PATHOGENS, PATHOBIONTS AND AUTOIMMUNITY

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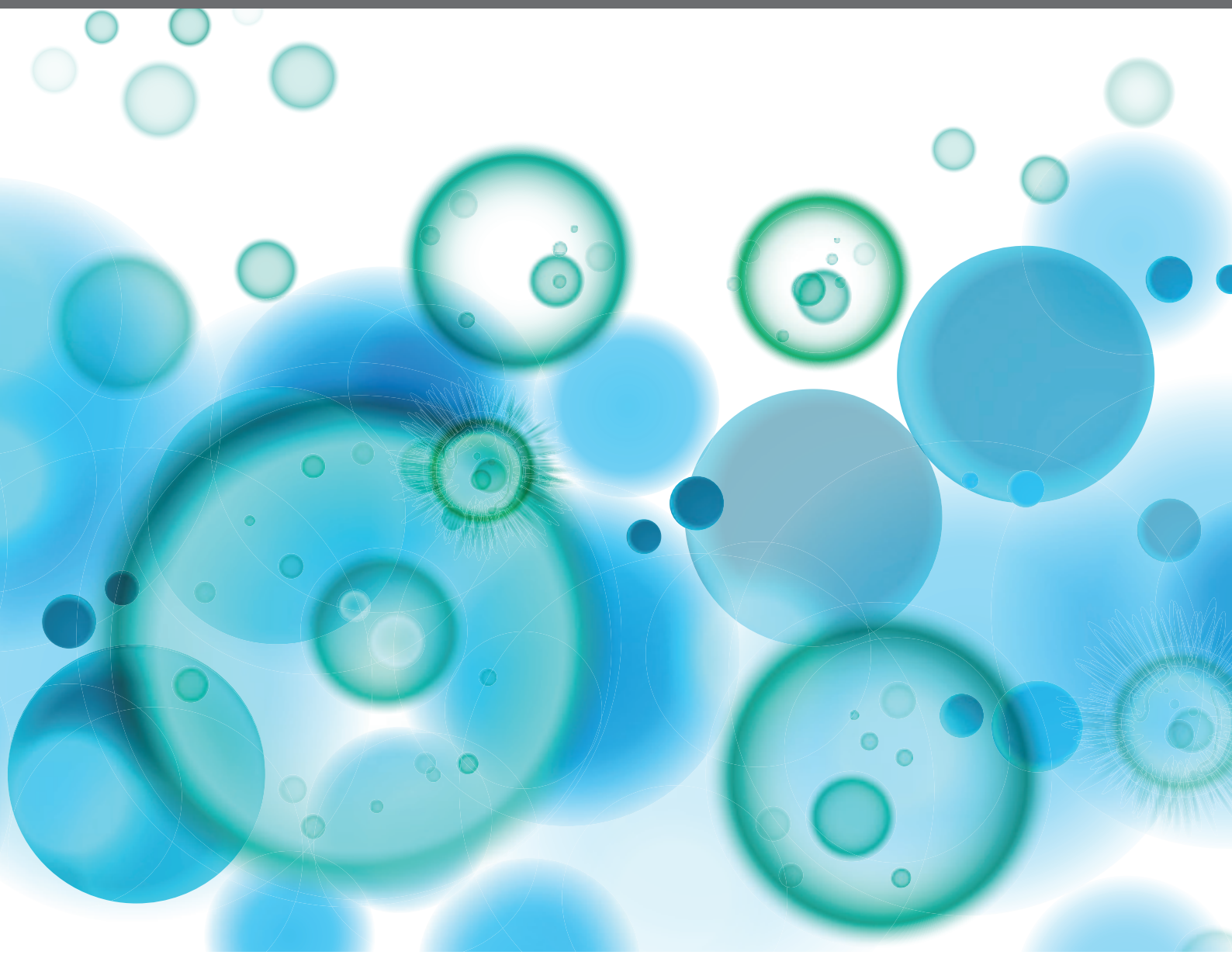

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PATHOGENS, PATHOBIONTS AND AUTOIMMUNITY

Topic Editors:
Linda Ann Spatz, City University of New York, United States
Judith A. James, Oklahoma Medical Research Foundation, United States
Gregg Joshua Silverman, New York University, United States

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EDITORIAL
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Editorial: Pathogens, Pathobionts, and Autoimmunity

Linda A. Spatz[1]*, *Gregg J. Silverman*[2] *and Judith A. James*[3]

[1] Department of Molecular, Cellular, and Biomedical Sciences, CUNY School of Medicine, The City College of New York, New York, NY, United States, [2] Departments of Medicine and Pathology, New York University (NYU) Grossman School of Medicine, New York, NY, United States, [3] Department of Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, Oklahoma City, OK, United States

Keywords: autoimmunity, autoimmune diseases, pathogens, viruses, molecular mimicry, SLE, pathobionts and gut microbiome, dysbiosis

