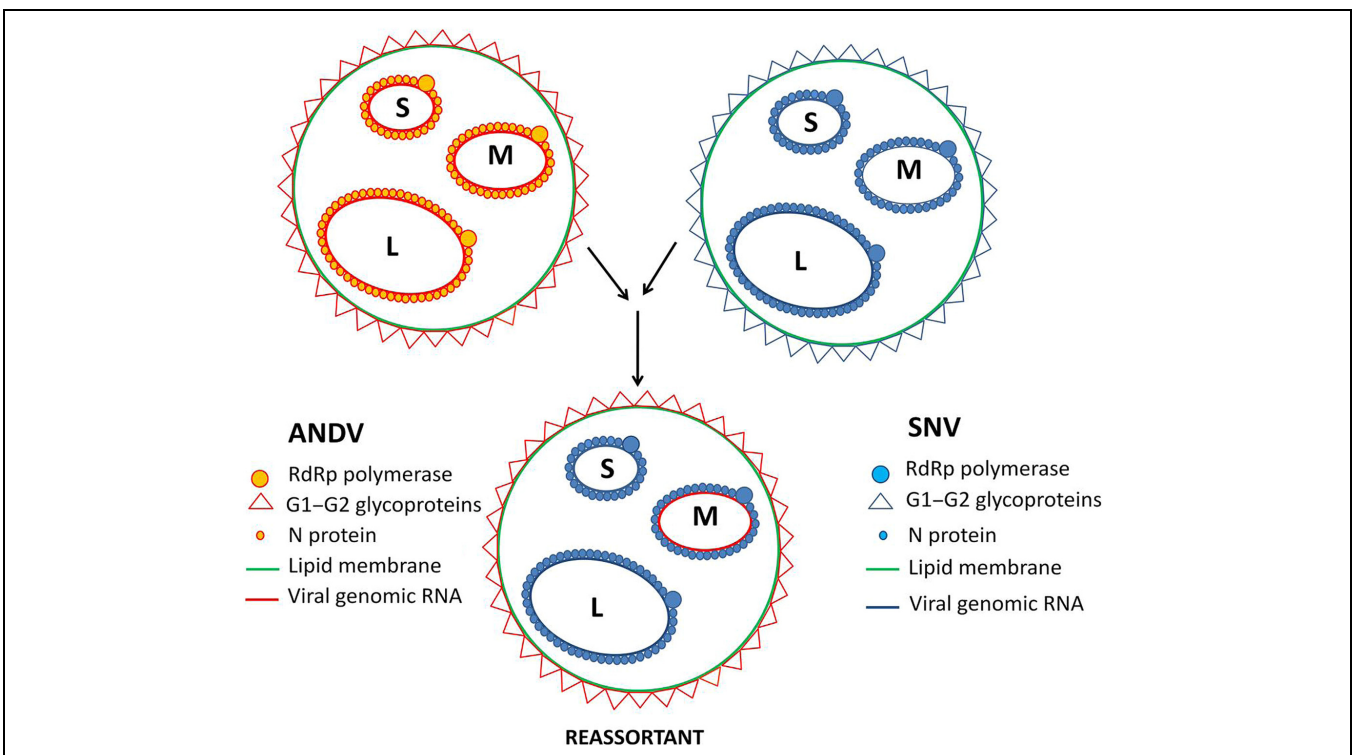

FIGURE 3 | Hantavirus reassortment. Infection of one host with two different Hantavirus strains may result in reassortment. Reassortment has been shown between ANDV and SNV resulting in reassortants possessing ANDV M segment and SNV L and S segment.

from both parental strains. Noteworthy, the resulting reassortant viruses always retained the S and L segments of the same parental Hantavirus, while the M segment was introduced from the other one. In spite of both viruses being *Sigmodontinae*-borne, their rodent hosts are separated geographically. SNV circulates in North America, while ANDV endemic for South America (Plyusnin and Morzunov, 2001). These data are in agreement with the previous finding published by Urquidi and Bishop (1992) demonstrating that the reassortant progeny between Bunyaviruses contains homologous S and L segments. Few progeny contain S and L segments that are heterozygous, i.e., virion contains corresponding segments from both parental strains. Virus progeny of the hantaviruses that are closely related have a different prevalence of homologous L and S segments from those that are distantly related (Rodriguez et al., 1998; Scmaljohn and Hopper, 2001).

The M segment plays an important role in Bunyavirus replication (Beaty et al., 1981) and is also known to alter the efficiency of virus budding. Glycoproteins encoded by the M segment take part in cell surface attachment, thus, they are essential for viral entry into the host cell. It is interesting to note that the stable reassortment progeny between SNV and ANDV contained the M segment from the virus which has higher capacity to replicate in the host cell type used in the experiments (Rizvanov et al., 2004). This data supports the notion that reassortment strategy utilized by segmented viruses may generate novel virus strains with higher capacity to propagate. Two additional conclusions that could be drawn from the reassortment experiments are: (i) the viral RdRp obviously works much better on the viral RNA template coated with the homologous N protein, and (ii) cytoplasmic tails of the Hantavirus G1/G2 glycoproteins seem to interact with the heterologous RNPs at least as efficiently as with the homologous ones.

Ability to generate reassortants between distantly related hantaviruses provides a tool for studying the role of each viral segment (and corresponding viral protein) in pathogenesis of infection. Also, reassortants can be used for analyzing the Hantavirus specificity to the animal host.

STRUCTURE AND PROPERTIES OF THE HANTAVIRUS N PROTEIN

The Hantavirus N protein consists of approximately 433 amino acid residues (about 50 kDa in size). The N protein appears to be highly conserved among different hantaviruses. It has been shown that large amounts of N protein are expressed early after infection (Vapalahti et al., 1995). Also, it has been demonstrated that early immune response in Hantavirus patients is directed mainly against N protein. Therefore, many virus diagnostics developed are based on detecting Hantavirus N protein or anti-N protein antibody (Amada et al., 2013; Yoshimatsu and Arikawa, 2014).

The N protein is expressed exclusively in the cytoplasm (Elliott et al., 2000) of the infected cell. Hantavirus N protein plays a pivotal role in the virus life cycle as it is required for encapsidating viral RNA, as well as regulating virus replication and assembly.

RNA BINDING AND RIBONUCLEOPROTEIN ASSEMBLY

The N protein protects viral genomic RNA from degradation by cellular nucleases by forming viral RNPs. The mechanisms of RNA encapsidation are not completely understood. It has been shown that the N protein selectively interacts with Hantavirus RNA, encapsidating vRNA (negative sense genomic) and cRNA (positive sense anti-genomic) while leaving viral mRNA free. Selective encapsidation is thought to be possible due to presence of the unique panhandle terminal structure formed by the self-complimentary terminal sequences of the full length vRNA and cRNA. It has been demonstrated that these 23 nucleotides-long terminal sequences can serve as a binding site for the viral RdRp and have high affinity to the N protein (Mir and Panganiban, 2004). In particular, the N protein of some hantaviruses, such as HTNV, has been proven to preferably bind to its S segment vRNA rather than to the S segment open-reading frame or non-specific RNA. This may suggest that the N protein recognition site resides in the non-coding region of HTNV vRNA (Severson et al., 1999). It was later reported that such binding depends on the 5′ end sequence of the S segment vRNA (Severson et al., 2001).

N PROTEIN INTERACTS WITH HUMAN MxA(p78) Protein

It has been shown that the efficiency of Hantavirus replication is inversely proportional to the ability of infected cells to activate MxA expression (Kanerva et al., 1996). MxA protein is a key component of the type I IFN-induced antiviral state providing resistance to a wide range of the RNA viruses (Pavlovic et al., 1993). There are two types of Mx proteins in humans, MxA and MxB (Horisberger et al., 1988), with only MxA known to possess anti-viral activity (Haller and Kochs, 2002). Interferon regulatory factor 3 (IRF-3) regulates activation of MxA gene transcription (Baigent et al., 2002). Generally, IRF-3 is present in the cytoplasm of the cell in a dormant state (Reich, 2002). However, upon infection, IRF-3 translocates into the nucleus, where it initiates transcription of MxA and other IFN inducible genes (Reich, 2002; Melchjorsen and Paludan, 2003). It has been shown that IRF-3 nuclear translocation can occur as early as 24 after Hantavirus infection (Khaiboullina et al., 2005).

MxA activation has been shown to vary in different cell types (Khaiboullina et al., 2005). For example, high MxA activation level was demonstrated in human umbilical cord endothelial cells (HUVECs), while ativation of MxA in VeroE6 cells was virtually undetectable. Further studies have shown that Hantavirus replication efficacy is inversely proportional to the ability of infected cells to activate expression of MxA protein (Kanerva et al., 1996; Khaiboullina et al., 2005). These data suggest that variations in Hantavirus replication may partially depend on ability of the particular cell types to activate MxA

protein. In turn, MxA protein is known to bind to the N protein forming an MxA/N protein complex (Khaiboullina et al., 2005). Formation of MxA/N complexes has been suggested for some other Bunyaviruses as a potential mechanism of MxA inhibition of viral replication (Kochs et al., 2002). Thus, it is very likely that in the case of hantaviruses the mechanism of MxA inhibition is similar.

HANTAVIRUS INFECTION ACTIVATES THE INNATE IMMUNE RESPONSE

Increased microvascular permeability is characteristic for hantavirus infections (Zhang et al., 1987; Enria et al., 2001; Hepojoki et al., 2014). However, permeability of endothelial cell monolayer did not change after Hantavirus infection *in vitro* (Khaiboullina et al., 2000; Sundstrom et al., 2001). Hantavirus infection is not cytopathic, therefore, it has been suggested that an increased microvascular leakage is most likely associated with cell response to infection, rather than related to virus replication. A DNA microarray conducted to determine changes in cell responses in Hantavirus infected cells showed that non-pathogenic (PHV) and pathogenic (SNV) hantaviruses have different effects on transcriptional activity in infected cells (Khaiboullina et al., 2004). In particular, it has been shown that PHV infection activates approximately five times less genes than the SNV infection does (36 genes were up-regulated in PHV-infected cells in comparison to 175 genes in SNV-infected cells). As infection progressed, more changes in transcriptional activation were detected.

Activation of nuclear and transcriptional factors was shown to vary in cells infected with pathogenic versus non-pathogenic hantaviruses (17 vs. 8; Khaiboullina et al., 2004). Also, Hantavirus infection activates IRF-7, IRF-1, and IRF-9 transcription factors (Khaiboullina et al., 2004). Interestingly, the transcriptional activity of these factors was lower in non-pathogenic (PHV) than in pathogenic (SNV) Hantavirus. Although no changes in transcriptional activity of IRF3 were noted, nuclear translocation of this factor in Hantavirus infected cells has been shown by immunohistochemistry (Khaiboullina et al., 2005). Nuclear translocation is essential for IRF3 activity which includes activation of IFN inducible genes as well as activation of cytokines. It has been demonstrated that IRF-3 controls activation of CCL5 gene transcription, while IRF-1 and IRF-3 regulate expression of MxA protein (Baigent et al., 2002). Up-regulation of CCL5 and MxA has been shown in Hantavirus-infected cells. Therefore, it could be concluded that Hantavirus-induced activation of IRF1 and IRF3 may lead to changes in cytokine and IFN inducible protein expression in infected cells.

DNA Array data have shown upregulation of several genes controlling processes of apoptosis, growth and proliferation. For example, upregulation of transcriptional activity of Bcl2 gene has been detected in Hantavirus infected HUVECs. Also, Hantavirus infected cells are characterized by transcriptional activation of vascular endothelial growth factor (VEGF), a survival factor for endothelial cells, which prevents apoptosis by inducing Bcl-2 expression. It has been shown that VEGF and Bcl2 cooperate to prevent apoptosis *in vitro*. For instance, increased expression of Bcl-2 was found in neuroblastoma cells treated with VEGF. Also, VEGF abrogates apoptosis induced by TNF-α-induced serum starvation (Pidgeon et al., 2001; Beierle et al., 2002). Therefore, it could be suggested that activation of Bcl2 and VEGF can explain absence of apoptosis in Hantavirus infected cells.

It has been suggested that cytokines play important role in pathogenesis of the vascular leakage in Hantavirus infected microvascular beds (Zaki et al., 1995). DNA Array data have shown an increased expression of a cluster of CC chemokine genes including RANTES (CCL5; Khaiboullina et al., 2004). Also, data presented by Geimonen et al. (2002) have demonstrated that transcriptional activation of CCL5 is characteristic for HTNV and PHV infection of endothelial cells. It is known that CCL5 plays a role in regulation of immune effectors migration to the site of infection (Schall et al., 1990). Interestingly, mononuclear leukocyte accumulation is a histological hallmark of Hantavirus infection (Zaki et al., 1995). One could suggest that increased traffic of immune effectors through the endothelial monolayer may lead to its damage and, thereby, making it more permeable (Schall et al., 1990).

N PROTEIN'S ROLE IN REGULATION OF ANTIVIRAL STATE

Expression of the glycoproteins of the pathogenic hantaviruses inhibits INF-β and TBK-1 induction via virulence determinants present on the G1 cytoplasmic tail. However, it has been suggested that, this alone may not be sufficient to make them virulent and some other virulence factors may play role (Matthys et al., 2011). Recently, it has been demonstrated that the ANDV N protein hinders autophosphorylation of TBK1 resulting in the inhibition of IRF3 phosphorylation and RIG-I/MDA5-directed type I IFN induction (Cimica et al., 2014). Additionally, the N protein can affect the protein kinase R (PKR) dimerization (Wang and Mir, 2015). It has been demonstrated that the Hantavirus N protein prevents PKR phosphorylation, which is essential for its enzymatic activity. PKR inhibits virus replication and is essential for establishing antiviral state (Goodbourn et al., 2000). PKR activates IFN via NF-κB and IRF1 up-regulation (Kumar et al., 1997). Additionally, PKR can activate apoptosis in infected cells (Gil and Esteban, 2000). Therefore, it could be suggested that the glycoproteins and the N protein may interfere with antiviral activity in infected cells, thus promoting viral replication.

SUMMARY

There are two clinical entities associated with Hantavirus infection, HFRS and HPS. The mortality rate may vary from 0.1 to 40% depending on the particular Hantavirus involved. The Hantavirus genome is composed of a three negative sense single stranded RNA segments coding for the N protein, G1 and G2 glycoproteins and viral polymerase. Genetic reassortment

between different hantaviruses has been documented both in nature and *in vitro*. Such reassortment could result in emergence of the novel Hantavirus strains with new virulence characteristics and/or new host range.

Emerging evidence suggests that the Hantavirus N protein plays a major role not only in virus replication, transcription and virus assembly, but also in establishing favorable environment for virus replication within the host cell. Pathogenic hantaviruses cause more pronounced changes in transcriptional activity of various cellular genes as compared to non-pathogenic strains. Activation of CCL5 may contribute to Hantavirus-induced leukocyte accumulation in infected tissue and, potentially, to pathogenesis of vascular permeability. The Hantavirus N protein interacts with host proteins interfering with activation of the antiviral pathways in infected cells.

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Early Life Factors Associated with Adult-Onset Systemic Lupus Erythematosus in Women

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Background: Exposure early in life can influence adult disease and immunity, but the role of early life exposures in risk of systemic lupus erythematosus (SLE) is not established.

Methods: Women in a national cohort (ages 35–74) provided data on perinatal, maternal, and sociodemographic factors, longest residence to age 14, and residential farm history of at least 12 months to age 18. Cases (*N* = 124) reported SLE diagnosed age 16 years or older with use of disease modifying antirheumatic drugs. Non-cases (*N* = 50,465) did not report lupus. Odds ratios (OR) and 95% confidence intervals (CI) were estimated by logistic regression adjusting for age and race/ethnicity.

Results: SLE was associated with low birthweight (data on 84 cases and 36,477 non-cases; <2,500 versus 3,000 to <3,500 g OR = 2.2; 95%CI 1.2, 3.9) and preterm birth (57 cases and 22,784 non-cases; ≥1 month early versus full-term OR = 3.4; 95%CI 1.6, 7.4). Considering longest childhood residence to age 14, SLE was associated with more frequent pesticide use (e.g., at least monthly OR = 2.3; 95%CI 1.3, 4.1). SLE was associated with having an early and extended childhood farm residence (i.e., prenatal/maternal farm exposure and longest childhood farm residence OR = 1.8; 95%CI 1.1, 3.0 versus neither). In those with a childhood-only farm residence of 12+ months, agricultural pesticide use was associated with SLE, with the strongest associations for direct personal exposures.

Conclusion: The association of SLE with preterm birth is consistent with studies in other populations and with an observed association with low birthweight. The associations of SLE with childhood exposure to residential and agricultural pesticides warrant further study.

Keywords: lifecourse epidemiology, pesticide exposure, birthweight, preterm birth, autoimmune disease

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by immune reactivity to multiple nuclear components and inflammation, resulting in diverse clinical features and multiple organ involvement. The causes of SLE are generally not known. Racial disparities and increased familial risk suggest a genetic predisposition. It is believed that environmental factors may contribute

to the development of disease, but knowledge on specific risk factors is mostly limited to occupational exposures (1).

The developmental origins hypothesis has been proposed for many adult-onset, chronic inflammatory diseases, including SLE (2–6). Exposures during and after gestation, including nutritional, infectious, chemical, and psychosocial factors (7–11), play a critical role in shaping future immunity. A few epidemiologic studies have examined perinatal and childhood factors associated with SLE, with results including evidence of an association with preterm birth and inconsistent associations with birthweight (12, 13).

Socioeconomic factors have been associated with disease severity, inflammation, and adverse outcomes in SLE (14, 15). Although socioeconomic disparities originating from childhood have been described across a variety of chronic inflammatory diseases (16), the role of childhood socioeconomic status (SES) and related adversities in risk of SLE is not known. Other early life immune modifying exposures (e.g., infections, vitamin D) have also been considered in relation to SLE risk (17).

History of farm occupation and pesticide exposures has been associated with SLE (18–20), but the role of childhood exposures have not been specifically examined. One past study suggested an association of SLE with mixing pesticides in a farm environment (21), while another showed a dose–response association personal pesticide in women use with rheumatoid arthritis (RA) and SLE that was highest in women with a farm history (18). Women who live or work on a farm as adults may be more likely to have grown up on a farm. Therefore, it is plausible that previous studies showing SLE associations with farm-related pesticide use may reflect to some extent an association with childhood exposures.

In this study, we examine associations between adult-onset SLE in a national cohort of women and a range of perinatal and early life exposures, some of which had previously been associated with RA in this same cohort (22). Investigating the role of early life factors in risk of SLE will help us to understand what factors underlie susceptibility for developing autoimmune disease in adulthood. Along with knowledge of genetic susceptibility, understanding the developmental origins of SLE risk may help identify opportunities for prevention and mechanisms contributing to immune dysregulation.

MATERIALS AND METHODS

Sample and Case Identification

We conducted a case–control analysis using baseline data from the NIEHS Sister Study cohort ($N = 50{,}884$ women ages 35–74, enrolled 2003–2009). Women were eligible if they had a sister with breast cancer, but did not have breast cancer themselves at enrollment (23). Participants were identified through self-referral following a broad advertising campaign in the U.S. and Puerto Rico. The Sister Study was approved by the Institutional Review Board of the National Institute of Environmental Health Sciences, NIH. Participants provided written informed consent.

Probable cases ($n = 124$) were classified based on a self-reported doctor's diagnosis of SLE at age 16 or older and reported use of disease modifying antirheumatic drugs (DMARDs). Cases with missing data on medications or not reporting DMARDs ($N = 153$), reporting non-specified or discoid lupus only ($N = 61$), or diagnosis before age 16 were excluded ($n = 6$). The most common DMARDs reported by probable cases were hydroxychloroquine (90% ever and 70% currently used) and methotrexate (11% ever and 10% current). No data on disease severity or nephritis were available; however, medications often used to treat nephritis were less common (e.g., Azothioprine; 8% ever used). Five probable cases (5% of 124) also reported a diagnosis and DMARD use for RA. Non-cases ($n = 50{,}465$) reported no lupus. Other possible cases of autoimmune disease were not excluded from the referent, but are unlikely to influence results due to their relative rarity in the overall sample.

Exposures and Covariates

Computer-assisted telephone interviews collected data on sociodemographic factors, childhood residential history, lifestyle factors, personal medical history, and medication use. Questions on sociodemographic factors included food insecurity ("When you were growing up, were there times your family didn't have enough to eat?"), highest household education level when respondent was age 13, and relative household income while growing up (well off, middle income, low income, or poor). Adult sociodemographic covariates included participant age (baseline), race/ethnicity (dichotomized for this analysis as non-Hispanic White and non-White, including black, Hispanic, and other racial and ethnic groups), and education level (dichotomized for this analysis as less than a college degree, including <12 years, high school degree or equivalent, some college but no degree, or associate degree/technical degree versus college degree, including college graduate/bachelor's degree and graduate/professional degree).