Editorial on the Research Topic

Pathogens, Pathobionts, and Autoimmunity

In this Research Topic on Pathogens, Pathobionts and Autoimmunity, we explore how viruses, bacteria, and commensals can contribute to autoimmunity. The articles presented are either reviews of the literature or original data on mechanisms by which pathogens and pathobionts may trigger autoimmune disease. Several articles are devoted to viruses that have previously been associated with autoimmunity. In particular, Epstein Barr virus (EBV) has frequently been implicated in a number of autoimmune diseases and several mechanisms have been identified. These are discussed in the two reviews by Houen et al. (Houen and Trier; Houen et al.) and by Jog and James and in original research articles by Munroe et al. and Farina et al. In their review entitled "Epstein-Barr Virus and Systemic Autoimmune Diseases", Houen and Trier offer possible mechanistic explanations for how EBV can trigger diverse disease manifestations in different individuals. They suggest that this may be influenced by the predominant cell types that are infected with EBV in different individuals, such as memory B cells or epithelial cells. In their review on "Epstein-Barr virus and Multiple Sclerosis", Houen et al. focus on recent studies suggesting that EBV-transformed B cells that enter the central nervous system are direct participants in Multiple Sclerosis (MS) pathogenesis and that monoclonal antibody therapy targeting CD20-positive memory B cells is an effective treatment for patients with relapsing remitting MS and with primary progressive MS.

In their review on "Epstein Barr Virus and Autoimmune Responses in Systemic Lupus Erythematosus", Jog and James discuss several mechanisms by which EBV can contribute to systemic lupus erythematosus (SLE), including molecular mimicry, dysregulated anti-EBV T cell responses, single nucleotide polymorphisms in the CD40, IL10, and CTLA4 genes associated with SLE that also facilitate EBV re-activation, and recently identified SLE risk alleles known to bind strongly to the EBV EBNA-2 protein (1–7). In addition, they describe how EBV viral proteins, such as viral IL-10 (vIL-10) and latent membrane protein 1 (LMP-1), which are homologues of human proteins that alter immune responses, can potentially lead to SLE and how noncoding EBV viral RNAs can lead to activation of IFN-α, a pro-inflammatory cytokine associated with SLE (8, 9). Finally, they summarize several animal models including humanized mice, that are currently being used to study the role of EBV in autoimmunity.

In a primary article in this Research Topic, Munroe et al. examine the role of EBV LMP1 in SLE. LMP1 is a functional mimic of CD40 that can activate B cells. Mice transgenic for a mCD40-LMP1 hybrid molecule have splenomegaly, lymphadenopathy, an expanded population of immature/activated B-lymphocytes and slightly elevated levels of autoantibodies (10). It has previously been shown that mice injected with EBV nuclear antigen 1 (EBNA-1) develop antibodies that cross-react

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Betty Diamond, Feinstein Institute for Medical Research, United States

***Correspondence:**
Linda A. Spatz
lspatz@med.cuny.edu

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with the ribonucleoprotein, Sm B due to molecular mimicry of a peptide in EBNA-1 that is highly homologous to a peptide in Sm B (11, 12). In the current study, Munroe et al. demonstrate that lymphocytes from mCD40-LMP1 mice injected with EBNA-1 develop significantly greater cellular and humoral immune responses to EBNA-1 and to Sm B than lymphocytes from wild type mice and have elevated ANAs and anti-dsDNA antibodies due to epitope spreading to DNA protein complexes. This suggests that EBV can accelerate autoimmunity in SLE by providing a combination of signals; one that leads to immune dysregulation and another that elicits a cross-reactive autoimmune response.

EBV is also associated with systemic sclerosis (SSc, scleroderma) pathogenesis. In their primary research article, Farina et al. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8089375/) demonstrate that EBV can indirectly infect endothelial cells (ECs) using monocytes bound to EBV as a shuttle. They previously demonstrated that monocytes from SSc patients carry EBV and that these monocytes can adhere to the endothelium (13). In the current report, they show that once in ECs, EBV can activate TLR9 and INF-α inducible genes and trigger an innate, pro-inflammatory response that can lead to vascular damage, seen in the early stages of SSc. Understanding the role of EBV in vascular injury in SSc, may lead to therapeutic strategies aimed at preventing viral infection.

In addition to EBV, other viruses have also been associated with autoimmunity. In an extensive review in this Research Topic, Mustelin and Ukadike discuss various mechanisms by which retroviruses and retrotransposons in our genome can contribute to rheumatological autoimmune diseases. A large part of the human genome consists of retroviral sequences or retrotransposons that have been reverse transcribed and integrated into our genome (14). Mustelin and Ukadike cite studies demonstrating that the expression of certain human endogenous retroviral RNAs (HERV-K and HERV-E) and retroviral proteins are increased in SLE and RA patients. These retroviral proteins are believed to elicit autoantibodies. Mustelin and Ukadike also describe studies demonstrating how elevated levels of retrotransposon RNA transcripts such as long interspersed nuclear elements (L1) in SLE patients, can bind the ribonucleoproteins Ro60 and La as well as the viral ORF1p protein to form complexes which can serve as target autoantigens in SLE. In addition, they review how these endogenous viruses can interact with RNA and DNA sensors to elicit the production of IFN-α and thereby promote SLE pathogenesis. Therapeutic strategies that target IFN-α, including the recently FDA-approved anifrolimumab, or that inhibit reverse transcriptase or DNA and RNA sensors, are also discussed.