Data on the longest childhood residence to age 14 years and childhood farm residence of 12 months or longer to age 18 were collected by computer-assisted telephone questionnaire. For the longest childhood residence, participants born in the U.S. were grouped by state into: (1) census regions (West, Midwest, Northeast, South, and Puerto Rico) and (2) stratified by 40th and 35th parallels (approximate according to U.S. state). Questions about the longest childhood residence included whether the residence was a farm, had been a farm, or was near a farm, and whether pesticides were used in and around the house or garden, including frequency of use. Women who did not report a farm as longest childhood, adult, or current residence were also asked whether they had lived on any other farm for 12 or more months. A detailed questionnaire then asked about pesticide use on crops and animals, including opportunities for personal exposures (i.e., personally mixed or applied, and presence in the field when pesticides were being sprayed) for all residential farm experience 12 months or more up to age 18. Similar questions were asked for residential and agricultural pesticides based on current and longest adult residence and any farm residence 12 months or more ages 18 years and older.

Participants were also mailed family history questionnaires that included assessment of perinatal factors and were encouraged to contact their mother or other relatives to assist with as needed to provide data on maternal age at participants' birth, prenatal, maternal, and household smoking, maternal farm residence or work on farm while pregnant, breast feeding, birthweight (pounds, ounces), and gestational age at birth. Participants unable to report exact birthweight were asked whether birthweight was

<5 pounds or at least 5 pounds, and responses were converted to grams and categorized (<2,500; 2,500–3,499; 3,400–3,999; and ≥4,000 g).

Birth order was calculated from brother birthdates reported in the family history questionnaires and sister birthdates reported during the computer-assisted telephone interviews. Other perinatal factors [multiple birth, soy formula, maternal/gestational diabetes, gestational hypertension or preeclampsia, and diethylstilbestrol (DES) exposure] were not considered due to low prevalence.

Analyses

Analyses were conducted in SAS (version 9.2, SAS Institute Inc., Cary, NC, USA). Logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI). Significant findings were those in which the 95%CI excluded the null (OR = 1.0), corresponding to a two-sided $p < 0.05$. Initial models were age-adjusted and secondary models also adjusted for race/ethnicity. We also evaluated associations with a childhood SES adversity score [a sum of four factors – young maternal age at birth (<20 years), low or poor relative childhood household income, maximum household education less than college, and childhood food insecurity], which was previously examined with respect to RA in this cohort (22). For significant associations, we examined possible confounding by education and conducted sensitivity analyses excluding non-Whites (a higher risk subgroup and potential effect modifier) and women over age 60 (with higher potential for errors in reporting of perinatal and childhood factors). Results were not substantially changed unless otherwise noted. Data on adult factors (education, residential pesticides, and farm residence) were used to explore potential joint effects and identify independent "childhood only" exposures.

RESULTS

Cases were slightly younger than non-cases and enrolled in the cohort within a median of 11 years since diagnosis (**Table 1**). Cases were more likely than non-cases to be of non-White race/ethnicity (including black or Hispanic) and were less likely to have graduated from college.

Systemic lupus erythematosus was associated with low birthweight, with a linear dose response (p-trend = 0.008) and with preterm (at least 1 month early) versus term birth (**Figure 1**). Excluding preterm births (seven of eight preterm cases also had a low birthweight), the overall trend was no longer significant ($p = 0.08$; not shown); however, when the sample was restricted to women under age 60, the trend across low birthweight categories became significant again ($p = 0.03$, not shown). For women providing exact birthweight, cases ($N = 84$) weighed 6.8 (SD 1.6) pounds at birth compared with 7.2 (SD 1.3) in non-cases ($N = n = 36{,}477$), for an adjusted OR = 0.80; 95%CI 0.69, 0.94 per pound.

TABLE 1 | Characteristics of probable SLE cases and non-cases in the Sister Study.

	Probable SLE *N* = 124	Non-cases *N* = 50,465
	Median years (IQR)	
Age at enrollment	54 (47, 60)	55 (60, 61)
Age at diagnosis	43 (33, 50)	N/A
Time since diagnosis	11 (3, 16)	N/A
	N (%)	*N* (%)
Race/ethnicity[a]		
White	86 (69)	42,269 (84)
Non-White	38 (31)	8,181 (16)
Educational attainment		
≤High school degree	16 (13)	7,725 (15)
Some college or technical degree	59 (48)	17,010 (34)
4-year college graduate	25 (20)	13,633 (27)
Professional or graduate degree	24 (19)	12,085 (24)

[a]*Non-Hispanic White and non-White (Black – 19% of cases versus 9% of non-cases, Hispanic 9% of cases versus 5% of non-cases, and other racial and ethnic groups – 3% cases and non-cases).*

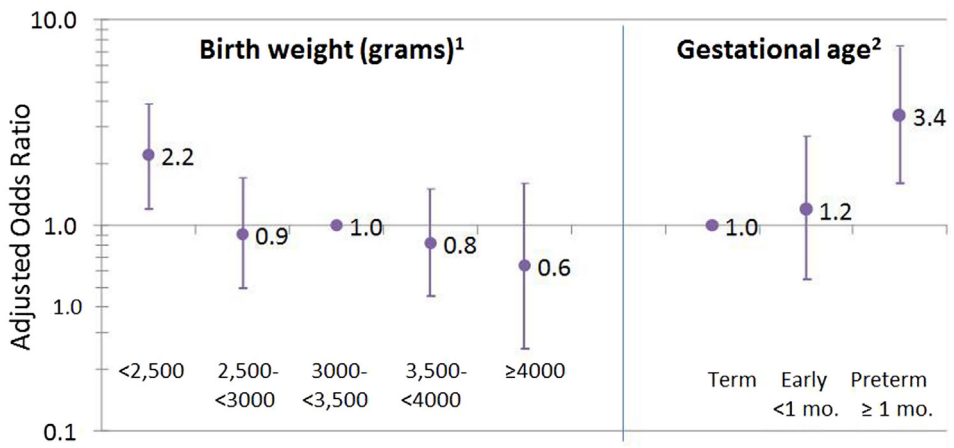

FIGURE 1 | Birthweight and gestational age associated with SLE. [1]Missing on 39 cases (31%) and 13,659 (27%) non-cases; test for trend across birthweight categories, $p = 0.008$. [2]Missing or do not know for 67 cases (54%) and 27,681 (55%) non-cases.

Systemic lupus erythematosus cases were significantly more likely to have been born to mothers who lived or worked on a farm while pregnant (**Table 2**). Birth season, prenatal smoking (maternal and household), being breastfed, firstborn, and sibling number were not significantly associated with SLE. Overall, SLE was not associated with individual childhood social factors. We saw no evidence of joint effects for combined lower adult educational attainment and increased SES score (Table S1 in Supplementary Material); in college educated women odds of SLE were almost doubled for women with two or more childhood SES adversity factors compared with only one or none (OR = 2.0; 95%CI 1.2, 3.6).

For longest childhood residence, apparent geographical differences in SLE by census region or latitude (a higher prevalence of SLE cases than non-cases in the South and lower latitude) were non-significant after adjusting for race/ethnicity (**Table 3**). Odds of SLE were somewhat elevated for living in a rural compared with a suburban environment; however, when compared with all other areas combined, the association of SLE with rural residence was significant (OR = 1.6; 95%CI 1.1, 2.3). An association was seen for SLE with use of residential pesticides in childhood, with a dose response for more frequent use (none, less than once per month, or at least once per month; p-trend < 0.0001). This association was not confounded by adult residential pesticide

TABLE 2 | Perinatal and childhood sociodemographic risk factors for SLE.

	Case *N* = 124	Non-case *n* = 50,465	Odds ratios[a] (95% CI; confidence intervals)
	N (%)	*N* (%)	
Birth characteristics and perinatal factors			
Mom lived or worked on farm while pregnant			
No	88 (75)	39,560 (82)	Referent
Yes	30 (25)	8,803 (18)	1.6 (1.0, 2.4)[b]
Prenatal smoking			
None	35 (32)	15,303 (34)	Referent
Mom only	9 (8)	3,406 (8)	1.2 (0.58, 2.5)
Household only	45 (41)	14,917 (34)	1.3 (0.86, 2.1)
Both	21 (19)	10,853 (24)	0.90 (0.53, 1.6)
Birth season			
Winter (December–February)	29 (24)	12,214 (24)	1.3 (0.76, 2.2)
Spring (March–May)	32 (26)	12,157 (24)	1.4 (0.83, 2.2)
Summer (June–August)	24 (19)	13,170 (26)	Referent
Fall (September–November)	39 (31)	12,924 (26)	1.6 (0.93, 2.6)
Breastfed			
No	61 (54)	22,526 (49)	Referent
Yes	51 (46)	23,339 (51)	1.2 (0.84, 1.8)
Firstborn			
No	98 (81)	37,936 (77)	Referent
Yes	23 (19)	11,412 (23)	0.90 (0.57, 1.4)
Sibling number[c]			
One	16 (13)	6,091 (12)	1.2 (0.71, 2.2)
2 or 3	48 (40)	21,824 (44)	Referent
4 or 5	28 (23)	12,181 (25)	0.95 (0.60, 1.5)
6 or more	29 (24)	9,515 (19)	1.1 (0.70, 1.8)
Early life social factors[d]			
Young maternal age	11 (9)	2,358 (5)	1.7 (0.91, 3.2)
Lower income	46 (37)	17,044 (34)	1.0 (0.71, 1.5)
Lower household education	72 (60)	27,041 (54)	1.1 (0.79, 1.7)
Food insecurity	15 (12)	4,753 (12)	1.1 (0.64, 1.9)
SES adversity factors[e]			
0	34 (29)	17,643 (36)	1.0 (referent)
1	40 (34)	16,319 (34)	1.2 (0.78, 2.0)
2	33 (29)	10,817 (22)	1.4 (0.90, 7.3)
3–4	11 (9)	3,613 (7)	1.2 (0.61, 2.5)

[a]*Odds ratios and 95% confidence intervals adjusted for age and race/ethnicity.*
[b]*p-Value <0.05.*
[c]*Women must have a sister to be enrolled in study. Thus, all have at least one sibling.*
[d]*Young maternal age of <20 years of age at birth, relative household income reported as low income or poor, and maximum household education in adults/parents less than a college degree.*
[e]*Socioeconomic status (SES) summary score based across four variables – young maternal age, lower household income and education, and food insecurity; p-trend = 0.26.*

TABLE 3 | Characteristics of longest childhood residence and associations with SLE.

	SLE		Odds ratios[b] (95% confidence intervals)[a]
	Probable *N* = 119	Non-case *N* = 48,950	
	N (%)	*N* (%)	
Longest childhood residence[a]			
U.S. Region			
West	18 (15)	7,032 (14)	1.3 (0.70, 2.3)
Midwest	32 (27)	17,443 (36)	Referent
Northeast	30 (24)	12,087 (25)	1.4 (0.83, 2.2)
South	36 (29)	11,428 (23)	1.4 (0.85, 2.3)
Puerto Rico	3 (3)	915 (2)	NC
Latitude			
≤35°	29 (24)	7,918 (16)	Referent
35–40°	30 (25)	11,461 (12)	0.90 (0.52, 1.5)
>40°	60 (50)	29,526 (60)	0.75 (0.46, 1.2)
Environment			
Urban	26 (21)	11,324 (23)	0.83 (0.49, 1.4)
Suburban/other	33 (27)	13,927 (27)	Referent
Small town	24 (20)	11,663 (23)	0.79 (0.47, 1.3)
Rural	39 (32)	12,851 (20)	1.4 (0.86, 2.0)
Residential pesticides[c]			
Never used	70 (61)	34,500 (79)	Referent
Infrequent (<monthly)	29 (25)	7,657 (17)	1.6 (1.0, 2.5)
Frequent (monthly+)	15 (13)	2,534 (6)	2.3 (1.3, 4.1)
Residence			
Was not a farm	67 (58)	28,960 (64)	Referent
Near a farm	15 (13)	6,213 (14)	1.1 (0.65, 2.6)
Used to be a farm	10 (9)	2,971 (7)	1.6 (0.80, 3.0)
Was a farm	24 (21)	7,093 (16)	1.6 (1.0, 2.6)[c]
Early life and extended childhood farm residence[d]			
No maternal or childhood	81 (74)	33,272 (77)	Referent
Only maternal/prenatal	6 (5)	3,293 (8)	0.70 (0.30, 1.6)
Only childhood	3 (3)	1,869 (4)	NC
Both maternal and childhood	20 (18)	4,963 (11)	1.8 (1.1, 3.0)

[a]*Residence location was missing or outside the U.S. for 5 cases and 1,560 non-cases. Women were grouped by state into census regions and by latitude based on majority of state area and/or population.*
[b]*Odds ratios calculated by logistic regression, adjusted for age and race/ethnicity; not calculated (NC) if 3 or fewer cases.*
[c]*Use by self or others; p-trend = 0.002.*
[d]*Mother lived or worked on a farm while pregnant and longest childhood residence was a farm; referent group includes women who lived near a farm or on a former farm and women with other short-term farm experiences.*

use (reported for current or longest held adult residence by 55% of cases and 45% of non-cases), even though use of childhood residential pesticides was more common in women who reported adult residential pesticides (not shown). Compared to women who did not report any residential pesticide use, SLE was significantly associated with having used pesticides in both childhood and as an adult (OR = 1.8; 95%CI 1.1, 3.1) and a non-significant association of similar magnitude was seen for childhood-only use (OR = 1.6; 95%CI 0.88, 2.9) (Table S2 in Supplementary Material), supporting an independent association with childhood-pesticide use regardless of adult use.

While having a long-term childhood farm residence was associated with SLE, when coupled with information on maternal farm history, this reflected the association with an early and extended farm experience (i.e., prenatal farm exposure and longest childhood farm residence was a farm OR = 1.8; 95%CI 1.1, 3.0) (**Table 3**). Few cases ($n = 3$) reported having their longest childhood residence on a farm in the absence of maternal/prenatal farm experience, and maternal/prenatal farm exposure alone was not associated with SLE. Based on data from the more detailed farm residence questionnaire, SLE was associated with living on a farm at least 12 months in childhood-only, but not as an adult (**Table 4**). In this context, SLE was associated with self-reported personal exposure to pesticides in childhood through application to crops. An elevated, non-significant association was seen for use on animals, and a stronger association was seen for exposure to pesticides from both animals and crops compared to either one alone. For those women with data on both longest childhood farm residence and agricultural pesticide use, the majority (80%) of cases and (70%) non-cases who reported agricultural pesticides also had an early and extended farm residence (from **Table 3**), so independent effects could not be assessed.

TABLE 4 | Farm residence and agricultural pesticide use associated with SLE.

	SLE		Odds ratios[a] (95% confidence intervals)
	Probable	Non-case	
	N = 124 *N* (%)	*N* = 50,465 *N* (%)	
Lived on farm 12+ months			
Never	87 (72)	37,288 (77)	Referent
Childhood only	28 (23)	7,348 (15)	1.7 (1.1, 2.7)
Child and adulthood	4 (3)	2,275 (5)	0.85 (0.31, 2.3)
Adult only	2 (2)	1,757 (4)	0.57 (0.14, 2.3)
Childhood-only farm pesticide exposures versus never lived on farm			
Never lived on farm	87 (76)	37,288 (85)	Referent
Farmed – pesticides on crops			
None used	8 (7)	3,132 (7)	1.2 (0.58, 2.5)
No personal exposures	6 (5)	2,479 (6)	1.1 (0.47, 2.5)
Personal exposures	13 (11)	1,288 (3)	4.2 (2.4, 7.7)
Farmed – animals			
No livestock contact	7 (6)	2,184 (5)	1.4 (0.66, 2.9)
Contact, no pesticides used	13 (11)	3,676 (8)	1.5 (0.90, 2.6)
Contact, pesticides used	7 (6)	1,118 (3)	2.1 (0.98, 4.6)
Personal pesticide exposures			
None	7 (6)	2,744 (6)	1.2 (0.55, 2.6)
Either crops or livestock	14 (12)	3,233 (7)	1.9 (1.1, 3.3)
Both crops and livestock	6 (5)	826 (2)	3.5 (1.5, 8.2)

[a]*Odds ratios calculated by logistic regression, adjusted for age and race/ethnicity; not calculated (NC) if 3 or fewer cases.*

DISCUSSION

These findings support the idea that perinatal and early life exposures may influence risk of developing SLE. Ours is the first study to report SLE associations with more frequent use of pesticides at the childhood residence and with maternal/prenatal and childhood farm residence. We also saw that SLE was associated with personal exposure to agricultural pesticides in women who had lived or worked on a farm for 12 months or more up to age 18 in the absence of adult farm exposures. Together, these results suggest a dose-related elevation in SLE risk associated with early life pesticide exposure. Prior studies have suggested associations of SLE with farming occupation or personal pesticide exposures SLE (18–21), but the relevant timing of exposures was not known. These novel findings warrant replication in other populations.

We also saw associations of SLE with low birthweight and preterm birth. The independent effects of preterm birth may be difficult to identify due to the close relationship between these two factors, however, the birthweight association remained consistent when excluding preterm births. Prior evidence on birthweight and SLE is inconsistent, with one study showing SLE associated with high birthweight in women (12) and null findings in other studies (13, 24). Reasons for an apparent inconsistency across studies are not clear. In the prior study (combining two cohorts of the Nurses' Health Study, NHS) (12), the association with high birthweight was most apparent for the older cohort (mean age of 46 in 1976), whereas the younger cohort (mean age of 35 in 1989) showed only modest and non-significant associations of SLE with both high (OR = 1.3) and low (OR = 1.5) birthweight. The current study participants were more similar to the younger NHS cohort; however, cases in the NHS cohorts were incident, whereas the current study case sample included prevalent cases and could be biased if higher birthweight was related SLE severity or other reasons for non-participation. In the most recent study, linking registry data on births and SLE diagnoses in Sweden (13), the population and cases were considerably younger (average age at onset of 21 years). However, this study did not rely on recall for birthweight and had a sufficient sample size to adjust for gestational age.

Prematurity has been associated with SLE in females and younger male cases (12, 13). In the present study sample, a similar proportion of cases and non-cases were missing data (compared with term birth OR = 1.1; 95%CI 0.71, 1.6 for "missingness" adjusted for age and race/ethnicity), so we did not impute missing values. Under the assumption that preterm birth is an event that is well reported if it occurs, we performed a sensitivity analysis assigning missing values to "term" birth, and results were unchanged. Many pathways and mechanisms might link birthweight and prematurity to increased risk of SLE (6, 25, 26). Limitations in the present study sample size and data precluded further investigations of life-course pathways, possibly operating through other developmental factors, such as age at menarche or adult obesity (27).

We saw no significant associations with social factors when race/ethnicity was included in the model, which is different from

our previous findings in RA (22). Minority race/ethnicity may contribute to social disparities, and associations with young maternal age and SES score were statistically significant when only adjusted for age (not shown). We also noted that childhood SES score appeared to be associated with SLE regardless of adult educational attainment (Table S1 in Supplementary Material). Lower adult education was also associated with SLE, but we did not see any stronger association for women with both low adult education and two or more childhood SES factors as we did for RA (22). We cannot rule out the influence of selection bias. Lower SES has been associated with higher morbidity and mortality in SLE (14, 28, 29), so one possibility is that SLE cases with, long-term lower SES and greater health disparities may have been unable to enroll in the cohort.

Few other perinatal factors were associated with SLE, including maternal or household smoking, birth season, breast feeding, being first born or number of siblings. Geographic differences in SLE across the U.S. have been previously reported, but are difficult to disentangle from racial differences (30, 31). In our analyses, higher latitude (proxy for lower UVB) was inversely associated with SLE risk in age-adjusted models (not shown), which was attenuated after adjusting for race/ethnicity.

We had no data on specific pesticides or types of pests treated. Organochlorines such as DDT have previously been linked to SLE risk (1). A causal association of organochlorine insecticides and SLE in susceptible individuals is supported by experimental studies, in which treatment with chlordecone, methoxychlor, or *o*,*p*′-DDT accelerated SLE disease onset, including earlier increases in autoantibody levels and renal impairment in lupus-prone mice, but not in a non-susceptible mouse strain. Many other pesticides impact the immune system through diverse mechanisms (32) and bear further investigation in experimental models also taking into account the question of timing relative to developmental windows of susceptibility.