Many bacteria have also been linked to autoimmune diseases especially those that populate the gut microbiome. Alterations in the types and concentrations of bacteria that normally inhabit the gut microbiome and changes in the environment in the gut have been observed to contribute to autoimmunity. In their review article, Wu et al. discuss how microbial dysbiosis in the gut and disturbances in several pathways that affect the interaction of gut microbes with the host, may play a role in disease pathogenesis in SLE, inflammatory bowel disease (IBD), rheumatoid arthritis (RA), MS, and type I diabetes (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7786055/). They note how a reduction in anti-inflammatory microbes within the *Lachnospiraceae* family and an increase in pro-inflammatory microbes such as *Ruminococcus gnavus* (that has been reassigned to the genus Blautia) are observed in a subset of patients with IBD, and have been linked to lupus nephritis pathogenesis (15). They also review studies demonstrating how molecular mimicry may be the mechanism by which the gut microbiota is linked to SLE. They describe how autoantibodies and autoreactive T cells specific for Ro60 may arise due to cross-reactivity with a Ro60 ortholog protein produced by gut commensals and how autoantibodies to lipoglycans in *Ruminococcus gnavus* are linked to the generation of anti-dsDNA autoantibodies (15).

In a primary research report, Bagavant et al. (https://pubmed.ncbi.nlm.nih.gov/34122404/), investigate a link between *Enterococcus gallinarum*, a gram-positive bacteria found in the gut, and SLE. They observe that SLE patients with antibodies to Ribosomal P (three phosphorylated proteins on the 60s subunit of ribosomes) have higher titers of IgG antibodies to *E. gallinarum* than healthy individuals. They also observe higher titers of serum IgG antibodies to E. gallinarum in SLE patients with antibodies to dsDNA, Sm, and RNA. They suggest that *E. gallinarum* may influence the subset of autoantigens targeted in lupus patients and infer that molecular mimicry may play a role.

Disruption of the intestinal barrier plays a role in inflammatory autoimmune responses as it allows bacteria to translocate into sites outside the gut that they don't normally inhabit. In one of two primary research reports in this Research Topic by Zhang et al. they demonstrate that intestinal barrier disruption and translocation of bacteria into the liver can trigger autoimmune hepatitis (AIH). They also show that liver macrophages activated by receptor interacting protein 3 (RIP3), a regulator of necrosis/necroptosis, can contribute to the liver damage observed in AIH by their secretion of pro-inflammatory cytokines. Furthermore, they demonstrate that antibiotic treatment can inhibit RIP3 accumulation and activation of liver macrophages, thereby ameliorating disease. This supports their hypothesis that microbiota play a role in AIH and suggests that RIP3 can be a potential therapeutic target in this autoimmune disease. In their second report in this Research Topic, Zhang et al. examine the role of the commensal gut bacterium *Bifidobacterium animalis* (B420) that is found in probiotics, in the treatment of experimental autoimmune hepatitis in mice (EAH). They observe pathobiont dysbiosis in EAH mice with a reduction in fecal anerobes and an expansion in potentially pathogenic bacteria such as *Bacteroides* and *Ruminococcus*. They also observe that certain short chain fatty acids (SCFAs) such as butyric acids are decreased in AIH patients and EAH mice and that RIP3 is increased. They now demonstrate that B420 treatment can improve the intestinal barrier, increase the level of butyric acids and lower the levels of RIP3, all of which have protective effects. These results reveal that B420 can ameliorate liver damage in EAH mice and suggest that modulation of the gut microbiota with probiotics has potential in the treatment of AIH.

Finally, an original and a review article in this Research Topic examines the role of the gut microbiome in ankylosing spondyloarthropathies. There is much overlap between spondyloarthropathies and gut inflammation, and many patients with a spondyloarthropathy also have gut inflammation, while some patients with IBD have spondyloarthritis (SpA) (16). Therefore, the

pathogenesis of these inflammatory diseases may be intertwined. Using metabolomics and metagenomics, Berlinberg et al. address the influence of gut dysbiosis on tryptophan metabolism and the relevance for SpA pathogenesis. They observe that there is an expansion in certain tryptophan metabolites such as indole-3-acetate (IAA) and indole-3-acetaldehyde (I3Ald) in individuals with SpA, irrespective of whether they also have associated Crohn's disease (CD), suggesting that the presence of these metabolites is specific to SpA. They also observe that the gut communities of SpA patients commonly have increases in the representation of microbial genes involved in tryptophan metabolism, whereas in healthy individuals there is instead an elevation in genes involved in tryptophan synthesis. The authors suggest that the influence of the overall composition of the gut microbiota communities may be more important in the alteration in tryptophan metabolism leading to SpA than individual bacterial species. This hypothesis is supported by Gill and Rosenbaum in their review on "Putative pathobionts in HLA-B27-associated spondyloarthropathy" https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7848169/. They discuss various bacterial, fungal, and viral pathobionts that are associated with HLA-B27 SpAs and overlapping inflammatory diseases of the gut. They observe that different microbes are associated with SpA and CD in different individuals and that there doesn't appear to be one consensus microbe. They conclude that the microbiome community and the interactions of the gut microbes with one another are what determines whether HLA-B27 individuals will develop inflammatory autoimmunity and that this is influenced by the genetics of the individual and the environment.

In summary, this Research Topic elucidates many different ways that viral pathogens and gut pathobionts can contribute to autoimmunity. Each of these mechanisms may offer individual pathways that can be targeted by therapeutic strategies.

AUTHOR CONTRIBUTIONS

There are 3 editors for this Research Topic. Each of the authors contributed equally in inviting contributors for this edition and in reviewing articles submitted for this edition. LAS wrote the editorial with assistance from her co-editors GJS and JAJ. All authors contributed to the article and approved the submitted version.

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Bifidobacterium animalis ssp. Lactis 420 Mitigates Autoimmune Hepatitis Through Regulating Intestinal Barrier and Liver Immune Cells

Hongxia Zhang[1†], Man Liu[1†], Xin Liu[1†], Weilong Zhong[1], Yanni Li[1], Ying Ran[1], Liping Guo[1], Xu Chen[1], Jingwen Zhao[1], Bangmao Wang[1] and Lu Zhou[1,2*]*

[1] Department of Gastroenterology and Hepatology, General Hospital, Tianjin Medical University, Tianjin, China, [2] Department of Gastroenterology and Hepatology, People's Hospital of Hetian District, Xinjiang Uygur Autonomous Region, China

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Edited by:
Gregg Joshua Silverman, New York University, United States

Reviewed by:
Angelica Thomaz Vieira, Federal University of Minas Gerais, Brazil
Jillian M Richmond, University of Massachusetts Medical School, United States

***Correspondence:**
Bangmao Wang
gi.tmuh@sohu.com
Lu Zhou
lzhou01@tmu.edu.cn

[†]*These authors have contributed equally to this work*

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Autoimmune hepatitis (AIH) is an immune-mediated inflammatory liver disease of uncertain cause. Accumulating evidence shows that gut microbiota and intestinal barrier play significant roles in AIH thus the gut–liver axis has important clinical significance as a potential therapeutic target. In the present study, we found that *Bifidobacterium animalis* ssp. lactis 420 (B420) significantly alleviated S100-induced experimental autoimmune hepatitis (EAH) and modulated the gut microbiota composition. While the analysis of clinical specimens revealed that the fecal SCFA quantities were decreased in AIH patients, and B420 increased the cecal SCFA quantities in EAH mice. Remarkably, B420 application improved intestinal barrier function through upregulation of tight junction proteins in both vitro and vivo experiments. Moreover, B420 decreased the serum endotoxin level and suppressed the RIP3 signaling pathway of liver macrophages in EAH mice thus regulated the proliferation of Th17 cells. Nevertheless, the inhibition effect of B420 on RIP3 signaling pathway was blunted *in vitro* studies. Together, our results showed that early intervention with B420 contributed to improve the liver immune homeostasis and liver injury in EAH mice, which might be partly due to the protection of intestinal barrier. Our study suggested the potential efficacy of probiotics application against AIH and the promising therapeutic strategies targeting gut–liver axis for AIH.

Keywords: autoimmune hepatitis, *Bifidobacterium*, intestinal barrier, macrophages, Th17 cells, gut–liver axis

INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease with increasing incidence, while the underlying mechanisms remain unclear (1). In addition to genetic factors, various environmental factors have been implicated in the development of liver diseases (2–4). Recently, the gut microbiota has been recognized as a major environmental risk factor for AIH, and the associated mechanisms include disruption of the intestinal barrier, intestinal bacterial translocation, and break of immune tolerance (5, 6).

Recently, intestinal dysbiosis was reported in patients with AIH. In a Chinese cohort, disease-associated dysbiosis in steroid treatment-naïve AIH patients was characterized by reduced

biodiversity and decreased abundance of anaerobes (6). Furthermore, Timur Liwinski et al. reported a disease-specific decline of the relative abundance of *Bifidobacterium* in patients with AIH (7), which suggested that probiotics might potentially exhibit a beneficial effect in AIH. *Bifidobacterium* is one of the most important bacterial groups found in the human intestinal tract and its characteristics and mechanism of action have been reported since 1950 (8–10). Moreover, *Bifidobacterium* has been clinically in some chronic diseases used to maintain the balance of intestinal microbiota without serious side effects (11–13). *Bifidobacterium animalis* subsp. lactis 420 (B420), known for its immunoregulatory properties and improving intestinal epithelial integrity in mice models, has been given to humans in earlier clinical trials (14–17). Therefore, we used B420 to explore the potential effects and application of probiotics in AIH in our experiment.

Short-chain fatty acids (SCFAs), primarily acetate, propionate, and butyrate, are the major products of the colonic microbial fermentation of undigested dietary fiber (18, 19). A double-blind and randomized clinical trial performed by Livia et al. proved that the intake of fermented milk containing *Lactobacillus* and *Bifidobacterium* seems to increase fecal SCFA (20). In particular, butyrate has potential immunoregulation properties and serves as the preferred metabolic substrate for intestinal epithelial cells (21, 22). Lipopolysaccharide (LPS), which is the major component of the outer membrane of most Gram-negative bacteria and is referred to as an endotoxin, plays a key role in gut–liver axis (23, 24). Interestingly, previous study found that SCFA could inhibit LPS-induced inflammatory responses, which indicated that SCFA might be an important protective metabolite in gut–liver interactions (25).