Our study has several limitations, one being the use of self-reported cases. Validation studies are costly, however, and can reduce sample size and generalizability due to biased participation in studies requiring release of medical information. Because self-report is notoriously non-specific for lupus, we increased specificity of our case definition by confirming probably clinical cases based on the self-reported use of DMARDs (33). This definition does not identify all cases, including those with incomplete medication histories or those receiving non-DMARD treatments. However, a prevalence of ~2 cases per 1,000 (e.g., 124/50,000) is reasonable for this cohort, given an estimated population prevalence of 1/1,000, with higher rates in women. If socioeconomic factors and other environmental exposures are related to severity and treatment with DMARDs, then our case definition may also lead to underestimated associations with disease. Disease severity may influence treatments, survival or participation in the cohort, limiting generalization of these findings to SLE in the broader population.

These analyses relied on self-reported data on perinatal and childhood factors. Sensitivity analyses were conducted, limiting the sample to women under age 60, as reporting accuracy is expected to diminish with age and years since exposure, and younger women may be more likely to be able to consult their mothers for assistance. In most instances, we did not see changes in associations. Difficulty in reporting birthweight and preterm birth was obvious, as evidenced by the high number of missing data. Other factors may be more easily and objectively reported, such as childhood farm residence. Some immune modifying factors were not considered because they were rare (e.g., pregnancy-related hypertensive disorder or DES exposure) or not assessed (e.g., maternal nutrition) (34). Reporting of childhood pesticides may be influenced by adult pesticide use, which in turn may be influenced by health status.

Strengths of the study include the broad range of early life data available in the cohort, and results showing internally consistent findings for residential and agricultural pesticides and birthweight/prematurity. Replication of these analyses using incident cases, using a life-course approach, is warranted.

AUTHOR CONTRIBUTIONS

CP conceived of the topic, obtained, and managed the data, designed and conducted the analyses, interpreted findings, and wrote the paper. AD and DS contributed expertise on exposures, obtained and manage data, assisted with analysis design and interpreted findings, and critically reviewed the paper prior to submission. All authors agreed to final draft and submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00103

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Silica, Silicosis, and Autoimmunity

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Inhalation of dust containing crystalline silica is associated with a number of acute and chronic diseases including systemic autoimmune diseases. Evidence for the link with autoimmune disease comes from epidemiological studies linking occupational exposure to crystalline silica dust with the systemic autoimmune diseases systemic lupus erythematosus, systemic sclerosis, and rheumatoid arthritis. Although little is known regarding the mechanism by which silica exposure leads to systemic autoimmune disease, there is a voluminous literature on silica exposure and silicosis that may help identify immune processes that precede development of autoimmunity. The pathophysiology of silicosis consists of deposition of silica particles in the alveoli of the lung. Ingestion of these particles by macrophages initiates an inflammatory response, which stimulates fibroblasts to proliferate and produce collagen. Silica particles are encased by collagen leading to fibrosis and the nodular lesions characteristic of the disease. The steps in the development of silicosis, including acute and chronic inflammation and fibrosis, have different molecular and cellular requirements, suggesting that silica-induced inflammation and fibrosis may be mechanistically separate. Significantly, it is unclear whether silica-induced inflammation and fibrosis contribute similarly to the development of autoimmunity. Nonetheless, the findings from human and animal model studies are consistent with an autoimmune pathogenesis that begins with activation of the innate immune system leading to proinflammatory cytokine production, pulmonary inflammation leading to activation of adaptive immunity, breaking of tolerance, and autoantibodies and tissue damage. The variable frequency of these immunological features following silica exposure suggests substantial genetic involvement and gene/environment interaction in silica-induced autoimmunity. However, numerous questions remain unanswered.

Keywords: silica, silicosis, autoimmunity, human, animal model

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INTRODUCTION

Environmental factors play a significant role in the development of human autoimmunity (1). These factors include the food we eat, the fluids we drink, the air we breathe, chemicals (natural and synthetic), infections, by-products of manufacturing processes, and radiation (2–4). A recent review of the epidemiologic evidence of environmental factors in human autoimmune diseases concluded that exposure to crystalline silica contributes to the development of a number of autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), and antineutrophil cytoplasmic antibody (ANCA)-related vasculitis (5). Despite this strong linkage of silica exposure with autoimmune diseases, there is little evidence of the possible mechanisms underlying this relationship (6, 7). This is due in large part to a lack of accepted criteria for diagnosis or classification of environmentally associated autoimmunity (8) as well as a

paucity of animal models that mimic features of silica exposure in humans (6). In contrast, there is a voluminous literature on silica exposure and the development of silicosis in humans and animal models (9–11). In this article, I provide a brief overview of the immunological consequences of silica exposure and discuss how an understanding of identified mechanisms and biological markers may contribute to an understanding of silica-induced autoimmunity.

SILICA AND INFLAMMATION

Silica (SiO_2) is an oxide of silicon and is most commonly found in nature as quartz. Silica exists in many crystalline forms (called polymorphs) with α-quartz being the most common form (11). Exposure to respirable crystalline silica (<10 μm in size) occurs most often in occupational settings, where materials containing crystalline silica are reduced to dust or when fine particles are disturbed. These occupations are often called the dusty trades and include abrasive blasting with sand, jack hammering, drilling, mining/tunneling operations, and cutting and sawing (10, 12). Inhaling crystalline silica dust can lead to silicosis, bronchitis, or cancer (10, 11). Silicosis is characterized by chronic inflammation and scarring in the upper lobes of the lungs and can be classified based on the quantity inhaled, time course, and length of exposure (10, 11, 13). Chronic simple silicosis is the most common form, occurring after 15–20 years of moderate to low exposures to respirable crystalline silica. The accelerated form occurs after 5–10 years of high exposures to respirable crystalline silica, and acute silicosis, or silicoproteinosis, occurs after a few months or as long as 5 years following exposures to extremely high concentrations of respirable crystalline silica. The acute form is the most severe form of silicosis. The pathophysiology of silicosis involves deposition of particles into alveoli where they cannot be cleared. Ingestion of these particles by alveolar macrophages initiates an inflammatory response, which stimulates fibroblasts to proliferate and produce collagen. The silica particles are enveloped by collagen leading to fibrosis and nodular lesions characteristic of the disease.

CELL AND MOLECULAR REQUIREMENTS FOR SILICA-INDUCED INFLAMMATION/ FIBROSIS

A number of studies in experimental animals have revealed differences in silica-induced inflammatory responses and silicosis (14–16), arguing that gene–environment interactions are important in the severity of disease. Gene deletion studies have identified a number of the cellular and molecular requirements for silica-induced inflammation and fibrosis. The inflammatory response following exposure to crystalline silica is mediated by NALP3 inflammasome-driven IL-1β (17). Inflammasome activation is argued to occur following uptake of silica by scavenger receptors, lysosomal rupture, and release of cathepsin B accompanied by production of reactive oxygen species (ROS) and potassium efflux (10, 17–19) (**Figure 1**). The binding of silica to scavenger receptors also results in apoptosis of macrophages and the release of mediators (e.g., proinflammatory cytokines) contributing to lung inflammation and fibrosis (20). However, scavenger receptors also play a significant role in clearing silica, and their absence enhances inflammation but not fibrosis (21, 22).

Consistent with the differential requirements for scavenger receptors, the steps in the development of silicosis, including acute and chronic inflammation and fibrosis, have different molecular and cellular requirements (**Figure 2**). Inflammation and fibrosis occur independently of T, B, NKT, and NK cells (23), although treatment with anti-CD4 antibodies reduces the severity of fibrosis (24). This may be explained by the presumptive role of T regulatory cells in fibrosis (25). Deficiency of IL-1α reduced IL-1β production and neutrophil accumulation following silica exposure (26), suggesting that release of endogenous IL-1α from alveolar macrophages promotes subsequent lung inflammation. Pulmonary inflammation is also dependent on IFN-γ (27), but not IL-4 or IL-13 (28) or IL-12 (29). In keeping with its anti-inflammatory potential, IL-10 helps limit the silica-induced inflammatory response but amplifies the fibrotic response (30). The role of IL-10 in fibrosis appears to be due to exacerbation of the Th2 response and the production of profibrotic IL-4 and IL-13 (31). Acute inflammation, but not chronic inflammation or fibrosis, requires IL-17 (32), conversely, chronic inflammation, but not acute inflammation or fibrosis, requires type I IFN and IRF7 (33). Additional studies have suggested that presence of innate immune response components (particularly IL-1 receptor, IL-1, ASC, NALP3, IL-18 receptor, IL-33 receptor, TRIF, and TLR2, 3, and 4) are not required for accumulation of collagen in the lung (fibrosis), while inflammation, neutrophil accumulation, IL-1β release, and granuloma formation did require MyD88 (25). In contrast, others have suggested that absence of NALP3 and ASC reduces collagen deposition (17). While these studies may question the role of innate immunity in fibrosis, it is becoming clear that silica-induced inflammation and fibrosis can be uncoupled as evidenced by the observation that steroid treatment reduced lung inflammation and proinflammatory cytokine expression (TNF-α, IL-1β) but had no significant effect on lung fibrosis or expression of fibrogenic cytokines (TGF-β and IL-10) (34).

PROPERTIES OF SILICA THAT INFLUENCE INFLAMMATION

Both amorphous (non-crystalline) silica particles and crystalline silica are phagocytosed by, and toxic to, macrophages leading to endolysosomal rupture and caspase-3 activation (35). Nevertheless, the size of silica particles can dramatically affect the inflammatory response. Amorphous silica particles of 30–1,000 nm in diameter induce greater inflammatory responses, as judged by lysosomal destabilization, proinflammatory cytokine expression, and pulmonary inflammation, than 3,000–10,000 nm particles (36). However, it is unclear if silica-induced lysosome destabilization is essential to NLRP3 inflammasome activation, IL-1β production, and inflammation. The binding of immobilized silica crystals to the cell membrane of macrophages was sufficient to induce IL-1β without evidence

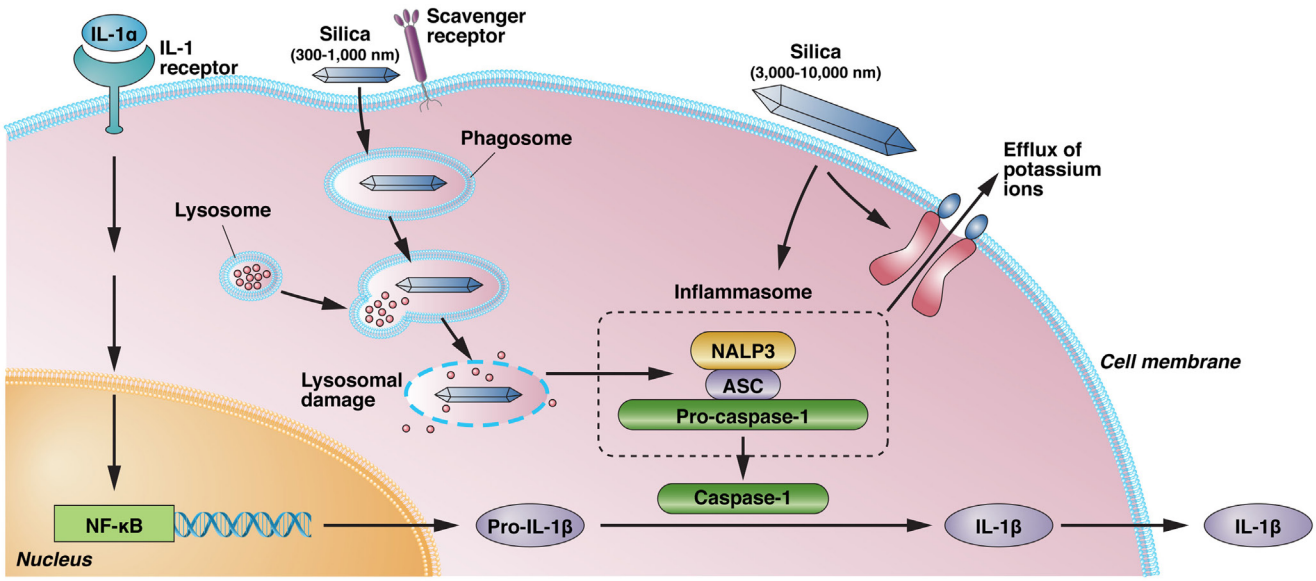

FIGURE 1 | Silica-induced activation of inflammasome and IL-1 production. IL-1α, released from alveolar macrophages following crystalline exposure, results in NF-κB activation and transcription and translation of pro-IL-1β. Phagocytosis of crystalline silica leads to phagosomal damage and release of phagosome contents into the cytoplasm. This results in the activation of NALP3 and its association with the intracellular adapter protein ASC, which combines with and activates pro-caspase-1. The resulting inflammasome cleaves pro-IL-1β to the proinflammatory IL-1β. However, binding of immobilized silica crystals to the cell membrane of macrophages is also sufficient to induce IL-1β without evidence of lysosomal damage. Activation of the NALP3 inflammasome by silica also results in efflux of intracellular potassium ions, suggesting a possible interaction of silica with a membrane-associated protein, but it is unclear if K^+ efflux following binding of immobilized silica crystals to the cell membrane results in inflammasome activation. Scavenger receptors have a role in the recognition and uptake of silica. NALP3, NACHT, LRR, and PYD domains-containing protein 3; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; NF-κB, nuclear factor-κB; IL, interleukin.

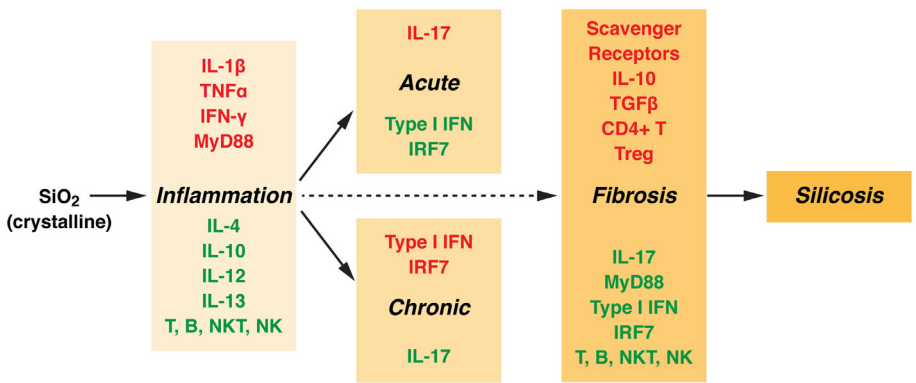

FIGURE 2 | Molecular and cellular components involved in silica-induced inflammation and fibrosis. Silicosis is marked by inflammation and fibrosis with the formation of nodular lesions in the upper lobes of the lungs. The collagen containing silicotic nodules are a specific response to crystalline silica. However, the cellular and molecular components responsible for the inflammatory and fibrotic responses are not the same. Components required for inflammation (acute or chronic) and fibrosis are highlighted in red, while those not essential have been highlighted in green (see text for details). TNF, tumor necrosis factor; IFN, interferon; MyD88, myeloid differentiation primary response gene 88; IL, interleukin; NK, natural killer; NKT, natural killer T cell; IRF, interferon regulatory factor; TGF, transforming growth factor.

of lysosomal damage or a requirement for cathepsin B (18) (**Figure 1**). Blocking K^+ efflux from the cell was sufficient to reduce IL-1β release although whether potassium efflux is directly responsible for NLRP3 activation remains unclear (18). An alternative explanation for silica-induced IL-1β expression argues that silica exposure results in release of IL-1α into the alveolar space, which then drives production of IL-1β and lung inflammation (26). This explanation is consistent with the concept that IL-1α functions as an alarm molecule and plays a critical role early in inflammation (37).

SILICA-ASSOCIATED AUTOIMMUNITY

A number of epidemiological studies support the association between occupational exposure to respirable crystalline silica dust and development of systemic autoimmune diseases (5, 12). Exposure to asbestos, another silicate that occurs in mining and construction, may be concurrent with crystalline silica exposure. While it can be difficult to assess the role of each separately, epidemiological data are too limited to argue for a strong association between asbestos exposure and autoimmunity (5). However, there is growing evidence that asbestos exposure may be associated with autoimmunity (e.g., hypergammaglobulinemia and autoantibodies) in the absence of confirmed autoimmune disease (38, 39). This is an important observation as several studies of crystalline silica exposure also point to the appearance of features of autoimmunity, especially autoantibodies, in exposed individuals in the absence of autoimmune disease (40, 41). This suggests that study of larger cohorts of asbestos exposed individuals may lead to stronger associations with autoimmune diseases.

For respirable crystalline silica dust, the prevalence of disease is increased when compared to the general population and shows evidence of strong occupational bias mostly associated with males (41). In high-level exposure, SLE is 10 times higher than the expected sex-specific prevalence in the general population (12, 41), but the strength of this association falls in both men and women as exposure is reduced (42). Moreover, there is evidence that disease features may differ between those with silica-induced systemic autoimmune disease and those with idiopathic disease (41, 42). Uranium miners with SLE had considerably less arthritis and also less photosensitivity compared to those with idiopathic SLE (41); there was also reduced prevalence of discoid lesions although this did not reach statistical significance. However, the silica group was all males with late onset disease, while the idiopathic disease group was 90% females and matched only for geographical location and ethnicity. Thus, it is unclear if the differences reflect silica exposure or sex and/or age differences. In the second study, demographic characteristics were more carefully controlled; however, the silica-exposed SLE patients were found to have reduced prevalence of anemia and leukopenia (42). Differences in autoantibodies have also been reported. Patients with silica-associated SSc had greater prevalence of anti-DNA topoisomerase 1 autoantibodies, and both silica-associated SSc and SLE had fewer patients with high titer antinuclear antibodies (ANA) (>1:1,280) compared to those with idiopathic disease (40).

Individual study populations have been found to have increased occurrence of different diseases suggesting a common underlying pathophysiology (43). This is supported by the observation that clinical features and autoantibodies specific to connective tissue diseases including anti-DNA, anti-SS-A/Ro, anti-SS-B/La, anti-centromere, and anti-topoisomerase 1 occur at higher frequency in exposed individuals without autoimmune disease compared to the general population (12, 40, 42). Although silicosis may be associated with immune abnormalities including autoantibodies, the association of silica exposure with expression of autoimmune disease can occur in the absence of silicosis (12, 41). Furthermore, while there is an association between intensity of exposure and autoantibodies including an association of high-level exposure with SLE, there is no relationship between autoantibodies and silicosis (12, 41, 44). This suggests that the development of fibrosis and nodular lesions may not be required for development of autoimmunity. Whether this reflects the recent suggestion that fibrosis is linked to T regulatory cells (25) is uncertain.

The variable frequency of disease features in silica-induced autoimmunity suggests significant genetic involvement and gene/environment interactions. Silicosis can occur in 47–77% of individuals with adequate follow-up after silica exposure (45). In patients with silicosis, hypergammaglobulinemia can occur in over 65% of patients (46). In silicosis, ANA prevalence can be 34% or higher (47). End-stage renal disease due to silica exposure occurs in about 5% of exposed individuals (45), and development of diagnostically definable systemic autoimmune disease is even less frequent (12). These findings are consistent with a disease progression that begins with silica-induced activation of the innate immune system leading to inflammation of the lung, activation of adaptive immunity, breaking of tolerance, and autoantibodies and tissue damage.

ANIMAL MODELING OF SILICA EXPOSURE TO MIMIC HUMAN AUTOIMMUNITY

Only a small number of animal studies have modeled silica-induced autoimmunity. Lupus-prone NZM2410 mice exposed to crystalline silica exhibit pulmonary inflammation and fibrotic lesions, autoantibodies, kidney deposits of IgG and C3, proteinuria, and reduced survival compared to controls (48). A follow-up study reported increased TNF-α in bronchoalveolar lavage fluid (BALF), B1a B, and $CD4^+$ T cells in lymph node as well as alteration in the ratio of $CD4^+$ to $CD4^+CD25^+$ T cells (49). A more recent study using lupus-prone NZBWF1 mice confirmed the exacerbation of SLE-like disease as well as identifying the formation of inducible bronchus-associated lymphoid tissue (iBALT) (50). Exposure to asbestos induces a similar spectrum of autoantibodies, kidney immune deposits, and changes in $CD4^+CD25^+$ T cells in non-autoimmune prone C57BL/6 mice (51). Eronite, an asbestos-like fibrous mineral, induced ANA, IL-17, TNF-α, and renal deposits of IgG in C57BL/6 mice (52). Asbestos exposure in Lewis rats failed to exacerbate arthritis induced by collagen or peptidoglycan–polysaccharide but did induce ANA, anti-histidyl tRNA synthetase antibodies, and proteinuria but showed no evidence of kidney immune deposits (53, 54). Non-autoimmune Brown Norway rats given sodium silicate ($NaSiO_4$) by subcutaneous injection developed ANA including anti-DNA, -Sm, -SS-A, and -SS-B (55). The ANA titers increased with time with the majority being positive for anti-RNP (56). These studies demonstrate that crystalline silica, and asbestos, can elicit autoimmunity in mice and rats and that non-crystalline silica can induce humoral autoimmunity in non-autoimmune prone rats, but they provide little evidence for possible mechanisms.