It is commonly accepted that macrophages are implicated in the pathological inflammation and fibrosis of liver diseases and activated macrophages are present in the portal area of AIH (26–28). Our previous studies have found that receptor-interacting protein kinase 3 (RIP3) signaling was involved in LPS-induced macrophage/monocyte activation in AIH (29). RIP3 kinase activity supports the recruitment of the mixed lineage kinase domain-like (MLKL) to trigger membrane leakage with the consequent production of pro-inflammatory cytokines (1, 30, 31). The composition of the local cytokine milieu dictates CD4+ Th cells to differentiate into specific T cell subsets, of which the Th17 cells are the main effector cells executing intensify inflammation and tissue injury functions in the live tissue of AIH (1, 32). Taken together, these findings indicated that environmental factors, especially intestinal microbiota, may involve in the activation of immune cells and loss of self-tolerance to autoantigens in persons genetically susceptible to AIH.

In the present study, we addressed the efficacy and associated mechanisms of probiotics on immune-mediated liver injury through B420 supplement. Our results showed that B420 alleviated liver injury in EAH mice, partly by modulating gut microbiota and RIP3 signaling of liver macrophages, and these effects were accompanied by the increase of cecal SCFA production, upregulation of intestinal tight junction proteins, repression of liver pro-inflammatory cytokines and a decrease of Th17 cells in liver and spleen. Collectively, these findings revealed that probiotics supplement might exhibit potential efficacy against AIH through targeting gut–liver axis.

MATERIALS AND METHODS

Ethical Approval Statement

All experimental procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at Tianjin Medical University and followed the International Association of Veterinary Editors guidelines for the Care and Use of Laboratory Animal. The animal use protocol listed below has been reviewed and approved by the Animal Ethical and Welfare Committee of Tianjin Medical University, Approval No. IRB2015-YX-009.

Animal Experiments

Twenty-six female SPF C57BL/6 mice (6 weeks of age) were purchased from Beijing Animal Study Centre, and maintained under specific pathogen-free conditions in Animal Centre of the Tianjin Medical University. All mice were randomly divided into three groups including control group (n=6), model group(n=6) and B420 group(n=6). The rest mice (n=8) were killed and hepatic antigen S100 were extracted after perfusion of livers with cold phosphate-buffered saline (PBS) as previous description (33). Briefly, the livers were cut into scrap and homogenized with cold PBS on ice. After ultrasonic grinding, the homogenate was centrifuged at 150 g for 10 min to remove nuclei. Next, the supernatants were centrifuged at 100,000 g for 1 h. The supernatants further ran through a 90-cm CL-6B Sepharose column (Pharmacia, Freiburg). The first nontoxic peak was acquired and concentrated to 0.5–2.0 g/L. The model group and B420 group were intraperitoneal immunized with 0.5 ml liver S100 antigen emulsified in an equal volume of complete Freund's adjuvant (CFA, sigma, USA) on day 7 and day 14 to induce experimental autoimmune hepatitis (EAH) and the control group was intraperitoneal injected with 0.5 ml sterile normal saline (NS) with an equal volume of CFA on day 7 and day 14. Mice of B420 group were treated with *B. animalis* ssp. lactis 420 (B420) (DuPont Nutrition & Biosciences, China; ATCC: SD6685, 10^9 CFU/200ul) dissolved in sterile NS *via* gavage and mice of the other two groups were gavaged 200ul NS every day for 4 weeks. On day 28, all the animals were sacrificed under anesthesia (**Figure 1A**).

Participants

A total of fourteen AIH patients and six controls were included. AIH patients were recruited from the Gastroenterology Department at Tianjin Medical University General Hospital. The diagnosis of AIH was made if patients conformed with (1) 1999 revised International Autoimmune Hepatitis Group (IAIHG) score ≥10 and/or (2) 2008 IAIHG simplified AIH score ≥6 and/or (3) histological features indicative of AIH (34, 35). All the patients were collected before corticosteroids therapy. Control subjects were selected from the health manage center of Tianjin Medical University General Hospital

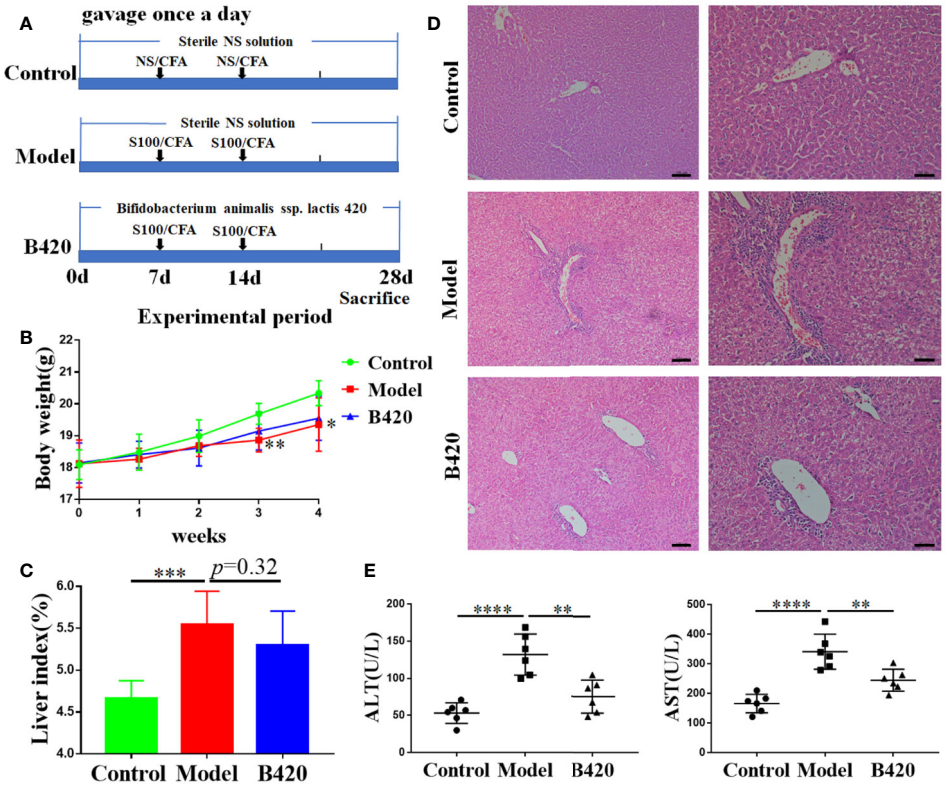

FIGURE 1 | B420 attenuated liver injury in EAH mice. **(A)** Modeling process of EAH and administration of B420. **(B)** Body weight of each group was recorded weekly. **(C)** The liver index (liver wight/body weigh) between groups was measured. **(D)** Representative H&E images of liver tissues were shown (Scale bar: left:100μm, right:50μm). **(E)** The serum concentrations of ALT and AST were assessed. In **(A–D)**, n = 6 in each group. The data were presented as means ± SD (Student's t-test, **p < 0.01, ***p < 0.001, ****p < 0.0001).

to match with AIH patients for age and gender. The control group had to fulfil the following inclusion criteria: (1) normal ranges of liver function test, (2) an absence of hepatitis B/C virus antigen, (3) normal abdominal ultrasound test, (4) an absence of autoimmune diseases and family history. The feces of the individuals were collected.

Liver and Ileum Histological Examination

Liver tissues and ileum tissues were collected and fixed in 4% paraformaldehyde after mice were sacrificed. The paraffin embedded liver tissues and intestinal tissue were sectioned at approximately 5μm and processed for staining with hematoxylin and eosin (H&E) according to the standard H&E protocol. The pathological change of the liver and intestinal tissue was evaluated by two independent and experienced pathologists. The liver histopathology index was measured according to the Ishak system including periportal interface hepatitis, confluent necrosis, focal lytic necrosis and portal inflammation (36).

Biochemical Analysis and Enzyme-Linked Immunosorbent Assay (ELISA)

The blood of the mice was centrifuged at 150 g for 10 min. The serum was then stored at −80°C. The serum concentration of LPS was quantified with the ELISA kits according to the manufacturer's instructions (eBioscience). The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were tested by using the automated chemistry analyzer (AU5800, Beckman Coulter, USA) from the clinical laboratory of the Tianjin Medical University General hospital.

Intestinal Microbiota Analysis

The 16S rRNA gene sequencing procedure was performed by the GENEWIZ Genomics Institute (Suzhou, China). Total fecal bacteria DNA extractions were acquired from cecal specimens of each 3-week old and 8-week old offspring by QIAamp ® Fast DNA Stool Mini Kit (QIAamp, Germany). The microbial 16S V3-V4 region was amplified with indexes and adaptors-linked universal primers (341F: ACTCCTACGGGAGGCAGCAG, 806R: GGACTACHVGGGTWTCTAAT). PCR was performed using KAPA HiFi Hotstart PCR kit high fidelity enzyme in triplicate. Amplicon libraries were quantified by Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, US) and then sequenced on Illumina HiSeq platform (Illumina, San Diego, US) for paired-end reads of 250 bp. After discarding the singletons and removing chimeras, tags were clustered into operational taxonomic units (OTUs) using USEARCH

(v7.0.1090) at 97% similarity. Afterwards, a representative sequence of each OTU was subjected to the taxonomy-based analysis using the RDP database. Heatmap was created using R. Cluster analysis. Alpha diversity and beta diversity were analyzed using QIIME. The relative abundance of bacteria was expressed as the percentage.

Cecal Short-Chain Fatty Acid Quantification

The SCFA concentrations were determined by gas chromatography (GC) as previously described (37). Briefly, the feces from participants and cecal contents from mice were diluted, acidified, and extracted ultrasonically on ice for 10 min. The samples were then centrifuged at 12,000 g and 4°C for 15 min. After the supernatant was mixed with ethyl acetate (1:1), the extract was filtered through a 0.22-μm pore-size filter and poured into an Agilent 7890A Series GC. The SCFA standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

In Vivo Permeability Assay

Intestinal permeability was determined by FITC-dextran assay. FITC-D (4000 MW, Sigma-Aldrich) dissolved in normal saline infusion (50 mg/mL) and was administrated to the mice by gavage at 6 mg/10 g body weight. Whole blood was collected 4 h after FITC-D administration using heparinized microhematocrit capillary tubes *via* eye bleed. Sera was extracted from the blood by centrifuging at 4°C for 10 min at 2,000 rpm. Fluorescence intensity analysis was carried out using a plate reader. The concentration of FITC-D of each mouse was detected based on the FITC-D standard curve.