When mechanism has been examined, the results point to a significant role for cell death as a source of immune stimulation. ANA from crystalline silica-exposed NZM2410 mice

preferentially bind to alveolar macrophage-like cells undergoing silica-induced apoptosis but not if apoptosis was inhibited by a caspase inhibitor (57), suggesting that silica-induced autoantibodies are directed against material from dying cells. This is supported by the observation that apoptosis induced by asbestos exposure results in surface blebs enriched in the autoantigen SSA/Ro52, which are bound by autoantibodies from asbestos-exposed mice (58). Rottlerin, which affects kinase and non-kinase proteins as well as activating K^+ channels (59), reduced silica-induced proteinuria, autoantibodies, and IgG and C3 kidney deposits in lupus-prone NZM2410 mice (60). These studies led to the hypothesis that silica-induced activation of alveolar macrophages leads to apoptosis and inflammation, ingestion of cellular debris, migration of activated antigen-presenting cells (APCs) to lymph nodes, and activation of T and B cells (61). However, it is unclear how this leads to breaking of self-tolerance.

CONCLUSION

In aggregate, the immunological consequences of silica exposure that lead to autoimmunity are consistent with a disease progression that begins with activation of the innate immune system resulting in proinflammatory cytokine production, inflammation of the lung leading to activation of adaptive immunity, breaking of tolerance, and autoantibodies and renal damage. However, numerous questions remain unanswered.

It is unknown if the early events leading to IL-1β expression (**Figure 1**) are required for silica-induced autoimmunity. Are there size, shape, surface area, or charge rules for silica-induced lysosomal destabilization, K^+ efflux, and inflammasome activation? Does K^+ efflux play a role in silica-induced inflammation/autoimmunity? The contribution of the inflammasome and IL-1 to systemic autoimmunity remains unclear (62) because while caspase 1 is required for pristane-induced autoimmunity (63), neither caspase 1 nor NALP3 is required for mercury-induced autoimmunity (64). Additional research is also needed to determine if nanoparticles and other non-crystalline forms of silica lead to autoimmunity.

Many of the genetic requirements for silica-induced inflammation (**Figure 2**) are also required for systemic autoimmunity. In particular, IFN-α/β and/or IFN-γ are required for idiopathic (65) and induced systemic autoimmunity (66, 67). Additionally, the role of IL-17 in autoimmunity continues to grow (68). Conversely, genetic elements required for silica-induced fibrosis may play little role in silica-induced autoimmunity. Deficiency of scavenger receptors exacerbates autoantibody responses (69). Moreover, the protective role of T regulatory cells and their cytokines IL-10 and TGF-β in systemic autoimmunity (70, 71) argues that the fibrotic process elicited by silica exposure may negatively regulate the development of autoimmunity. It remains to be determined which of the molecular and cellular components that drive silica-induced inflammation and fibrosis explain the variable frequency of immunological features found in silica-induced autoimmunity.

Finally, a significant concern for future research is the paucity of animal models of silica-induced autoimmunity (6). Susceptibility to silicosis varies among inbred mouse strains (15) and no single inbred mouse strain mimics the genetic or disease heterogeneity found in humans. Considerable effort will be needed to identify an appropriate experimental model so that studies can be "shaped by what is observed in humans, not by what is possible in mice" (72).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Orally Administered *Salacia reticulata* Extract Reduces H1N1 Influenza Clinical Symptoms in Murine Lung Tissues Putatively Due to Enhanced Natural Killer Cell Activity

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Influenza is a major cause of respiratory tract infection. Although most cases do not require further hospitalization, influenza periodically causes epidemics in humans that can potentially infect and kill millions of people. To countermeasure this threat, new vaccines need to be developed annually to match emerging influenza viral strains with increased resistance to existing vaccines. Thus, there is a need for finding and developing new anti-influenza viral agents as alternatives to current treatments. Here, we tested the antiviral effects of an extract from the stems and roots of *Salacia reticulata* (SSRE), a plant rich in phytochemicals, such as salacinol, kotalanol, and catechins, on H1N1 influenza virus-infected mice. Following oral administration of 0.6 mg/day of SSRE, the incidence of coughing decreased in 80% of mice, and only one case of severe pulmonary inflammation was detected. Moreover, when compared with mice given *Lactobacillus casei* JCM1134, a strain previously shown to help increase *in vitro* natural killer (NK) cell activity, SSRE-administered mice showed greater and equal NK cell activity in splenocytes and pulmonary cells, respectively, at high effector cell:target cell ratios. Next, to test whether or not SSRE would exert protective effects against influenza in the absence of gut microbiota, mice were given antibiotics before being inoculated influenza virus and subsequently administered SSRE. SSRE administration induced an increase in NK cell activity in splenocytes and pulmonary cells at levels similar to those detected in mice not treated with antibiotics. Based on our results, it can be concluded that phytochemicals in the SSRE exerted protective effects against influenza infection putatively *via* modulation of the immune response, including enhancement of NK cell activity, although some protective effects were not necessarily through modulation of gut microbiota. Further investigation is necessary to elucidate the molecular mechanisms underlying the protective effects of SSRE against influenza infection.

Keywords: *Salacia reticulata* extract, H1N1 influenza virus, *Lactobacillus casei* JCM1134, antibiotics, gut microbiota, natural killer cells, splenocytes, pulmonary cells

INTRODUCTION

Influenza is a leading cause of respiratory tract infection (1). Influenza viruses are categorized as A, B, and C (2), and influenza A virus can be further subtyped as H3N2, H2N2, and H1N1. Every year, approximately 10% of the world's population is infected with influenza (3) that results in 250,000–500,000 deaths (4), but in most cases, the infection only lasts for 1–2 weeks without the need for hospitalization (4). Nonetheless, influenza viruses periodically cause epidemics in humans, such as the 2009 H1N1 pandemic in Mexico (5), which can potentially infect and kill millions of people. Thus, medication with antiviral agents that trigger an immune response and inhibit viral replication in infected patients is required to prevent further viral spread and higher mortality rates among the population.

Following an influenza viral infection, natural killer (NK) cells are reportedly activated by proinflammatory cytokines, such as interleukin-12 (6) and/or type I interferons (7), and cytotoxicity of NK cells is stimulated by interferon-gamma (IFN-γ) (8, 9). Since toxicity receptors NKp46 and NKp44 on human NK cells readily identify hemagglutinin and neuraminidase on the surface of influenza virus, NK cells alone have the potential of destroying infected cells (9). Nonetheless, impaired cytotoxicity and depletion of NK cells that lead to higher morbidity and mortality rates are often observed in influenza virus-infected subjects for reasons not yet fully understood (10). Hence, presently influenza viral infections are most efficiently prevented and controlled by vaccines that are mainly designed to mobilize a strain-specific antibody response to viral surface hemagglutinin or neuraminidase (2). Nevertheless, every year, new vaccines need to be developed to match emerging influenza viral strains with increased resistance to existing vaccines (11, 12). This challenge highlights the need for finding and developing new antiviral agents as alternatives to those currently available.

Although the human gut microflora is remarkably stable under dietary changes (13) and its composition is highly individualized (14), administration of lactic acid bacterial (LAB) strains, such as those found in fermented food products (15–17), has been shown to protect experimental models against influenza viral infections by enhancing the immune response (16–20). For example, continual consumption of a milk-based drink containing a *Lactobacillus brevis* strain was reported to lower the incidence of influenza in unvaccinated schoolchildren in Japan (15). These findings emphasize the potential of probiotics as complement to conventional vaccine-based approaches for treatment of influenza infection (21). Nonetheless, inconsistency in the survival rate of strains (22, 23) and lack of consensus on the effective doses (24, 25) are issues needed to be addressed before LAB can be considered reliable as therapeutic agents against viral infections.

A new generation of antiviral extracts from biological sources has shown promising effects against influenza virus. For example, Ladania067 from the leaves of black currant (*Ribes nigrum folium*) showed potent antiviral activity against influenza A/Regensburg/D6/09 (H1N1) (26), although efficacy observed 24 h after intranasal application *in vivo* was not higher than 85%. Likewise, a crude extract from *Crytoporus volvatus*, a polypore fungus, showed strong activity against H1N1/09 influenza viral infection in mice. Nonetheless, while *C. volvatus* extract indeed reduced virus loads in lungs and protected from a lethal viral dose, it failed to substantially decrease the virus titer (27). Although *Salacia* species have long been used as therapeutic agents in traditional medicine in Asia for treating disorders, such as diabetes, cancer, and immunosuppression (28, 29), the properties of *Salacia* species against viral infections have not been well characterized. Recently, in our premises, we observed that an extract from the bark and roots of *Salacia reticulata* induced an increase in activity of immune-related genes, including Cd26, IgG2a, TNF-α, and Ccl5 in intestinal epithelial cells (IEC) of Sprague-Dawley rats (30). Cd26 and IgG2a are involved in cell-mediated immunity (31) and the humoral immune response (32), and TNF-α and Ccl5 in the antiviral defense response (33), respectively, upon a challenge with influenza virus. Thus, it is likely that extracts from *S. reticulata* may induce a similar increase in immune cell activity after influenza virus infection.

In the present study, we first evaluated the effect of administration of an *S. reticulata* stem and root extract (SSRE) on lung damage caused by influenza viral infection. Next, we measured NK cell activity in spleen and lungs of influenza virus-infected mice to further assay the protective effect of administration of SSRE against influenza viral infection, and compared this effect with that exerted by a commercially available LAB strain. We also investigated the effect of SSRE on NK cell activity in spleen and lung cells of mice given a powerful antibiotic cocktail and inoculated with H1N1 influenza virus.

MATERIALS AND METHODS

Animals

Six-week-old ddY mice were purchased from Japan SLC (Shizuoka, Japan) and bred in a pathogen-free animal facility. Mice had *ad libitum* access to regular rodent chow (Lab MR stock; Nihon Nosan Kogyo, Tokyo, Japan) and water. Prior to experiments, all mice were allowed to acclimatize for 7 days. The experimental animals were handled in accordance with the guidelines for animal studies issued by the Experimental Animal Committee of Kyoto Prefectural University (Approval No. KPU270406).

Bacterial and Viral Strains

Lactobacillus casei JCM1134 was purchased from Japan Collection of Microorganisms (Tsukuba, Japan). *L. casei* was grown under anaerobic conditions at 37°C overnight in 10-mL Hungate tubes, with de Man, Rogosa, and Sharpe (MRS) broth as medium.

A murine influenza virus, A/PR/8/34/2009 (H1N1), was used for this study. Madin–Darby canine kidney cells were infected with the virus and after several passages, viral solutions were prepared and titrated as the tissue-culture-infective dose ($TCID_{50}$), according to a standard procedure (34, 35).

Preparation of the Hot Water Extract of *S. reticulata*

The stems and roots of *S. reticulata* grown in and imported from Sri Lanka were dehydrated and pulverized. The powdered

material was boiled in water at 90°C for an hour. The concoction was then filtered to remove any solid material, and the resulting filtrate was spray-dried (ADL-310, Yamato Science, Tokyo, Japan). The SSRE was stored at 4°C until further use.

Experimental Design

Experiment 1

Twelve mice were equally divided and allocated to two experimental groups. Mice were orally administrated treatments as follows.

(1) Control group treatment: 500 μL of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4; pH 7.4).
(2) SSRE group treatment: 500 μL of SSRE solution (1.2 mg of SSRE/mL of PBS).

Experiment 2

Eighteen mice were equally divided and allocated to three experimental groups. Mice were orally administrated treatments as follows.

(1) Control group treatment,
(2) SSRE group treatment,
(3) Probiotic group treatment: 10^8 cfu of JCM1134 in 500 μL of PBS.

Experiment 3

Eighteen mice were equally divided and allocated to three experimental groups. Mice were orally administrated treatments as follows.

(1) Antibiotic–control group (baseline group) treatment: antibiotics in drinking water [1 g/L of ampicillin (Nacalai Tesque, Kyoto, Japan), 500 mg/L of vancomycin (Wako Pure Chemicals, Osaka, Japan), 1 g/L neomycin sulfate (Sigma-Aldrich, Tokyo, Japan), and 1 g/L of metronidazole (Nacalai Tesque, Kyoto, Japan)] + control group treatment.
(2) Antibiotic–SSRE group treatment: antibiotics + SSRE.
(3) Normal microbiota group treatment: control group treatment.

In Experiment 3, administration of antibiotics started 3 days prior to administration of SSRE. Treatments in all experiments were administered once a day for 2 weeks. The humane endpoint was established at a body weight loss >20%, at which mice would be killed, as previously suggested (36).

Experimental Influenza Viral Infection to Mice

After completion of the treatment period, mice from all experiments were intranasally inoculated with 10^5 $TCID_{50}$ of A/PR/8/34/2009 (H1N1) virus. Five days post-inoculation, mice were killed with an overdose of sodium pentobarbital (Schering-Plough, K.K. Osaka, Japan). Except for tissues from Experiment 1, which were immersed immediately after removal in 10% neutral-buffered formalin and used for histopathology analysis, all murine lungs and spleen tissues of mice were collected and immediately used for further analyses.

Histopathology

Lung tissues of mice from Experiment 1 were used for this experiment. Lung tissues were embedded in paraffin, and 5-μm sections were excised from bilateral posterior lobes and stained with hematoxylin and eosin (H&E). All samples were randomly numbered and examined by an experienced pathologist blinded to the study conditions. The pathologist used an Olympus microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with a 100× magnification lens and scanned the entire surface area of each lung section.

Isolation of Splenocytes and Pulmonary Cells

Lung and spleen tissues were sectioned into small pieces and immersed in ice-cold Hanks buffered solution (HBSS, Nacalai Tesque, Kyoto, Japan). The pieces were gently pressed against sterile 70-μm cell strainers (BD Biosciences Japan, Tokyo, Japan) to obtain single-cell suspensions, which were centrifuged at 500 × *g* for 4 min at 4°C. The cell supernatant was retrieved, resuspended in ACK lysis buffer (0.5 mol/L of NH_4Cl, 10 mmol/L of $KHCO_3$, and 0.1 nmol/L of Na_2EDTA; pH 7.2), and incubated at room temperature for 5 min to remove erythrocytes. The remaining cells were washed twice with HBSS and resuspended in 1 mL of culture medium [RPMI medium (Nacalai Tesque) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL, Sigma-Aldrich Japan, Tokyo, Japan), and streptomycin (100 μg/mL; Sigma-Aldrich, Japan)]. Splenocytes and pulmonary cells were stained with trypan blue and counted with a TC10 automated cell counter (Bio-Rad, Richmond, CA, USA).

NK Activity of Splenocytes and Pulmonary Cells

A murine T cell lymphoma cell line, YAC-1, was obtained from RIKEN Bio-Resource Center (Ibaraki, Japan). YAC-1 cells were cultured in 75-cm^2 culture flasks containing 10 mL of culture media and incubated in a humidified chamber (37°C, 5% CO_2). After 3-day incubation, YAC-1 cells were stained for 15 min at 37°C with 10-μM Dioc18 [3,3′-dioctadecyloxacarbocyanine perchlorate (Life Technologies Japan, Tokyo, Japan)] to cause green fluorescence of cell membranes. YAC-1 cells were then washed twice with 1-mL HBSS and seeded in 100 μL of culture media in 96-well culture plates (Orange Scientific, Tokyo, Japan) in a concentration of 1.0×10^4 cells (for coculture with splenocytes) or 5.0×10^3 cells (for coculture with pulmonary cells) per well.

Natural killer cell activity was assessed by a flow-cytometric method, as previously described (37, 38). For the NK activity assays, YAC-1 cells were used as target cells, and splenocytes and pulmonary cells as effector cells. One hundred microliters each of splenocyte and pulmonary cell suspensions were added to each well containing DioC18-labeled YAC-1 cells to achieve 25:1 and 10:1, and 10:1 and 5:1 effector cell:target cell (E:T)

ratios, respectively. After incubation for 4 h, 10 μL of ethidium monoazide bromide (EMA, 0.5 ng/mL, Sigma-Aldrich, Japan) solution was added to each well for nuclear staining of dead cells. The ethidium was bound to nuclei by photo affinity using a 26-W fluorescent light (5 cm distance) for 10 min at room temperature. After washing twice with PBS containing 0.5% BSA (Nacalai Tesque), the cells were fixed with 2% PFA overnight. Each sample was incubated in duplicate and analyzed using an Accuri C6 flow cytometer (Beckton Dickinson). During the data acquisition, a live gate was set in the FL1 histogram on the green fluorescent target cells to discriminate effector and target cells (**Figures 1A,B**). At least 2,000 target cells per sample were collected. The gated target cells were further analyzed with a dot plot of FL1 (green fluorescence) versus FL3 (red fluorescence) to calculate the percentage of EMA-positive dead cells.

The percentage of spontaneously lysed target cells was determined for DioC18-labeled YAC-1 cells incubated for 4 h in the absence of effector cells (**Figure 1C**), and the value was subtracted from the data of each sample. Spontaneous lysis of target cells ranged from 3 to 10% depending on the experiment. DioC18-labeled YAC-1 cells treated with 3% saponin were also stained with EMA and further analyzed to set the gate for discrimination of live and dead cells (**Figure 1D**). A representative dot plot image of the target cells after incubation with effector cells is shown in **Figure 1E**.

Statistical Analysis

Data of NK activity were analyzed by one-way ANOVA followed by Tukey HSD *post hoc* test to determine significant differences among the treatment groups. In all statistical analyses, differences were considered significant if P values were <0.05. All data were analyzed using freely available R software version 3.1.2 (https://www.r-project.org/).

RESULTS

Effect of *Salacia* Extract

Five days post-infection, mice infected with influenza virus showed noticeable clinical symptoms, including sneezing and coughing. Nonetheless, upon oral administration of 0.6 mg/day of SSRE, 80% of mice showed a decrease in the incidence of coughing (**Table 1**).

Histopathology

Histopathologically, pulmonary tissues of control mice showed typical acute influenza-associated pneumonia, as neutrophilic and lymphocyte infiltration was prominent in the alveoli and peri-bronchi (**Figure 2A**). Edema and cell debris were also noted. In contrast, pulmonary inflammation was clearly inhibited in mice in the SSRE group (**Figure 2B**), and the influenza cases in this group decreased from five severe to one severe and four moderate (**Table 1**).

NK Cell Activity Assays

Comparison between the Effects of *Salacia* Extract and Lactic Acid Bacteria Preparation

At the E:T ratio of 25:1, NK cell activity in splenocytes of SSRE-administered mice was significantly higher than that observed in splenocytes of mice given JCM1134 or in the control group

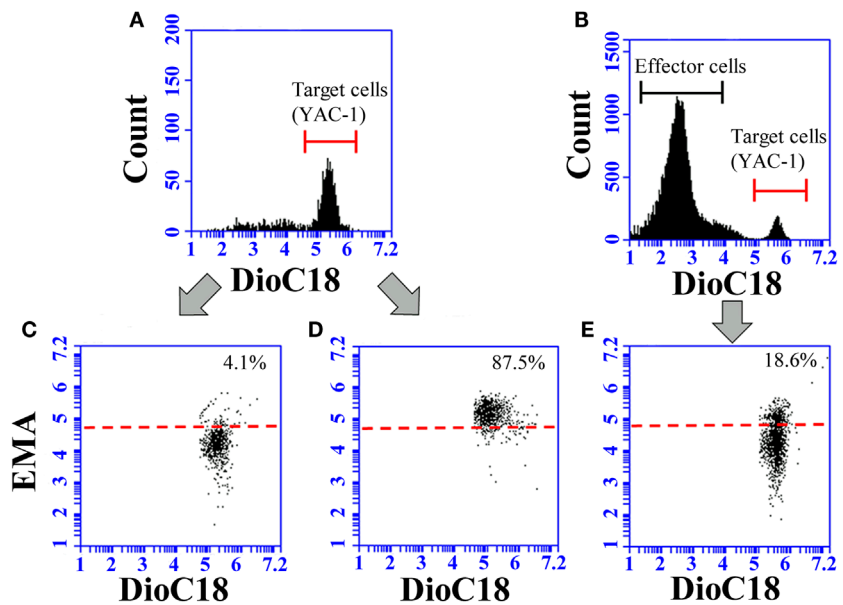

FIGURE 1 | Representative images of flow-cytometric analysis for the determination of NK activity. To discriminate the target cells, live gate (red line) was set in the FL1 histogram on the green fluorescent **(A,B)**. The target cells were further analyzed with a dot plot of FL1 and FL3 **(C–E)**. Spontaneous lysis of YAC-1 cells during the incubation was determined for DioC18-labeled YAC-1 cells incubated in the absence of effector cells **(C)**. The gate for the discrimination of live and dead target cells (red dotted line) was set in accordance with the red fluorescence of DioC18-labled YAC-1 cells treated with 3% saponin **(D)**. The target cells lysed by effector cells appear in the gate for dead cells **(E)**.

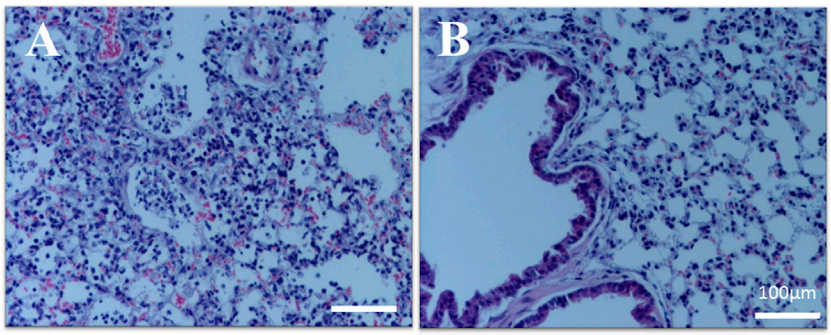

FIGURE 2 | Pulmonary histopathology of influenza virus-infected mice. Influenza virus-infected mice were administered an *S. reticulata* stem and root extract (SSRE), and their lung tissues were examined histopathologically 5 days post-viral infection. **(A)** Control group: pulmonary tissue sections from control mice show acute influenza pneumonia. In addition, infiltration of inflammatory cells, such as neutrophils and macrophages, can be observed in the alveoli, along with associated edema and cell debris. **(B)** SSRE group: only slight inflammation was observed in the pulmonary tissue sections of SSRE-administered mice.

TABLE 1 | Effect of *Salacia* stem and root extract on influenza virus-infected mice.

Treatment	Concentration (mg/day)	Infected mice	Viral dose ($TCID_{50}$)	Number of mice				
				Symptoms		Pulmonary inflammation		
				Sneezing	Coughing	Normal	Moderate	Severe
Control	0	5	10^5	1	5	0	0	5
SSRE	0.6	5	10^5	1	1	0	4	1

SSRE, S. reticulata stem and root extract.

(**Figure 3A**). At the E:T ratio of 10:1, splenocytes of SSRE-administered mice had higher NK cell activity than did those of mice administered JCM1134, although this difference was not significantly higher than the NK cell activity detected in splenocytes of control mice (**Figure 3B**).

Regarding the NK cell activity in pulmonary cells, at the E:T ratio of 10:1, it increased in mice administered both SSRE and JCM1134 when compared with the NK cell activity in pulmonary cells of mice in the control group (**Figure 4A**). However, at the E:T ratio of 5:1, the NK cell activity was comparable across all treatment groups (**Figure 4B**).

Effect of *Salacia* Extract upon Administration of an Antibiotic Cocktail

The body weight of mice drastically decreased after antibiotic administration. As a result, most tissues, including spleen, were substantially small and thus, collecting a sufficient number of cells from them for the analyses proved difficult. Hence, to ensure reliability and based on the results from Experiment 2, it was decided to use splenocytes and pulmonary cells only at E:T ratios of 25:1 and 10:1, respectively.

At the 25:1 E:T ratio, SSRE administration induced an increase in NK cell activity in splenocytes of antibiotic-administered mice that reached a level similar to that detected in splenocytes of mice not treated with antibiotics (**Figure 5A**). Likewise, at the 10:1 E:T ratio, NK cell activity in pulmonary cells of antibiotic-treated mice was increased by SSRE administration to a level similar to that detected in splenocytes of mice not given the antibiotic cocktail (**Figure 5B**).

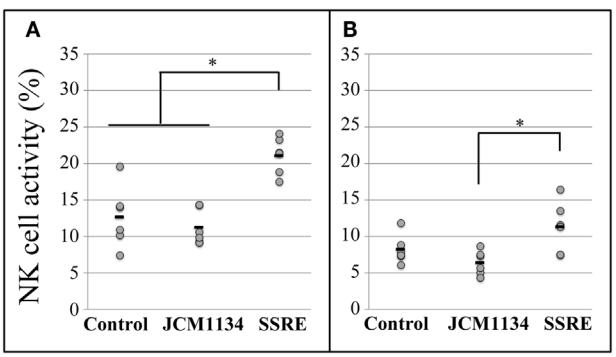

FIGURE 3 | Natural killer cell activity in splenocytes of influenza-infected mice administered either *L. casei* JCM1134 or *S. reticulata* stem and root extract. **(A)** NK cell activity in splenocytes of mice at an effector cell:target cell (E:T) ratio of 25:1. **(B)** NK cell activity in splenocytes of mice at an E:T ratio of 10:1. Control: mice given 500 μL of phosphate-buffered saline (PBS). JCM1134: mice administered 10^8 cfu of *L. casei* JCM1134 in 500 μL of PBS. SSRE: mice administered 500 μL of *S. reticulata* stem and root extract in PBS (1.2 mg/mL of PBS). Each horizontal bar represents the mean value for six mice. *$P < 0.05$.

DISCUSSION

Salacia species were previously found to have potential immunomodulatory properties for treating influenza *in vitro* (39, 40). At these premises, we also observed that administration of a bark

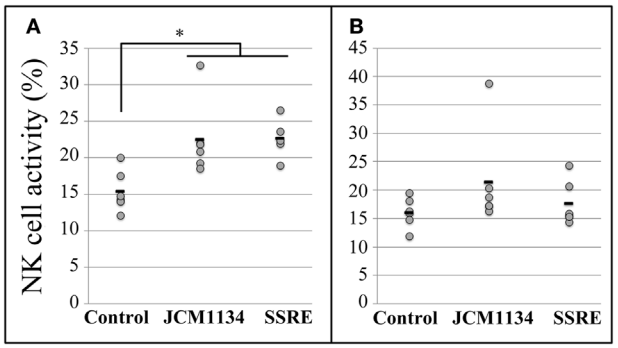

FIGURE 4 | Natural killer cell activity in pulmonary cells of influenza-infected mice administered either *L. casei* JCM1134 or *S. reticulata* stem and root extract. **(A)** NK cell activity in pulmonary cells of mice at an effector cell:target cell (E:T) ratio of 10:1. **(B)** NK cell activity in pulmonary cells of mice at an E:T ratio of 5:1. Control: mice given 500 μL of phosphate-buffered saline (PBS). JCM1134: mice administered 10^8 cfu of *L. casei* JCM1134 in 500 μL of PBS. SSRE: mice administered 500 μL of *S. reticulata* stem and root extract in PBS (1.2 mg/mL of PBS). Each horizontal bar represents the mean value for six mice. *$P < 0.05$.

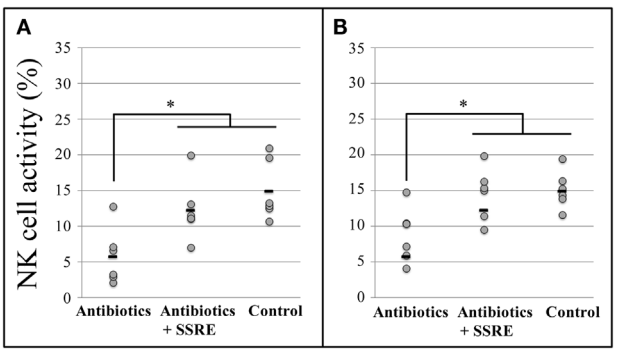

FIGURE 5 | Natural killer cell activity in splenocytes and pulmonary cells of influenza-infected mice administered *S. reticulata* stem and root extract after given an antibiotic cocktail. Antibiotic administration drastically decreased the body weight, and hence, the size of tissues of mice also decreased. As a result, the number of splenocytes and pulmonary cells for the analyses was insufficient. Thus, splenocytes **(A)** and pulmonary cells **(B)** were used only at effector cell:target cell (E:T) ratios of 25:1 and 10:1, respectively. Antibiotics: mice given an antibiotic cocktail in drinking water consisting of 1 g/L of ampicillin, 500 mg/L of vancomycin, 1 g/L neomycin sulfate, and 1 g/L of metronidazole + 500 μL of phosphate-buffered saline (PBS). Antibiotic–SSRE: mice administered the antibiotic cocktail + 500 μL of *S. reticulata* stem and root extract in PBS (1.2 mg/mL of PBS). Control: mice with intact microbiota given 500 μL of phosphate-buffered saline (PBS). Each horizontal bar represents the mean value for six mice. *$P < 0.05$.

and root extract from *S. reticulata* triggered an antiviral immune response in rat ileum involving Cd26 and IgG2a (30). Therefore, we wanted to test whether SSRE would trigger similar immune responses in H1N1 influenza-inoculated mice.

Excessive production of immune cells, such as neutrophils and lymphocytes, during influenza infection often leads to severe lung inflammation (41). In contrast, our results showed that upon SSRE administration, pulmonary inflammation of lung tissues markedly decreased (**Figure 2B**) and cough scores improved (**Table 1**) in influenza-inoculated mice. Similar effects were previously reported in influenza virus-infected mice that administered a compound prepared with extracts from plant roots and rice grains (42). It is very likely that SSRE was effective in reducing production of immune cells by inhibiting influenza viral replication. For example, in previous work, it was reported that *Salvia chinenesis* inhibited the binding of viral double stranded RNA and non-structural protein 1 (39) expressed in nuclei of H5N1 virus-infected cells (43). Non-structural protein 1 is essential for the virus replication cycle (44), blocks gene expression (45), and antagonizes innate immune response (46) initiated in host cells.

In the present study, we focused on NK cell activity to further evaluate the protective effect of SSRE against H1N1 influenza virus-infected mice. NK cells are the first line of defense against influenza infection (7). However, if NK cell activity is not regulated, healthy cells may also be damaged by NK cell cytotoxicity (47). While we observed that SSRE administration substantially enhanced NK cell activity, histological observation confirmed that there was no excessive infiltration of cells in lung tissues of SSRE-administered mice. Hence, we believe that although SSRE administration induced an increase in NK cell activity, it also activated regulatory processes that maintained the number of NK cells produced under control. A preliminary experiment conducted in our laboratory provided further evidence for this theory, as it suggested that SSRE administration did not alter NK cell activity in splenocyte and pulmonary cells of healthy mice, that is, mice not infected with influenza virus (data not shown). In other words, this result seems to indicate an absence of overstimulation by SSRE of NK cell activity under healthy conditions. Our results also showed that at high E:T ratios, NK cell activity was enhanced in splenocytes and pulmonary cells of SSRE-administered mice, but at lower E:T ratios, NK cell activity was not different across treatment groups. During viral infections, cell-to-cell contact between NK cells and infected cells downregulates receptors on NK cell surface, such as NKG2D and NKp30, which inhibits NK cell activity *in vivo* (48). Thus, high effector/target ratios at primary infection sites are required for an effective immune protection (49), which is consistent with our results.

In Experiments 1 and 2, it is very probable that gut microbiota of mice played a role in the effects observed after administration of SSRE. Evidence suggests that during viral infection, gut microbiota helps reduce host susceptibility by exerting *colonization resistance* (50) to prevent adhesion of pathogens and maintain immune homeostasis. Indeed, gut microbiota has been suggested to upregulate the expression of NK cell surface ligands on IEC when microbiota imbalance and intestinal inflammation are detected (51). Influenza virus infection, however, can severely alter the composition of gut microbiota and disrupt gut homeostasis. For example, previous studies showed that influenza virus infection increased exposure of galactose and mannose residues on IEC surfaces and hence overexpression of glycoreceptors, which increased adhesion of pathogens and caused an elevated production of proinflammatory cytokines (52).

Certain LAB strains with probiotic activity also show antiviral properties. Moreover, LAB supplementation to influenza-infected models has been reported to help protect against influenza infections. For example, LAB preparations were demonstrated to modulate immune responses in influenza virus-infected mice (16–19), which resulted in lower virus titer (16) and improved clinical symptoms (20). Therefore, to estimate the immunomodulatory properties of SSRE, in Experiment 2, we tested SSRE against JCM1134, a bacterial strain previously used elsewhere as adjuvant to help increase *in vitro* NK cell activity (53). Interestingly, our results showed that NK cell activity induced by SSRE was similar to or greater than that induced by JCM1134, especially in spleen, which suggests that SSRE could be used as an alternative or complement to LAB supplementation to treat influenza infections.

Although detailed investigation of the molecular mechanisms of action of individual active ingredients in SSRE was beyond the scope of the present work, two routes can be theorized. *S. reticulata* is rich in phytochemicals with immunomodulatory properties, such as polyphenols salacinol, kotalanol (54, 55), epigallocatechin, and epicatechin (56). Thus, one route may have been an indirect effect by salacinol and kotalanol. Salacinol and kotalanol likely inhibited disaccharidases, including α-glucosidase, in the gut of mice (54, 55, 57), which altered the composition of gut microbiota, such as the ratio between firmicutes and bacteroidetes (30). This shift in microbial composition resulted in an increased release of activated cytokines, such as NKT cell-induced IFN-γ in IEC (9).

An alternative route may have been a more direct effect of phytochemicals in SSRE, as they were likely absorbed unaltered. Indeed, phytochemicals, such as catechins, may have stimulated NK cell immune surveillance levels (58) which, upon detection of viral particles, inhibited further replication of influenza virus (59). To test this alternative route, in Experiment 3, mice were administered SSRE after being given a powerful antibiotic cocktail and inoculated with influenza virus. We confirmed by fecal smear assays that the antibiotic cocktail removed most gut microbiota, because at the start and end of SSRE/PBS administration, only a negligible number of bacteria were found in feces of antibiotic-administered mice (data not shown). Moreover, as expected, the sole administration of antibiotics drastically inhibited NK activity in mice (**Figure 5**), the result of which was chosen as baseline to test the effect of SSRE administration. The significant reduction in NK activity detected in microbiota-deprived mice was likely due to the microbiota composition was severely altered by the antibiotic administration (60), which in turn caused downregulation of gene expression and disruption of the immune response (61). Antibiotics, such as azithromycin (62) and neomycin (63), reportedly suppress NK cell cytotoxicity and vancomycin decreases IFN-γ levels (51). Thus, the fact that the administration of SSRE increased NK cell activity to normal levels in splenocytes and pulmonary cells of microbiota-deprived mice was somewhat unexpected (**Figures 5A,B**). These results strongly suggest that phytochemicals in SSRE, possibly catechins, stimulated without mediation of gut microbiota an increase in NK cell activity in spleen and lungs. Moreover, these results are in concordance with previous work reporting the effectivity of a mixed extract from three edible plants, namely, *Angelica radix*, *Cnidium rhizome*, and *Paeonia radix*, in helping restore the proliferative response, cytokine production, and NK cell activity in splenocytes of aged mice with impaired immune response (64). Further investigation of the antiviral activity of phytochemicals in *S. reticulata* is needed to determine the underlying molecular mechanisms of action against influenza infection.

In summary, in the present work, we assayed the antiviral properties of an extract from stems and roots of *S. reticulata*. We showed that SSRE administration markedly reduced clinical symptoms and immune cells infiltration in lung tissues of influenza virus-infected mice. In addition, SSRE administration induced an increase in NK cell activity in splenocytes and pulmonary cells of mice equal to or greater than that observed in JCM1134-administered mice. The enhancement of NK cell activity by SSRE may contribute at least to some extent to the attenuation of lung damage observed in Experiment 1. Furthermore, when SSRE was given to mice deprived of gut microbiota by an antibiotic cocktail, NK cell activity was increased to a level similar to that observed in mice not given antibiotics. It can be reasonably concluded that at least some of the protective effects exerted by phytochemicals in SSRE against influenza infection were not necessarily through modulation of gut microbiota, but the underlying molecular mechanisms need to be further elucidated.

AUTHOR CONTRIBUTIONS

GR-P: wrote the paper, analyzed the data, helped complete the experiments, and prepared and edited figures and tables. ME, YH and TTsuruta: carried out the experiments. YO and FU: conceived the experiment and prepared the experimental material (*Salacia*). TTsukahara: conceived and co-designed the experiment. YT: conceived and co-designed the experiment and prepared influenza virus inoculant. RI: conceived and co-designed the experiment, supported during the experiments, co-wrote the paper, and supervised data analysis.

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OPINION
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Probiotics as a Potential Alternative for Relieving Peripheral Neuropathies: a Case for Guillain-Barré Syndrome

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Keywords: Guillain–Barré syndrome, peripheral neuropathies, neuronal autoimmunity, probiotics, microbiota, gut–brain axis

INTRODUCTION

Trillions of microorganisms form the "natural flora" of the human body. These microorganisms outnumber the host cells 10 to 1, essentially making humans a microbial ecosystem of sorts. Before the advent of the high-throughput sequencing technologies such elusive microorganisms were uncultivable and could not be studied using traditional microbiological techniques. However, since the past decade, with the establishment of the Human Microbiome Project reference database in 2012, research in this area has grown logarithmically (Grogan, 2015). The human microbiota is introduced from mother to the fetus and even infants, and remains with the growing individual for the rest of his/her life, being effected by and also affecting the individual's lifestyle, food habits, and metabolism. Friendly microbes have been shown to protect from several diseases and a dysbiosis in the microbiota has been linked to infections by pathogens, autoimmunity and lifestyle disorders. The establishment of the brain-gut axis (reviewed in Carabotti et al., 2015) in the past few years has even shown that gut microbiota and brain affect each other (Carabotti et al., 2015). A very recent study established the missing link between the central nervous system (CNS) and the immune system i.e., the CNS lymphatic vessels, thus completing the microbiota-neuronal-immune system triangle (Louveau et al., 2015). Even stress, and behavioral changes can result in an altered microbiota and vice–versa (Schmidt, 2015). This has brought microbiologists and neuroscientists together to look into the probable mechanisms in which dysbiosis of gut microbiota may affect development of neurodegenerative diseases and even neuronal autoimmunity.

Autoimmune diseases of both the central and the peripheral nervous system (PNS) have been linked to microbiota, although the extent to which the link has been established is very different. While some like multiple sclerosis (MS) has working mice-models, others like Guillain–Barré syndrome (GBS), a rare autoimmune disorder of the PNS has no defined models, although experimental autoimmune neuritis (EAN) may be used to study some aspects of GBS, especially macrophage mediated demyelination (Kieseier et al., 2012). Even so, we have been warned against the fallacies of studying such models in isolation, as there is no strict one model-one disorder kind of relationship between human autoimmunity and mouse/rat model (Gold et al., 2000). Since probiotics have been found to be useful in the treatment of autoimmune diseases like Inflammatory Bowel Disease (IBD) (Sheil et al., 2007), type1 diabetes (Calcinaro et al., 2005) and even extra-intestinal neuronal and systemic-autoimmune disorders like MS (Lavasani et al., 2010) and systemic lupus erythematosus (SLE) (Manirarora et al., 2008; Alard et al., 2009), testing the efficacy of probiotics in the treatment of GBS might offer a less invasive and more acceptable and economical treatment alternative.

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PROBIOTICS MODULATE THE GUT MICROBIOTA TO AFFECT THE HOST IMMUNE SYSTEM

Altered gut microbiota or "gut dysbiosis" has been linked to autoimmune responses both intra-intestinally as well as extra-intestinally. Recently concluded and active research has helped detail the mechanisms of interaction between the gut microbiota and the immune system (Maynard et al., 2012). Attempts to understand the mechanisms of restoration of healthy gut microbiota (eubiosis) by probiotics has started to reveal the complex interactions and signaling involved between the host cells including components of the immune system, the probiotic strain and the other commensal or pathogen residing in the host system (Wagner et al., 2009; Wine et al., 2009; Maynard et al., 2012; Bollrath and Powrie, 2013). It is generally accepted that adaptive immunity to microbes may be mediated by Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and NOD-like receptors (NLRs), which are present on the epithelium, endothelium, and lymphoid tissue of the host. These receptors might signal downstream depending upon the situation of eubiosis (homeostasis)/dysbiosis (Walker, 2008; Maynard et al., 2012). Traditionally, two T-cell fates have dominated the immune system related signaling *viz.* T-helper cell (T_H1) and T_H2. T_H1 type response is elicited on exposure to pathogens and leads to their phagocytosis, causing inflammation to the surrounding host tissue and even autoimmune responses. T_H2 type of response is elicited mainly on exposure to environmental cues and also some pathogenic proteins, leading to the production of IgE and release of histamines that counter T_H1 response (Berger, 2000). Later studies have, however, revealed a much more complex picture of autoimmunity and has introduced new types of $CD4^+$ T-cell *viz.* T_H17 (Damsker et al., 2010) and $CD25^+$ $FoxP3^+$ regulatory T-cells (Treg) (Sakaguchi et al., 2009). During immune homeostasis the concentration of Interleukin (IL-10) (also secreted by T_H2 cells) secreting Treg cells dominate the intestinal mucosa. In most of the autoimmune conditions, a common feature seems to be diminished presence of Treg cells in the intestinal mucosa of the host resulting in a shift toward IL-17 secreting T_H17 cells and this shift in the T-cell population seems to be dependent on the intestinal cytokine Transforming Growth Factor (TGF-β). Interestingly, in the face of pathogenic infection or an inflammation caused by the resident microbiota, the IL-10 secreting Treg cells are also converted to IL-17, Retinoic acid-related Orphan Receptor (RORγT), and Interferon (IFN-γ) producing T_H17 cells (Xu et al., 2007) and vice–versa (Gagliani et al., 2015). Research exploring the gut-brain axis has lead to an understanding that altered gut microbiota plays a role not only in gastrointestinal autoimmunity but also extra-intestinal autoimmunity. Systemic and neuro-autoimmunities like SLE and MS have been linked to gut dysbiosis, making a strong case for linking GBS to altered microbiota.