Quantitative Realtime PCR (qRT-PCR)

Total RNA was extracted using the RNeasy mini kit (Qiagen, Carlsbad, CA, USA) followed by cDNA reverse transcription using the TIANScript RT Kit (TIANGEN, Inc. Beijing, China) according to the manufacturer's protocol. Realtime-PCR analysis was performed using Taqman Gene Expression Master Mix and primes (GENEWIZ, Inc. Beijing, China). The Oligonucleotide primers for target genes were listed in **Table 1**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an endogenous control. The relative mRNA expression levels of the target gene were evaluated by calculating the fold-changes normalized to the GAPDH for each sample using $2^{-\Delta\Delta Ct}$ method. All cDNA samples were analyzed in triplicate.

Western Blotting

The liver and intestinal tissues were dissolved in RIPA buffer with protease inhibitors (Solarbio, Beijing, China). After homogenization, the protein concentrations were determined by Bicinchoninic acid protein assay (Thermo Scientific Inc). Proteins were separated using SDS-polyacrylamide gel electrophoresis system and then blotted onto a polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, USA). Afterwards, the primary anti-RIP3 (ab62344, Abcam, Cambridge, MA, USA), anti-MLKL (ab196436, Abcam, Cambridge, MA, USA), anti-ZO-1 (ab96587, Abcam, Cambridge, MA, USA), anti-Occludin (ab216327, Abcam, Cambridge, MA, USA), and anti-GAPDH (rabbit, antimouse, Cell Signaling Technology) antibody were applied; anti-GAPDH antibody was employed as the loading control. After incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies (Cell Signaling Technology), the chemiluminescent signal was detected. The intensity of the band was determined by image processor program (Image J).

TABLE 1 | The Oligonucleotide primers used in realtime-PCR analysis.

Murine gene	Primer sequences (5′- 3′)
GAPDH	Forward primer: TGTGTCCGTCGTGGATCTGA
	Reverse primer: CCTGCTTCACCACCTTCTTGA
ZO-1	Forward primer: GGGCCATCTCAACTCCTGTA
	Reverse primer: AGAAGGGCTGACGGGTAAAT
Occludin	Forward primer: ACTATGCGGAAAGAGTTGACAG
	Reverse primer: GTCATCCACACTCAAGGTCAG
TNF-α	Forward primer: ACTCCAGGCGGTGCCTATG
	Reverse primer: GAGCGTGGTGGCCCCT
IL-6	Forward primer: CCAGTTGCCTTCTTGGGACT
	Reverse primer: GGTCTGTTGGGAGTGGTATCC
IL-1β	Forward primer: GTGGCTGTGGAGAAGCTGTG
	Reverse primer: GAAGGTCCACGGGAAAGACAC
RIP3	Forward primer: GAAGACACGGCACTCCTTGGTA
	Reverse primer: CTTGAGGCAGTAGTTCTTGGTGG
MLKL	Forward primer: CCTTGCTTGCTTGCTTTT
	Reverse primer: TTTCCTTGAGTTTGAGCCA
CCL2	Forward primer: ACCTTTTCCACAACCACCT
	Reverse primer: GCATCACAGTCCGAGTCA
CCR2	Forward primer: AAGGGTCACAGGATTAGGAAG
	Reverse primer: ATGGTTCAGTCACGGCATA

Immunofluorescence

Immunofluorescence analyses for tight junction proteins of the mice intestinal tissue were performed were performed with 4mm-thick frozen sections. Slides were fixed with acetone and blocked with 5% bovine serum albumin then incubated with antibodies against ZO-1 (Abcam, USA) or Occludin (Abcam, USA) overnight at 4°C. Subsequently, the sections were washed with PBS for 5 min three times and incubated with Alexa Fluor 488 (Santa Cruz Biotechnology, Inc) at room temperature in the dark for 60 min. Nuclear staining was achieved by 4', 6-diamidino-2-phenylindol (DAPI). Double immunofluorescence analyses for macrophage of liver tissue were performed as shown above. Slides were fixed with acetone and blocked with 5% bovine serum albumin then incubated with antibodies against F4/80 (ab16911, Abcam, Cambridge, MA, USA) and RIP3 (Abcam, USA), or F4/80 (Abcam, USA) and MLKL (Abcam, USA), further incubated with Alexa Fluor 488 (Santa Cruz Biotechnology, Inc) and Alexa Fluor 568 antibody (Santa Cruz Biotechnology, Inc). DAPI was lastly applied to dye the nucleus. Fluorescence photographs were obtained under fluorescence microscope DM5000 B (Leika, Germany).

Cell Isolation and Flow Cytometry Analysis

Single-cell suspensions of lymphocyte were harvested from spleen and liver of mice. Prior to intracellular cytokine staining, cells were stimulated with PMA and ionomycin (BD Bioscience) in the presence of brefeldin A (BD Bioscience) for 5h.

Cells were collected, washed by PBS, and stained with APC-conjugated anti-mouse CD4 antibody in the presence of FcR-Block (BD Bioscience) in dark for 30 min. After the wash, cells were fixed by CytoFix/Cyto Perm buffer (BD Bioscience) and stained with PE-conjugated anti-mouse IL-17A (BD Bioscience) anti-body or isotype control antibody for 30 min. Data were obtained on a FACS Calibur (BD Bioscience) and analyzed using FlowJo 7.6 software.