Our current understanding of the link between gut eubiosis and immune homeostasis, and the effect of probiotics on the former have been reviewed elsewhere (Hemarajata and Versalovic, 2013; Kueffel et al., 2013). Various studies have already tipped the balance toward the use of probiotics for providing relief in a number of diseases and disorders including extra-intestinal autoimmunities. As briefly mentioned above, a study has pointed toward the therapeutic effect of probiotic consisting of a mixture of *lactobacilli* on experimental autoimmune encephalomyelitis (EAE; an experimental model of MS) through IL-10 secreting Treg cells (Lavasani et al., 2010). In a later study, it has been shown that pro-inflammatory T-cell response consisting of IL-17A and IFN-γ producing T_H17 cells caused EAE in germ-free mice upon infection from Segmented Filamentous Bactria (SFB) (Lee et al., 2011), further linking gut dysbiosis and neuronal autoimmunity. Immunopathology of GBS too is complicated by T_H17 cells and the corresponding cytokines, thus making it a balance between T_H1/T_H17 and T_H2, although mechanistic details are yet to be elucidated (Liang et al., 2012; Li et al., 2012, 2014). Functional aspects of T_H17 cells and associated cytokines and their rational drug targeting with respect to GBS is discussed in some details elsewhere (Wang et al., 2013; Wu et al., 2015). Studies on the lines of drug trials have shown that in most stomach related ailments; probiotics do confer a health benefit (Mack, 2005). Some small studies in model organisms show that probiotics confer prophylactic or curative benefit against diabetes, certain cancers, Human Immunodeficiency Virus (HIV), rheumatoid arthritis, and MS among several other conditions (Erickson and Hubbard, 2000; Matsuzaki et al., 2007).

GUILLAIN–BARRÉ SYNDROME: PROTOTYPE OF AN IMMUNE MEDIATED PERIPHERAL NEUROPATHY

GBS is a post-infection autoimmune monophasic disorder of the PNS and is characterized by acute flaccid paralysis. The syndrome has variants categorized depending upon the kind of neuropathy *viz.* demyelinating or axon degenerating. These have been described in detail by Dimachkie and Barohn (2013) and have been summarized in the form of a table in this article (**Table 1**). The immunopathology of the GBS is not well understood however it is known that the syndrome occurs post-infection by *Campylobacter jejuni* (Kaldor and Speed, 1984; Allos, 1997), *Mycoplasma pneumonia* (Goldschmidt et al., 1980), certain Herpesviridae (Jacobs et al., 1998), HIV (Pontali et al., 2011) and flu viruses (Sivadon-Tardy et al., 2009) or in some cases after the administration of flu vaccine (Geier et al., 2003), most probably due to an imbalance in the various T-helper cell populations described above. Proinflammatory, T_H1 type cytokines like Tumor Necrosis Factor (TNF-α), IFN-γ, and IL-1 have implicated in triggering mechanism of EAN/GBS. Other proinflammatory cytokines like IL-12, TNF-α/β, and IL-2 are also detected during the course of the disease progression. T_H17 cytokines IL-17 and IL-22 have also been detected in the serum of GBS patients thus confirming the role of T_H17 mediated pro-inflammatory response (Li et al., 2012). Interestingly, proinflammatory cytokines IL-4, IL-5, and IL-6, may be produced by cells expressing the rival T_H2 phenotype, contributing to GBS.

TABLE 1 | Variants of Guillain–Barré Syndrome.

GBS variant	Symptoms	Mechanism of autoimmunity	Autoantigens implicated	Remarks/references
AIDP	Areflexia, mild sensory changes, distal paresthesisas, loss of tendon reflexes, ascending paralysis, respiratory failure	Macrophages, T-cell mediated demyelination		Dimachkie and Barohn, 2013
AMSAN	Loss of deep tendon reflex, distal weakness and sensory aymptoms	Auto antibodies against nerve gangliosides	GD1a, GM1, GM1b	Shoenfeld and Meroni, 2012; Dimachkie and Barohn, 2013
AMAN	Accute, flaccid ascending paralysis, high protein in the CSF, dysphagia, dysarthria, total loss of refexes, and respiratory failure in advanced cases	Auto antibodies against nerve gangliosides	GD1a, GM1, GM1b, GalNac-GD1a	Discovered in China, less common than AIDP in the West (Shoenfeld and Meroni, 2012; Dimachkie and Barohn, 2013)
MFS	Ophthalmoplegia, ataxia, areflexia	Auto antibodies against nerve gangliosides	GQ1b	5–10% cases in West but up to 25% in Japan (Shoenfeld and Meroni, 2012; Dimachkie and Barohn, 2013)

However, T_H2 type cytokines IL-10 and TGF-β are associated with receding EAN/GBS symptoms (Zhu et al., 1998). Both T-cell mediated and humoral immunity seems to be playing a role in GBS. In Acute Inflammatory Demyelinating Polyneuropathy (AIDP) variant, activated T-cells, upon antigen presentation by macrophages, cross the blood-nerve barrier (BNB) and release cytokines that activate endoneural macrophages, damaging the compact myelin. Alternatively either by directly recognizing a pathogenic epitope or elicited by T-cell, B-cells produce antibodies to the cross-reactive axolemmal autoantigens and by fixing a complement leads to the damage of Schwann cells and/or axons, leading to Acute Motor Axonal Neuropathy (AMAN), the more severe Acute Motor and Sensory Axonal Neuropathy (AMSAN), or Miller Fisher Syndrome (MFS) (Hughes and Cornblath, 2005). The most common autoantigens are GM1, GM1a, and GD1a (Dimachkie and Barohn, 2013). Due to these variants and their diverse pathology and pathogenesis, GBS is now considered a group of heterogeneous conditions with similar clinical phenotypes (Kieseier et al., 2012). GBS has been considered a unique autoimmune disorder due to the fact that unlike most other neuronal autoimmune disorders, it is monophasic and that its occurrence has been found to correspond to immunosuppression in the patient (Steiner et al., 2010). The antecedent causative organisms mostly linked with GBS include *C. jejuni* (≈38%) (Kaldor and Speed, 1984), *M. pneumonia* (≈21%) (Sharma et al., 2011). Infections by most or all of these agents are characterized by immunosuppression either as a primary result of the infection or as a secondary effect.

EUBIOTIC GUT TO CURE GUILLAIN-BARRÉ SYNDROME

Infection by *C. jejuni*, *M. pneumonia,* and viruses mentioned above also shows a differential effect on Treg and T_H1 cell populations with infected individuals showing a reduced Treg cell presence (Steiner et al., 2010). Besides, there is evidence of *C. jejuni* colonization being exacerbated by a shift in the microbiota (characterized by increased *Escherichia coli* load in the gut) upon infection by *Toxolasma gondii* (Haag et al., 2012) and its alleviation by probiotic strain *Lactobacillus helveticus* strain R0052 (Wine et al., 2009). With studies pointing toward probiotic assisted increase in the population of anti-inflammatory Treg cells (Smits et al., 2005; Kwon et al., 2010), and probiotics modulating T_H1(and T_H17)/T_H2 balance (Torii et al., 2007; Tanabe, 2013) it may be worth testing whether this increase in Treg cells reduces post infection autoimmunity in any way. It may not be wrong to expect a Treg cell mediated immune homeostasis of both the humoral (Wing and Sakaguchi, 2014) and cellular kind (Shevach, 2009), produced as a result of probiotic induced gut eubiosis.

CONCLUSION

GBS is a rare autoimmune disease effecting 2–4 people per 100,000. Being a rare condition it is neglected by the big pharmaceutical companies, as well as academic researchers searching for better treatments. Although the treatments are available in the form of intravenous immunoglobulin (IVIg) administration and plasma exchange, they become quite expensive, especially in the developing countries. Added to it the poor prognosis of the disease, there is a need for cheaper alternatives to the currently available treatments. Probiotics have been shown to be effective in treatment of several intestinal and extra-intestinal autoimmunities, as they act by replenishing the Treg cells in the immune system that have been shown to promote the immune system homeostasis. There is ample reason to believe that GBS also is a result of altered immune homeostasis caused by infection due to an antecedent infection. Thus, promoting the production of Treg cells may help cure GBS by acting against both T- cell mediated and humoral autoimmunity of the PNS.

AUTHOR CONTRIBUTIONS

The author AS claims the sole responsibility of researching and writing this article.

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The article is dedicated to Ms. Priyanka Saxena, my cousin, diagnosed with GBS and went through a painful recovery

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In silico Analysis Revealed High-risk Single Nucleotide Polymorphisms in Human Pentraxin-3 Gene and their Impact on Innate Immune Response against Microbial Pathogens

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Pentraxin-3 (PTX-3) protein is an evolutionary conserved protein that acts as a soluble pattern-recognition receptor for pathogens and plays important role in innate immune response. It recognizes various pathogens by interacting with extracellular moieties such as glactomannan of conidia (*Aspergillus fumigatus*), lipopolysaccharide of *Pseudomonas aeruginosa*, *Streptococcus pneumonia* and *Salmonella typhimurium*. Thus, PTX-3 protein helps to clear these pathogens by activating downstream innate immune process. In this study, computational methods were used to analyze various non-synonymous single nucleotide polymorphisms (nsSNPs) in PTX-3 gene. Three different databases were used to retrieve SNP data sets followed by seven different *in silico* algorithms to screen nsSNPs in PTX-3 gene. Sequence homology based approach was used to identify nsSNPs. Conservation profile of PTX-3 protein amino acid residues were predicted by ConSurf web server. In total, 10 high-risk nsSNPs were identified in pentraxin-domain of PTX-3 gene. Out of these 10 high-risk nsSNPs, 4 were present in the conserved structural and functional residues of the pentraxin-domain, hence, selected for structural analyses. The results showed alteration in the putative structure of pentraxin-domain. Prediction of protein–protein interactions analysis showed association of PTX-3 protein with C1q component of complement pathway. Different functional and structural residues along with various putative phosphorylation sites and evolutionary relationship were also predicted for PTX-3 protein. This is the first extensive computational analyses of pentraxin protein family with nsSNPs and will serve as a valuable resource for future population based studies.

Keywords: PTX-3, nsSNPs, *In silico, Aspergillus fumigatus*, galactomannan, lipopolysaccharide

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INTRODUCTION

Single nucleotide polymorphisms (SNPs) are the single base change in coding or non-coding DNA sequence and are present in every 200–300 bp in human genome (Lee et al., 2005). So far, 5,000,000 SNPs have been identified in the coding region of human population responsible for genetic variation (Rajasekaran et al., 2008). Among all SNPs, non-synonymous SNPs (nsSNPs) are present in exonic part of genome, which often leads to change in amino acid residues of gene product. Substitution of amino acid in protein may alter the stability and protein structure, affecting

the function. Alteration in protein function due to nsSNPs has been reported to various diseases in human (George Priya Doss et al., 2008; Dabhi and Mistry, 2014; Nagasundaram et al., 2015). Recently many studies have been focused on deleterious nsSNPs associated with immunity related genes especially genes associated with innate immune response (Davis et al., 2010; Lin et al., 2012; Ovsyannikova et al., 2014). In addition, recently limited studies have been carried using *in silico* approaches to understand how nsSNP could be deleterious at protein/functional level (Dabhi and Mistry, 2014; Kelly and Barr, 2014).

Human pentraxin proteins are conserved pattern recognition protein receptors, that play a crucial role in innate immunity response (Mantovani et al., 2008). Based on the size, pentraxin proteins are divided into long pentraxins (PTX-3, PTX-4, Neuronal pentraxin-1 and 2) and short pentraxins (C-reactive protein and serum amyloid protein; Garlanda et al., 2002). Pentraxin proteins contain conserved pentraxin-domain at C-terminus and multiple domain sites at N-terminus. C-reactive protein and serum amyloid are well studied member of pentraxin protein family and they have the ability to recognize polysaccharides from bacteria such as *Streptococcus pneumonia* and provide resistance against microbial infections (Garlanda et al., 2002; Martinez de la Torre et al., 2010). Furthermore, PTX-3 protein is a key soluble pattern recognition receptor of innate immunity in lung infections caused by *Salmonella typhimurium, Pseudomonas aeruginosa* and *Aspergillus fumigatus* (Garlanda et al., 2002; Chiarini et al., 2010; Thakur et al., 2015). Invasive infection has been associated to *Aspergillus* species at different organ sites (Kidney, Brain) with predominantly T_H1 immune response (Anand et al., 2013, 2015; Thakur et al., 2015).

PTX-3 protein is produced by various immune cells particularly dendritic cells, macrophages and neutrophils as 10–20 subunit multimer protein in response to various inflammatory mediators such as bacterial lipopolysaccharide, galactomannan of *A. fumigatus* conidia, interlukin-1, and tumor-necrosis factor-α, respectively (Rovere et al., 2000; Balhara et al., 2013). N-terminus and C-terminus of PTX-3 protein overlap and act as pathogen recognition receptors. They recognize various moieties of microbes and facilitate their opsonization (Garlanda et al., 2002). Pentraxin conserved domain binds with C1q and activates the classical complement pathway to signal innate immunity in response to infection (Inforzato et al., 2006). PTX-3 protein inhibits the colonization of various microorganisms in the respiratory tract and lung that includes fungi such as *Candida albicans* and *A. fumigatus* and virus like *influenza virus* (Reading et al., 2008; Cunha et al., 2014). It has been previously reported as a vital component of antifungal innate immune response (Salvatori and Campo, 2012). PTX-3 protein is also suggested as a biomarker to monitor immunopathological conditions of the patients (Balhara et al., 2013). Despite, the importance of PTX-3 gene and its high polymorphic nature it is still unclear how nsSNPs could affect its protective functions against human pathogens, which colonize in respiratory tract and lungs. Taking these into consideration, multiple *in silico* methods were chosen to identify nsSNPs in pentraxin-3 gene, and to predict structure, and function of pentraxin protein. Our analysis showed 10 high- risk nsSNPs in pentraxin-domain. Out of them, 4 nsSNPs were present in highly conserved amino acid residues and such pentraxin-domain variants are considered for putative structure analysis that resulted in alteration in domain possibly affecting their functions. The *in silico* analysis of pentaxin-3 gene of innate immunity paved a foundation for population based studies on predicted deleterious nsSNPs.

MATERIALS AND METHODS

SNP Data Mining for Pentaxin-3 Human Gene

SNPs and protein sequence for PTX-3 gene were collected from different web-based data sources such as dbSNP databases: the NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/SNP), UniProt data base (UniProtKB ID P26022) and the Ensembl genome browser (http://www.ensembl.org/index.html; Sherry et al., 2001; Flicek et al., 2014; Uniport, 2014). These SNPs were used for bioinformatics analysis.

Prediction of Functional Consequences of nsSNPs

nsSNPs were collected from retrieved SNPs and their functional consequences were predicted by using different *in silico* algorithms: Sorting Intolerant from Tolerant (SIFT) server was used to predict the deleterious effects of nsSNPs (http://sift.jcvi.org/). SIFT utilizes the sequence homology of the proteins and by aligning the natural occurring nsSNPs with paralogous and orthologous protein sequences to predict the deleterious effect of nsSNPs. The SIFT score less than 0.05 indicates the harmful effect of nsSNPs on protein function. Another algorithm used for the prediction of functional consequences of nsSNPs was polyphen-2 (http://genetics.bwh.harvard.edu/pph2). Polyphen-2 software uses the protein sequence as well as the amino acid variant position in protein sequence to predict the effect of nsSNP on protein structure and function.

If there is a change in amino acid or mutation in protein sequence, it is evaluated as "possibly damaging" (probabilistic score > 0.15), "probably damaging" (probabilistic score > 0.85) and "benign" (remaining mutations). PolyPhen-2 also calculates the PISC score for each amino acid substitution in protein. The PSIC score difference among variants directly indicates the functional consequences of nsSNPs on protein function. Another algorithms used for the prediction of nsSNPs effects on protein function were SNAP (https://www.broadinstitute.org/mpg/snap/), PhD-SNP (http://snps.biofold.org/phd-snp/phd-snp.html), MAPP (http://www.ngrl.org.uk/Manchester/page/missense-prediction-tools) and PANTHER (http://www.pantherdb.org/). In total, seven different SNP prediction algorithms were used. nsSNPs predicted to be deleterious by at least five different *in silico* SNP algorithms were categorized as high-risk nsSNPs. Because each algorithm uses different parameters to assess the nsSNPs, hence, nsSNPs with more positive results in SNP algorithms are more likely to be deleterious. 10 nsSNPs predicted to be deleterious by SNP prediction algorithms were subjected for further analyses.

Conservation Profile and Phylogenetic Analysis of PTX-3 Protein

To carry out phylogenetic analyses, human protein sequence for PTX-3 (GenBank Accession Number: NP_002843.2) and protein sequences for other eight species of Hominidae family such as *Pan troglodytes* (XP_516838.2), *Mesocricetus auratus* (XP_005077995.1), *Gorilla gorilla gorilla* (XP_004037950.1), *Cavia porcellus* (XP_003476359.1), *Bos Taurus* (NP_001069727.1), *Bison bison bison* (XP_010838164.1), and for Muridae family, *Mus musculus* (CAA58580.1) and *Rattus norvegicus* (NP_001103006.1) were retrieved from the National Center for Biotechnology Information (NCBI) and subjected for their evolutionary conservation. Protein sequence alignment was performed using ClustalX software version 1.8331 (http://www.clustal.org/) and by using multiple sequence comparison by log-expectation (MUSCLE; Edgar, 2004; Larkin et al., 2007). Multiple alignment files saved by ClustalX and MUSCLE in the ClustalX and MUSCLE format were converted to the MEGA format (*.meg) using the MEGA version 6 (http://www.megasoftware.net/; Tamura et al., 2013). Evolutionary conservation of amino acid residues in PTX-3 protein was carried out by ConSurf web server (consurf.tau.ac.il/; Ashkenazy et al., 2010). Phylogenetic analysis was performed by the Maximum Parsimony (MP) method using MEGA version 6 that helps to construe ancestral affiliations and calculate the rates of molecular evolution. The corresponding parameters of the MP algorithm were set at "complete deletion," and the "protein: p-distance" model. Bootstrap method (1000 bootstrap replicates to generate statistically significant phylogenetic tree)' was used (Tamura et al., 2013).

Prediction of Change in Protein Stability due to nsSNPs

To predict the change in protein stability due to mutation, we used I-Mutant version 2, a support vector machine based tool server. I-Mutant version 2 predict the Gibbs free energy change (DDG) by subtracting the mutated protein unfold Gibbs free energy (ΔG) from wild type proteins unfold Gibbs free energy (ΔG). Prediction of energy change can be performed by use of either protein sequence or structure.

I-Mutant version 2 also predict the sign of decrease (Dec.) or increase (Inc.) in Gibbs free energy with Reliability Index (RI) for change in amino acid, where RI-0 indicates lowest reliability and RI-10 indicates highest reliability. The value of change in free energy (DDG) below 0 (<0) indicates decrease in protein stability and value higher than 0 (>0) indicates increase in protein stability. During prediction of energy change, the pH and temperature was set as 7 and 25°C, respectively, for all nsSNP submissions.

Prediction of nsSNPs Positions in Different Protein Domains

To locate the nsSNPs position in different domains of PTX-3 protein structure, we used NCBI Conserved Domain Search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and InterPro (http://www.ebi.ac.uk/interpro/; Hunter et al., 2009; Marchler-Bauer et al., 2015). These tools take either protein sequence in FASTA format or protein ID as query sequence to deduce their domain and motifs. Both of these tools provide functional analysis of different proteins by classifying them into different families as well as domains or important sites.

Molecular Effects of High-Risk nsSNPs On Protein Structure

Three dimensional (3D) structure analyses were done for pentraxin-domain (wild type) and each of the high-risk nsSNP located within this domain. Homology modeling of pentraxin-domain was carried out to predict 3D-structure. The protein sequence of human PTX-3 protein was retrieved from UniProt data base (UniProtKB ID P26022). Homology modeling was done by Web based server Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2). This server uses Hidden Markov Method to generate alignments of submitted protein sequence against proteins with published protein structure. The resulting alignments are then used to produce homology based models of the query sequence to predict its three-dimensional structure. This server also uses an ab-initio folding simulation called Poing to model region of a query sequence with no detectable similarities to known structures. It combines multiple templates of known structure to produce final model of the query sequence. The 3D-structure model for pentraxin-domain was developed by submitting FASTA sequence of pentraxin-domain region to Phyre2 server under intensive mode. The Phyre2 server has used these templates having PDB ID (% identity); {1sac (24%), 1b09 (24%), 4pbo (23%), and 3flp (22%)} for the modeling of 3D-structure of pentraxin-domain. When the degrees of similarity of these templates were analyzed using BLASTp, human's templates 1sac and 4pbo showed 51% similarity with 70% positives. The structure refinement of the predicted model was carried out by ModRefiner by submitting PDB file of model structure (Xu and Zhang, 2011). Then energy minimization of model was performed with YASARA force field minimization tool to improve quality of predicted model of pentraxin-domain (Krieger et al., 2009). Further, model structure of pentraxin-domain was validated by using RAMPAGE server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). RAMPAGE is a popular program used to check the stereo-chemical quality of a protein structure. A Phi/Psi Ramachandran plot was obtained from RAMPAGE to validate the structure of pentraxin-domain.

Modeling of Amino Acid Substitution and Energy Minimization

To generate the mutated models of pentraxin-domain for corresponding amino acid substitutions, Swiss-PDB Viewer was used (Guex and Peitsch, 1997). The PDB model generated from Phyre2 server was used for mutated model generation of pentraxin domain. Swiss-PDB "mutation tool" was used to replaces the wild type amino acid with a new amino acid." The mutation tool facilitates the replacement of the native amino acid by the "best" rotamer of the new amino acid. The ".pdb" files were saved for all the models. Then to improve the quality of predicted model, energy minimization was performed with the YASARA force field minimization server (Krieger et al., 2009). The 3D-structure models were viewed using PyMOL

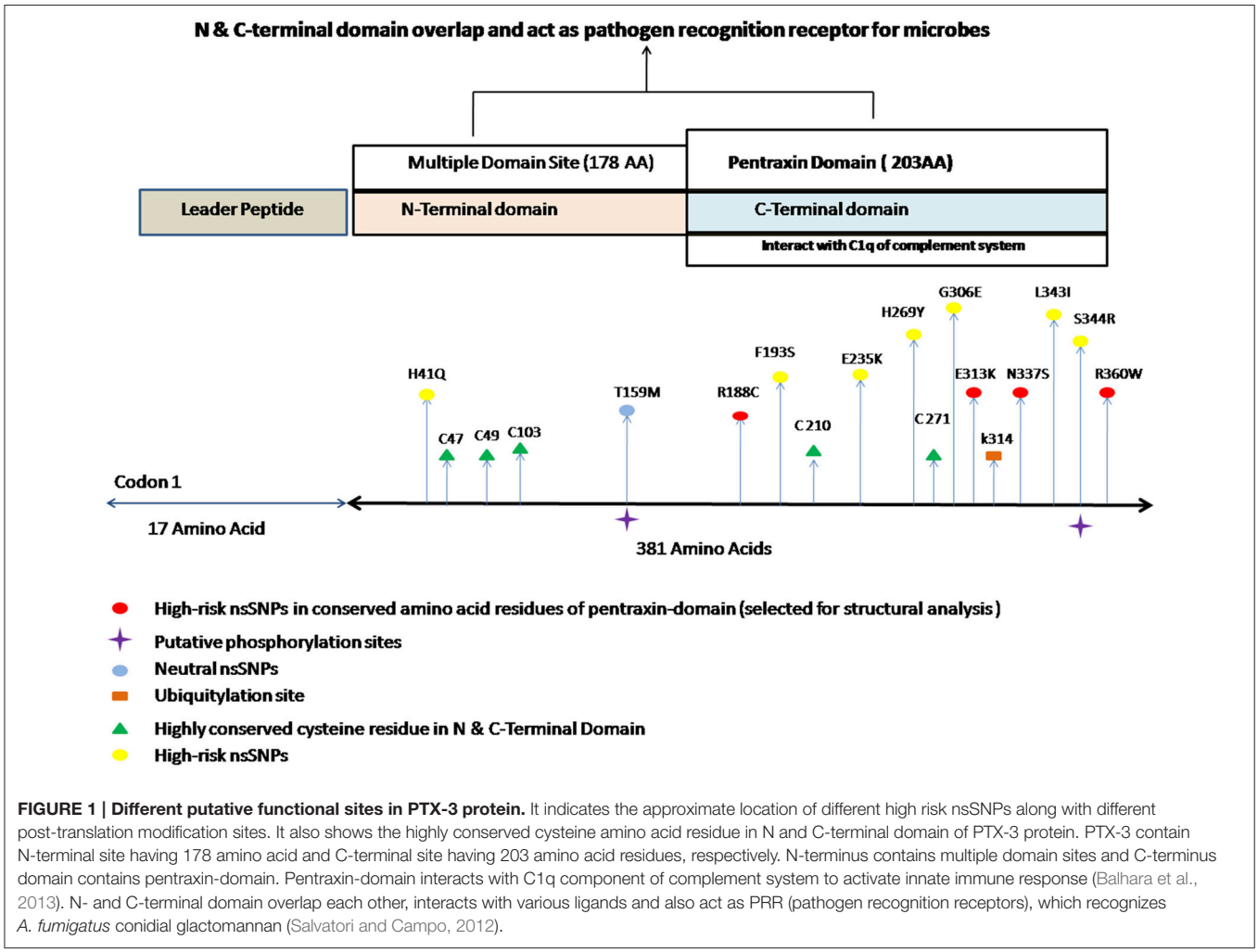

FIGURE 1 | Different putative functional sites in PTX-3 protein. It indicates the approximate location of different high risk nsSNPs along with different post-translation modification sites. It also shows the highly conserved cysteine amino acid residue in N and C-terminal domain of PTX-3 protein. PTX-3 contain N-terminal site having 178 amino acid and C-terminal site having 203 amino acid residues, respectively. N-terminus contains multiple domain sites and C-terminus domain contains pentraxin-domain. Pentraxin-domain interacts with C1q component of complement system to activate innate immune response (Balhara et al., 2013). N- and C-terminal domain overlap each other, interacts with various ligands and also act as PRR (pathogen recognition receptors), which recognizes *A. fumigatus* conidial glactomannan (Salvatori and Campo, 2012).

(https://www.pymol.org/). Further, Tm-score, and Root Mean Square Deviation (RMSD) was estimated for each mutated models using TM-Align (http://zhanglab.ccmb.med.umich.edu/TM-align/; Zhang and Skolnick, 2005).

Prediction of Putative Post-Translation Modification Sites in PTX-3 Protein

Different post-translation modification sites like wise putative ubiquitylation, sumoylation, phosphorylation, and glycosylation sites were predicted using different programs. Putative phosphorylation sites were predicted using NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) and GPS 2.1 (http://gps.biocuckoo.org/). For GPS 2.1 analysis, cut-off values ranging from 0.776-11 were selected. In NetPhos 2.0, threonine, serine, and tyrosine residues with a score of 0.5 were considered phosphorylated (Blom et al., 1999; Xue et al., 2011). Putative sumoylation sites were predicted using SUMOsp 2.0 (http://sumosp.biocuckoo.org/) program and SUMOplot (http://www.abgent.com/sumoplot) program. For SUMOplot, only high probability motifs with a score 0.5 were considered sumoylated. Medium level threshold with a 2.64 cut-off value was selected for SUMOsp 2.0 analysis, respectively (Gill, 2003; Xue et al., 2006). Whereas, putative ubiquitylation sites were predicted by using BDM-PUB (bdmpub.biocuckoo.org) and UbPred (www.ubpred.org) programs (Radivojac et al., 2010). For BDM-PUB, the only balanced cut-off value option was taken and in case of UbPred, the lysine amino acid having 0.62 score was considered as ubiquitylated in PTX-3 protein.

TABLE 1 | Predicted results for nsSNPs in PTX-3 gene using different algorithms.

Prediction	nsSNPs (%)						
	SIFT	PP-2	PP-1	MAPP	PhD-SNP	SNAP	PANTHER
Deleterious	25(38)	27(41)	21(32)	37(57)	18(28)	29(45)	7(11)
Neutral	40(62)	38(59)	44(68)	28(43)	47(72)	36(55)	58(89)

It shows percentage of deleterious and neutral nsSNPs out of total nsSNPs (65) by seven different algorithms; PP-2, polyphen-2, and PP-1, polyphen-1.

Prediction of Protein–Protein Interactions for PTX-3 Protein

Protein-protein interaction networks are important to reveal and interpret all functional interaction among cellular proteins.

In the current study, the online database resource "STRING" Search Tool for the Retrieval of Interacting proteins (http://string-db.org/) was used. This tool provides unique coverage and ease of access to both theoretical and experimental interaction evidence of PTX-3 protein of human. The input options for STRING database include protein sequence, protein name, and multiple sequences. STRING database is presently equipped with 5,214,234 proteins belonging to 1133 organisms. The interaction studies were performed in various modes. The different modes include evidence view, confidence view, interactive mode, and action view to deduce the most appropriate interactions among nodes in PTX-3 protein interaction network (Szklarczyk et al., 2011).

RESULT AND DISCUSSION

Retrieval of SNP Datasets

SNPs from dbSNP (National Center for Biotechnology Information, UniProt database, and Ensembl genome browser; Sherry et al., 2001; Flicek et al., 2014; Uniport, 2014) showed human PTX-3 gene contains 32 SNPs in 3′UTR region, 4 SNPs in 5′UTR region, 170 SNPs in non-coding region (Intron), and 65 missense variants. We subjected these 65 missense mutations or nsSNPs to various *in silico* SNP prediction algorithms that are summarized in **Table 1**, which characterizes nsSNPs as neutral or deleterious to structure and function of PTX-3 protein.

Analysis of Functional Consequences of nsSNPs

Seven *in silico* SNPs prediction algorithms were used; SIFT, PP-2, PP-1, MAPP, PhD-SNP, SNAP and PANTHER (see **Supplementary Table 1**). SIFT analysis uses sequence homology and characterized the effect of amino acid substitution on protein function (Capriotti et al., 2006; Bromberg et al., 2008; Kumar et al., 2009; Adzhubei et al., 2013). As each of these algorithms use different parameters to assess the nsSNPs to be deleterious or neutral, the percentage of deleterious or neutral nsSNPs in PTX-3 by different algorithms have been summarized in **Table 1**.

Furthermore, by using NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchler-Bauer et al., 2015) and InterPro (http://www.ebi.ac.uk/interpro/) (Hunter et al., 2009) tools, pentraxin-domain was predicted in PTX-3. We found 10 deleterious nsSNPs present in pentraxin-domain predicted by at least five different *in silico* SNP prediction algorithms (**Table 2** and **Figure 1**) and these were considered as high-risk nsSNPs. It has been reported, that N-terminal domain overlaps with C-terminal domain (pentraxin-domain) and both of them act as PRR (Pathogen Recognition Region; Martinez de la Torre et al., 2010). nsSNPs E313K, R188C, N337S, and R360W present in pentraxin-domain are highly conserved functional residues (**Table 3**) and hence, selected for structure prediction followed by comparison analysis with the wild type structure.

Recent studies showed nsSNP A48D (rs3816527) in PTX-3 gene is associated with colonization of *Pseudomonas aeruginosa* in cystic fibrosis patients (Chiarini et al., 2010). Cunha et al., has reported nsSNP A48D (rs3816527) in PTX-3 gene in patients of invasive aspergillosis that undergo hematopoietic stem cell transplantation and suggested substitution of amino acid may affect interaction of PTX-3 protein with other proteins due to change in electrostatic potential (Cunha et al., 2014). In our

TABLE 2 | Prediction of deleterious nsSNP in PTX-3 gene.

nsSNP ID	Mutation position	Domain	Deleterious prediction
rs564774580	R188C	PTX	5
rs529759691	F193S	PTX	5
rs532972316	E235K	PTX	5
rs190837481	H269Y	PTX	5
rs144979346	G306E	PTX	5
rs4478039	E313K	PTX	5
rs76994524	N337S	PTX	5
rs146705881	L343I	PTX	5
rs140073706	S344R	PTX	5
rs138818541	R360W	PTX	5

*It represents the use of seven different SNP algorithms with different parameters to evaluate the nsSNPs. nsSNPs predicted to be deleterious by at least five different in silico SNP algorithms were categorized as high-risk nsSNPs. Because each algorithm uses different parameters to assess the nsSNPs, hence, nsSNPs with more positive results in SNP algorithms are more likely to be deleterious. 10 nsSNPs predicted to be deleterious in pentraxin-domain by at least five different SNP algorithms (**Figure 1**).*

TABLE 3 | Conserved amino acids in PTX-3 protein that coincide in location with high-risk nsSNPs.

nsSNPs ID	Amino acid position	CS	ConSurf prediction
rs564774580	R188C*	9	Highly conserved and Exposed (F)
rs529759691	F193S*	8	Highly conserved and Buried
rs532972316	E235K*	9	Highly conserved and Exposed (F)
rs190837481	H269Y*	9	Buried (S)
rs144979346	G306E*	9	Highly conserved and Buried (S)
rs4478039	E313K*	9	Highly conserved and Exposed (F)
rs76994524	N337S*	9	Highly conserved and Buried (S)
rs146705881	L343I*	9	Highly conserved and Buried (S)
rs140073706	S344R*	7	Exposed
rs138818541	R360W*	9	Highly conserved and Exposed (F)

It indicates the conservation score of different amino acid residues calculated by ConSurf: Conservation score (1–4 variable, 5-Average, 6–9 conserved); F, functional; S, structural predicted sites by use of ConSurf Web Server. Asterisk indicates mutated sites at pentraxin conserved domain.

TABLE 4 | High-risk nsSNPs taken for structure analysis.

nsSNP ID	Mutation	Deleterious prediction	ConSurf prediction
rs4478039	E313K	6	9 Exposed (F)
rs1536891	R188C	6	9 Exposed (F)
rs76994524	N337S	5	9 Buried (S)
rs138818541	R360W	6	9 Exposed (F)

This table indicated the high-risk nsSNPs that are coincide with in pentraxin-domain of PTX-3 protein and highly deleterious predicted by more than four algorithms. Conservation score was calculated using ConSurf. Conservation (1–4 variable, 5-Avg, 6–9 conserved).

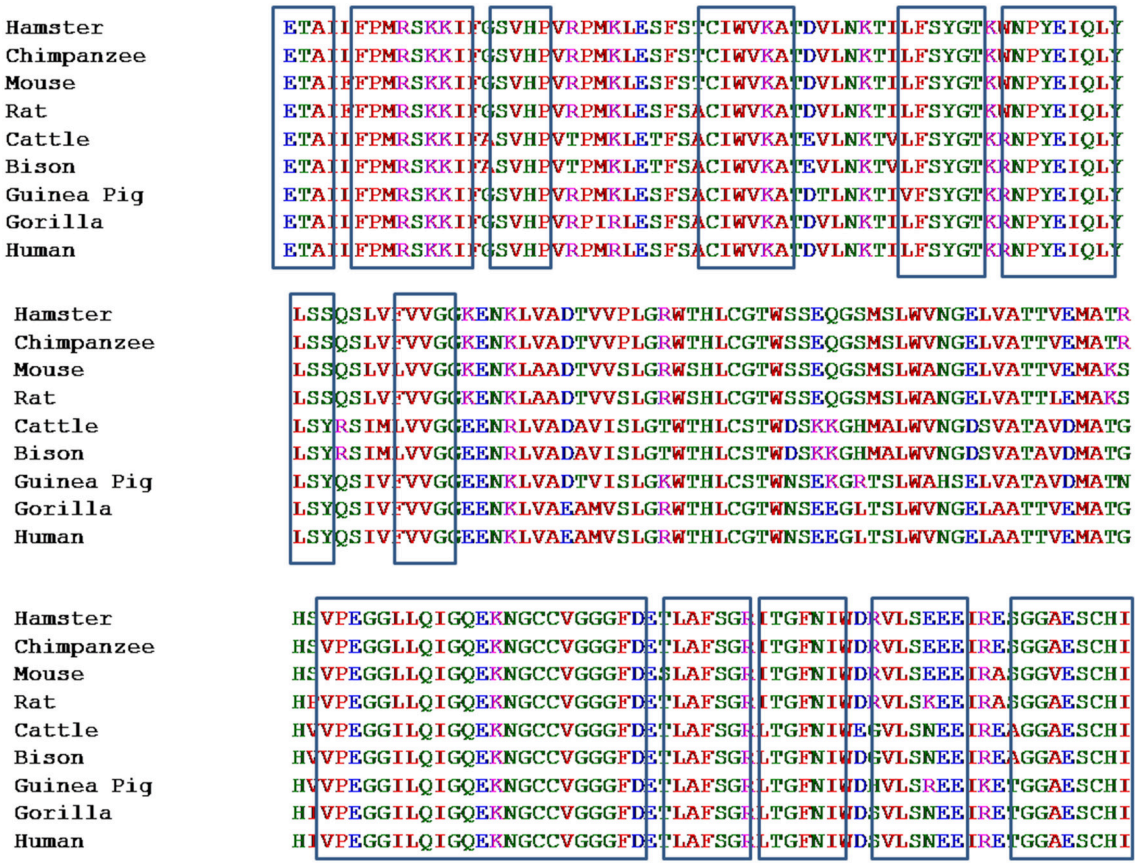

FIGURE 2 | Multiple sequence alignment of pentraxin-domain of PTX-3 protein among nine species taking human PTX-3 protein as reference. It represents the Multiple Sequence Alignment of conserved pentraxin-domain of PTX-3 protein of 9 different species. This was carried out using multiple sequence comparison by log-expectation (MUSCLE), which uses log-expectation scores and fast fourier transform methods, respectively. Program was used with default parameters. Human PTX-3 sequence was taken as a reference and is shown at the bottom of MSA. Areas in boxes represent various conserved regions in pentraxin domain.

TABLE 5 | Analysis of Ramachandran plot of modeled structures by using RAMPAGE server.

Model	Residues in most favored regions		Residues in allowed regions		Residue in outlier region	
	No. of residues	% of residues	No. of residues	% of residues	No. of residues	% of residues
Wild type	197	98	3	1.5	1	0.5
R188C	189	94.01	10	5	2	1
E313K	190	94.5	8	4	3	1.5
N337S	188	93.5	10	5	3	1.5
R360W	186	92.5	12	6	3	1.5

analyses, we also observed nsSNP A48D (rs3816527) in PTX-3 as a deleterious nsSNP by only SNAP nsSNP prediction algorithm.

Conservation Profile of High-Risk nsSNPs and Phylogenetic Analysis of PTX-3 Protein

Conserved amino acids in proteins are involved in various cellular processes in a biological system including genome stability (Greene et al., 2001). Amino acids that are located at enzymatic sites or required for protein-protein interaction become more conserved than other amino acids in protein (Williamson et al., 2013). Due to this reason, the nsSNPs that are located in conserved region are more deleterious as compared to nsSNPs that are located in variable regions in a protein because they destabilize their structure and function. For evaluating the harmful effects of 10 high-risk nsSNPs in the PTX-3 protein, we predicted the evolutionary conservation profile of PTX-3 gene using ConSurf web browser, which use Bayesian method to determine evolutionary conserved amino acid residues in proteins and also identify functional and structural residues

(Ashkenazy et al., 2010). ConSurf analysis predicted 10 highly conserved amino acid residues in pentraxin-domain of PTX-3 protein (**Table 3**, **Supplementary Figure 1**) and two amino acid residues in N-terminal domain site (multidomain site; **Table 3**). We also observed 4 highly conserved amino acids (R188C, E235K, E313K, and R360W) that are exposed functional-residues and 3 highly conserved amino acids (G306E, N337S, and L343I) that are buried structural-residues in PTX-3 protein. Furthermore, to identify the functional and structural sites in protein sequence ConSurf software was used, which uses evolutionary conservation data along with solvent accessibility prediction. Using these parameters, highly conserved amino acids (conservation score 8–9) were predicted to be functional and/or structural amino acid residues in PTX-3 protein based on their location relative to core-protein or protein-surface. Based on analyses, our data suggest that nsSNPs E313K, R188C, N337S, and R360W (**Tables 2, 4**) may alter the structure and function of PTX-3 protein especially to the pentraxin-domain (C-terminal domain). Among four high-risk nsSNP, we found R188C present in uterine corpus endometrioid carcinoma patients, amino acid mutation id-COSM1040427 available at catalog of somatic mutation in cancer (COSMIC) genome browser.

Pentraxin-3 gene is present on chromosome number 3 (q22–28) and contains three exons. First two exons encode for leader and N-terminus, whereas third exon encodes for C-terminus known as pentraxin-domain. Human pentraxin-domain of PTX-3 protein is highly conserved as compared to N-terminal domain (Breviario et al., 1992; Martinez de la Torre et al., 2010). The human and mouse PTX-3 proteins share 93% sequence similarity (Garlanda et al., 2002). Evolutionary relationship of PTX-3 proteins among nine different primates' species was carried out using human PTX-3 protein as a reference sequence. The multiple sequence alignment (MSA) generated form ClustalX and MUSCLE tools for the PTX-3 protein sequences were quite similar and various conserved regions were identified among nine species including human (**Figure 2**). In **Figure 2** the highly conserved regions are shown in black box. Also, multiple sequence alignment showed PTX-3 protein is conserved throughout nine species (**Figure 2**).

Furthermore, phylogenetic analysis was done for PTX-3 protein between nine species of primates including human (**Figure 3**). The phylogenetic tree generated form MEGA version 6 help to understand the evolutionary relationship among different species. Evolutionary tree demonstrates that human and chimpanzees lies close to each other suggesting that the PTX-3 gene is identical in these primates and could be originated from same ancestors. From phylogenetic tree analysis; we concluded that the PTX-3 is highly conserved in primates including humans (**Figure 3**).

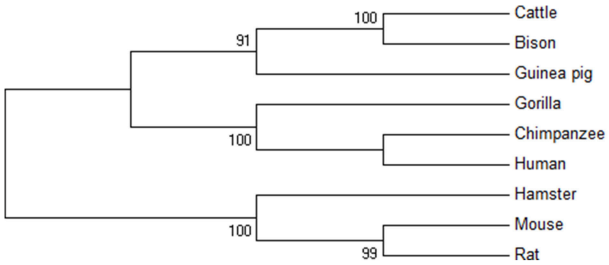

FIGURE 3 | Phylogenetic analysis of PTX-3 protein. A phylogenetic tree of the amino acid sequences of PTX-3 protein was constructed using the maximum parsimony (MP) method. The value on each branch is the estimated confidence limit (expressed as a percentage) for the position of the branches, as determined by bootstrap analysis. Only values exceeding 70% are shown.

Comparative Structure Modeling of Deleterious High-Risk nsSNPs

Deleterious high risk nsSNPs predicted by analysis were subjected to 3D-structure analysis. The ability of proteins to perform various functions or to interact with other molecules depends upon its tertiary structure (Alshatwi et al., 2011). To predict the 3D-structure of pentraxin-domain homology modeling was performed by using Phyre-2 server. Following templates e.g., 4pbo, 1b09, 1sac, and 3flp has been used by Phyre-2 server for the modeling of 3D-structure of pentraxin-domain. 4pbo is a zebrafish short-chain pentraxin proteins, 1b09 is a human C-reactive protein, 1sac is a human serum amyloid p-component, and 3flp is a heptameric SAP like pentraxin protein from *Limulus polyphemus*. These proteins are short-chain pentraxin proteins, where as PTX-3 is a long-chain pentraxin due to additional N-terminal domain. C-terminal domain (Pentraxin-domain) of PTX-3 is homologous to short-chain pentraxin proteins and are involved in recognition of pathogenic bacteria and fungi to activate the classical complement pathway via C1q, a complement pathway component (Inforzato et al., 2013; Chen et al., 2015). Predicted model structure of pentraxin-domain was submitted to structure refinement and energy minimization using ModRefiner and YASARA force field minimization server (Krieger et al., 2009; Xu and Zhang, 2011). The energy minimization repaired the distorted geometries of pentraxin-domain. After this step, model validation was done using RAMPAGE. The Ramachandran plot was obtained from RAMPAGE for energy minimized pentraxin-domain structure predicted by Phyre-2. The structure stability of pentraxin-domain was confirmed by Ramachandran plot. 197 (98%) residues were found in favored region, 3 (1.5%) residues were in allowed region, and 1 (0.5%) residue was found in outlier region. Further, to generate mutated model structure of pentraxin-domain for corresponding amino acid substitutions, Swiss PDB viewer was used (**Figure 4**). Mutated model structure of high-risk nsSNPs (R188C, E313K, N337S, and R360W) was subjected to RAMPAGE for stability assessment (**Supplementary Figure 2** showed RAMPAGE for wild type PTX-3 model protein structure and **Supplementary Figures 3–6** showed RAMPAGE for mutated model structure of PTX-3 protein having R188C, E313K, N337S and R360W, respectively). Results of the Ramachandran plot analysis for each of the high-risk nsSNP model structure was given in **Table 5**. To extend these analyses, we further calculated the root mean square deviation (RMSD) and Tm-score for R188C, E313K, N337S, and R360W high-risk nsSNPs. RMSD is used to measured

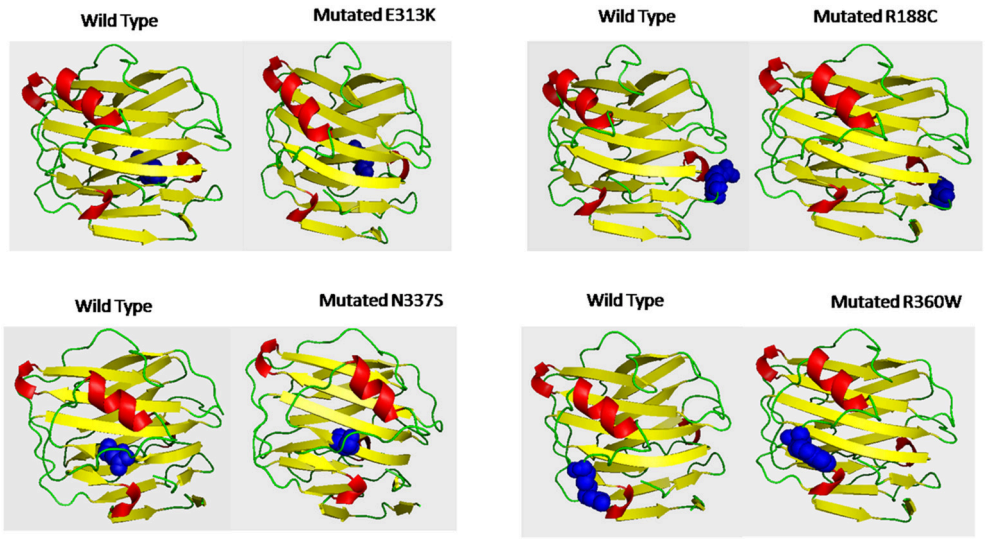

FIGURE 4 | Shows the 3D-putative structure of wild type pentraxin-domain and mutated type domain generated by Phyre-2 version 2.0, which search multiple sequence databases and build 3D-structure based on homolog of known structure. Putative models were viewed by PYMOL and SWISSPDB viewer. Putative 3-D structures were drawn for four high-risk nsSNPs located in pentraxin-domain. Left side of each box shows 3-D structure of wild type pentraxin-domain and at right side shows 3-D putative structure of mutated pentraxin-domain. The location of each mutated amino acid in mutated putative 3-D structures and in wild type pentraxin-domain shown in blue color.

average distance between alpha carbon backbone of wild type and mutant models of protein, where Tm-score is used to determine topological similarity between wild type and mutant model (Zhang and Skolnick, 2005; **Table 6**). A higher RMSD value indicated the deviation in mutant structure as compared to wild type. The maximum RMSD value was found in R360W (0.99) followed by R188C (0.97) and N337S (0.95). This result further indicates that the high-risk nsSNPs significantly alter the structure stability of pentraxin-domain of PTX-3 protein.

Pentraxin-domain of PTX-3 protein is essential for its function as it interacts with various components of innate immunity, strongly with C1qA, C1qC, and CFH to activate the classical complement pathway of innate immunity (Balhara et al., 2013). If the structure of pentraxin-domain is altered, could be possible that it may not be able to interact with these components of complement pathway. Thus, humans with these nsSNPs in their genome may be susceptible to various pathogens particularly *A. fumigatus*, *P. aruogenisa, S. pneumonia, S. typhimurium,* and *Influenza virus* (Reading et al., 2008; Chiarini et al., 2010; Cunha et al., 2014). It is also possible that these high-risk nsSNPs may impair the function of PTX-3 protein as pathogen recognition receptor recognizes several moieties present on cell wall of the pathogens and allow these pathogens for clearance by phagocytic cells.

Further, we used I-mutant to identify the stability of PTX-3 protein containing high-risk nsSNPs. I-mutant is a machine based tool to measure the free energy change caused by single amino acid in protein sequence (Capriotti et al., 2005). High-risk nsSNPs N337S and R360W were predicted to be less stable than the wild type protein, with free energy change values of −0.81 and −0.34, respectively, and having high conserved value predicted by ConSurf (**Table 7**).

Prediction of Putative Phosphorylation, Ubiquitylation, and Sumoylation Sites in PTX-3 Protein

Post-translation modifications were investigated in PTX-3 protein as these modifications control various biological processes in cell such as signaling in innate immune pathway and protein–protein interactions (Perkins, 2006; Liu et al., 2013). We used various *in silico* tools to predictive putative post-translation modification sites in PTX-3 protein. To identified amino acids in PTX-3 protein that may undergo phosphorylation, we use NetPhos 2.0 and GPS 2.1 servers. NetPhos 2.0 server predicted 5-serine and threonine- specific sites in PTX-3 protein. No tyrosine site was predicted by this server in PTX-3 protein. Whereas, GPS 2.1 servers predicted 22-serine and 20-threonine specific sites in PTX-3 protein (**Table 8**). Many of these phosphorylated amino acid residues undergo mutation (**Figure 1**) and are highly conserved in PTX-3. Some of them are predicted to be functional and structural residue by ConSurf.

In addition to phosphorylation site, we also identified ubiquitylation in PTX-3 protein by use of UbPred and BDM-PUB servers. Only single amino acid residue 314 was found, which undergo ubiquitylation (**Table 9**). We also screened the PTX-3 protein for sumoylation sites using SUMOsp 2.0 and SUMOplot but none of the amino acid was found, which undergo sumoylation but only amino acids that undergo sumo interaction were identified by SUMOsp 2.0 (**Table 9**).

TABLE 6 | TM-score and RMSD (Å) value for the high risk nsSNPs in pentraxin-domain of PTX-3 protein calculated by use of TM-align calculator.

nsSNP ID	Mutation position	TM-Score	RMSD(Å)
rs1536891	R188C	0.97	0.97
rs4478039	E313K	0.98	0.92
rs76994524	N337S	0.98	0.95
rs138818541	R360W	0.97	0.99

It represents, that Tm-score is used to assess topological similarity between wild type and mutant protein model. TM-align first generate optimized residue-to-residue alignment based on structural similarity using dynamic programming iterations. An optimal superposition of the two structures, as well as the Tm-score value which scales the structural similarity, will be returned. TM-score has the value in (0, 1), where 1 indicates a perfect match between two structures. Whereas, RMSD (root mean square deviation) is used to measure distance between α-carbon backbone of wild type and mutant models. A higher RMSD value indicates greater deviation between wild type and mutant structure.

TABLE 7 | Free energy change prediction in some selected non-synonymous SNPs (nsSNP) using I-Mutant in PTX-3 protein.

nsSNP ID	Mutation position	DDG	Sign of DDG	RI	PTM	ConSurf
rs34655398	H39Q	−1.97	Dec	8		3e
rs143387231	P40S	−1.41	Dec	9		5e
rs148384694	T41I	−0.56	Dec	7		5e
rs3816527	A48D	0.50	Dec	1		7e
rs367899909	V80A	−1.68	Dec	6		2b
rs557539937	A148T	−1.32	Dec	8		1b
rs572907291	A151T	−1.12	Dec	8		3e
rs112277608	T159M	0.43	Dec	0	Yes	3b
rs370211025	L184S	−2.21	Dec	9		8b
rs564774580	R188C	−1.15	Dec	4		9e(F)
rs529759691	K190E	0.64	Dec	3		6e
rs529759691	F193S	−2.58	Dec	8		8e
rs532972316	E235K	−0.87	Dec	6		9e
rS190837481	H269Y	0.51	Dec	1		9b(S)
rs56265729	V246M	−1.03	Dec	8		7b
rs144979346	G306E	0.14	Inc	2		9b(S)
rs4478039	E313K	−0.15	Dec	2		9e(F)
rs76994524	N337S	−0.81	Dec	8		9b(S)
rs146705881	L343I	−0.93	Dec	9		9b
rs140073706	S344R	−0.99	Dec	2		7e
rs373203093	I348V	−1.48	Dec	8		8b
rs138818541	R360W	−0.34	Dec	5		9e(F)

It shows DDG-change in free energy between mutant and wild; RI, reliability; PTM, Post-translation modification; ConSurf, ConSurf web server used to predict conservation (1–4 variable, 5-Avg, 6–9 conserved) e, exposed; b, indicates buried, and F, S indicates functional or structural residue according to the neural-network algorithm.

Previously, it has been observed that glycosylation of 220 amino acid residues of PTX-3 protein leads to increase in molecular weight of this protein from 40 kDa to 45 kDa. Unique glycosylation sites patterns are associated with different inflammatory cell and induce PTX-3 production by different innate immune cell (Balhara et al., 2013). Thus, post-translation modification sites are important for PTX-3 protein to regulate innate immune response.

TABLE 8 | Putative phosphorylation sites in PTX-3 protein.

NetPhos.2			GPS 2.1		
Serine	**Threonine**	**Tyrosine**	**Serine**	**Threonine**	**Tyrosine**
28(6b)	44(4e)	Not detected	13(4b)	41(5e)*	Not detected
45(5e)	89(4e)		20(2e)	45(5e)	
171(3e)	159(3b)*		54(7e)	77(1b)	
260(5e)	223(6b)		*66(9b)S**	114(1e)	
274(9e)F	281(2b)		98(1e)	124(4e)	
			115(1e)	159(3b)*	
			173(1e)	181(8b)	
			189(9b)S	*216(9b)S**	
			195(8e)F	*222(9b)S*	
			206(7b)	**229(9e)F**	
			208(9b)	268(3b)	
			226(9b)S	273(5e)	
			241(6b)*	281(2b)	
			244(7b)	292(4b)	
			263(2b)	293(2b)	
			276(9e)F	298(1b)	
			282(6b)*	326(4b)	
			330(8e)F	**334(9e)F**	
			341(4e)	351(4e)	
			344(7e)*	*369(9b)S*	
			356(7e)		
			380(5e)		

It represents the different amino acids with phosphorylation sites. Conservation scores were calculated by ConSurf and given under brackets along with exposed and buried indication. Functional putative residues indicated as bold and structural putative residues indicated as italicized. nsSNPs indicated as asterisk (*).

TABLE 9 | Putative sumoylation and ubiquitylation sites in PTX-3 protein.

Sumoylation		Ubiquitylation	
SUMOplot	**SUMOsp 2.0**	**BDM-PUB**	**UbPred**
58(9e)F	**237(9e)F**	190(6e)	58(9e)F
191(8e)F	**238(9b)S**	191(8e)F	190(6e)
314(8e)F	**239(7b)**	221(8e)F	191(8e)F
	240(8b)*	230(7e)	214(9e)F
	241(6e)*	255(3e)	221(8e)F
			230(7e)
			255(3e)
			314(8e)F

It indicates the amino acids with sumoylation and ubiquitylation. No sumoylation of any amino acid residue detected by SUMOplot. Whereas in case of SUMOsp 2.0 only amino acids undergo sumo interaction were detected and indicated in bold. Only one putative ubiquitylation site was detected by two programs, indicated in bold. Conservation score for amino acids given in bracts, where "F" indicates functional and "S" indicates structural residue according to the neural-network algorithm. nsSNPs indicated as asterisk (*).

Protein-Protein Interactions Study of PTX-3 Protein

STRING database was used to annotate PTX-3 protein interaction with other proteins. In interactive mode of STRING database prediction, the binding interaction of PTX-3 protein

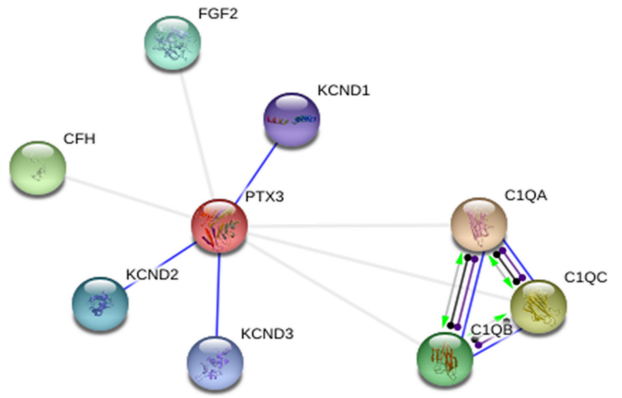

FIGURE 5 | PTX-3 protein–protein interactions in action view. It showed predicted binding interaction of PTX-3 with KCND1 (potassium voltage-gated channel, Shal-related subfamily, member (1), KCND2 (potassium voltage-gated channel, Shal-related subfamily, member (2), and KCND3 (potassium voltage-gated channel, Shal-related subfamily, member (3). Association of PTX-3 was observed with C1qA (complement component 1, q subcomponent, A chain), C1qB (complement component 1, q subcomponent, B chain), C1qC (complement component 1, q subcomponent, C chain), CFH (complement factor H), and FGF2 (fibroblast growth factor 2).

was observed with only KCND1, KCND2, and KCND3 (Potassium voltage-gated channel, Shal-related subfamily, and members). Whereas, in action view showed association of PTX-3 protein with following proteins; C1qA (complement component 1, q subcomponent, A chain), C1qB (complement component 1, q subcomponent, B chain), C1qC (complement component 1, q subcomponent, C chain), CFH (complement factor H) and FGF2 (Fibroblast growth factor 2) (**Figure 5**). Further, Nauta et al showed PTX-3 protein interacts with complement pathway components C1q and activates the classical complement pathway (Nauta et al., 2003). If high risk deleterious nsSNP is present in pentraxin-domain, interaction of PTX-3 protein with C1q component could be affected.

CONCLUSION

We conclude that several nsSNPs are present in PTX-3 gene. Most of these nsSNPs are located into pentraxin-domain of PTX-3 protein. Structural analysis of selected high-risk nsSNPs showed that the amino acid residue substitutions in pentraxin-domain had the deleterious impact on the stability of the PTX-3 protein. Amino acid residues which undergo substitution were E313K (rs4478039), N337S (rs76994524), R188C (rs564774580) and R360W (rs138818541). Overall, these nsSNPs showed deleterious effect on structure and subsequently to the function of PTX-3 protein. As, pentraxin-domain of pentraxin protein family previously known to interact with components of complement pathway and activates them and also function as pathogen recognition receptor for these pathogens e.g., *A. fumigatus* conidia, *P. aeruginosa*, *S. pneumonia,* and *S. typhimurium.* Due to the presence of these high-risk nsSNPs, it could be possible that the domain may not be functionally active and humans with these nsSNPs in their genome may be susceptible to infection for selected pathogens. Finally, we propose that these nsSNPs should be considered for risk assessment against infectious microbes in a population based study.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: RT and JS. Performed the experiments: RT. Analyzed the data: RT and JS. Contributed reagents/materials/analysis tools: JS. Wrote the paper: RT and JS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00192

Supplementary Figure 1 | Consurf results showing conservation and functional profile for PTX-3 protein.

Supplementary Figure 2 | Rampage result of wild type PTX-3 protein structure.

Supplementary Figure 3 | Rampage result of mutated R188C PTX-3 protein structure.

Supplementary Figure 4 | Rampage result of E313K PTX-3 protein structure.

Supplementary Figure 5 | Rampage result of N337S PTX-3 protein structure.

Supplementary Figure 6 | Rampage result of R360W PTX-3 protein structure.

Supplementary Table 1 | Prediction of deleterious nsSNP in PTX-3 using seven different algorithms.

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