Bacterial Culture and Bifidobacterium Supernatants

B420 was supplied by DuPont Nutrition & Biosciences and incubated in brain heart infusion medium under anaerobic conditions for 24 h at 37°C until the logarithmic phase of growth with a bacterial density of 0.5 at optical density (OD) 600. The culture suspensions were centrifuged at 5,000 × g for 10 min at 4°C, then the supernatant (B420-s) was collected and filter-sterilized through 0.22 μm filters. The B420-s was diluted to three concentration gradients (1:100, 1:50,1:20) with complete culture medium.

Cell Cultures

The mouse macrophage cell line RAW264.7 (ATCC SC-6003) was cultured in a Dulbecco modified Eagle medium (DMEM) (Gibco) in 10% fetal bovine serum (FBS) (Gibco), 50 U/ml penicillin and 50 U/ml streptomycin (all from Invitrogen, USA) in a humidified incubator containing 5% CO2 at 37°C. RAW264.7 cells were seeded in a 12-well plate at a density of 1×10^5 cells per well. Human Caco-2 cells (BNCC 338148) were cultured in Modified Eagle's Medium (MEM) (Gibco) supplemented with 20% fetal bovine serum and a penicillin-streptomycin solution. The cells were incubated under same conditions as above and were seeded in a 12-well plate at a density of 1×105 cells per well. In the LPS experiments (LPS group), the cells were treated with LPS (3mg/ml, Solarbio Biotech) for 12 h. In the (B420-s) experiments (LPS-B420-s group), the cells were pre-treated with B420-s of three concentration gradients (1:100, 1:50,1:20) for 3h, then treated with LPS (3 mg/ml) for 12 h.

Statistical Analysis

Data were presented as the mean ± SD. The statistical significance of differences was assayed by one-way ANOVA in multiple groups, and t-tests for paired samples using SPSS 22.0 (SPSS, Chicago, IL, USA). All the differences were considered as statistically significant at $p < 0.05$.

RESULTS

B420 Attenuated Liver Injury in EAH Mice

Previous research found that a reduced number of fecal anaerobes, represented by the disease-specific decline of *Bifidobacterium*, occurred in AIH patients (7, 38). Our preliminary study found that early intervention of B420 could alleviate liver injury in concanavalin A (Con A) induced hepatitis mice model and decrease the expression of proinflammatory cytokines in the liver tissues (**Supplementary Figures 1A–C**). To further explore the therapeutic potentials and mechanism of probiotics on AIH, we gavage the EAH mice with B420 (**Figure 1A**). In this study, we found that the mice in the model group had decreased body weight at 3–4 weeks of the modeling process, with no significant difference between model group and B420 group (**Figure 1B**).

The general view of liver was shown in **Supplementary Figure 2A**. The liver index was significantly increased in the model group compared to the control group, but there was no statistical difference observed between the model and B420 groups (**Figure 1C**). Significantly, the representative images of liver tissue (as indicated by H&E staining) showed that the model group had severe infiltration of inflammation cells in the portal area and B420 supplement alleviated liver inflammation (**Figure 1D**). Similarly, we also evaluated the general view of spleen and spleen index and results showed that the model group had significantly increased spleen index compared to the control group, and B420 treatment decreased the spleen index (**Supplementary Figures 2A, B**). Accordingly, the model group had higher alanine transaminase (ALT) and aspartic transaminase (AST) levels compared to control group and B420 supplement significantly decreased the transaminase levels (**Figure 1E**). Together these findings indicated that B420 supplement in early stage of AIH contributed to attenuate the infiltration of inflammatory cells and liver injury in EAH mice.

B420 Altered Composition and Diversity of Gut Microbiota in EAH Mice

Emerging findings demonstrated intestinal dysbiosis in autoimmune disease and alterations of intestinal microbiota, such as depletion of obligate anaerobes and expansion of potential pathobionts, have been reported in AIH patients (6, 38). To further explore the effect of B420 on gut–liver axis, we studied the fecal microbiomes of the mice. The comparison of the OTUs among the three groups revealed 227OTUs in the control group, 238 OTUs in the EAH group and 245 OTUs in the B420 group, and a total of 203 OTUs were shared by the three groups (**Figure 2A**). The gut microbiota of all the samples was dominated by three major phyla: Bacteroidetes, Firmicutes and Proteobacteria. Notably, compared to the control group, a higher abundance of Bacteroidetes and a lower abundance of Firmicutes and Proteobacteria were observed in model group, which resulted in a decreased Firmicutes/Bacteroidetes (F/B) ratio compared to that in the control group. However, B420 did not statistically affect the F/B ratio (**Figure 2B**). The genus-level analysis revealed that the model group had increased relative abundance of potential pathogenic bacteria, such as Bacteroides and Ruminococcus, whereas B420 weakened this increase. Additionally, a relatively lower abundance of *Lactobacillus* was observed in mice of model group compared to the control group, and B420 treatment restored the abundance of *Lactobacillus* (**Figure 2C**). The Chao1 and Fisher index revealed that the model group had significantly decreased alpha diversity

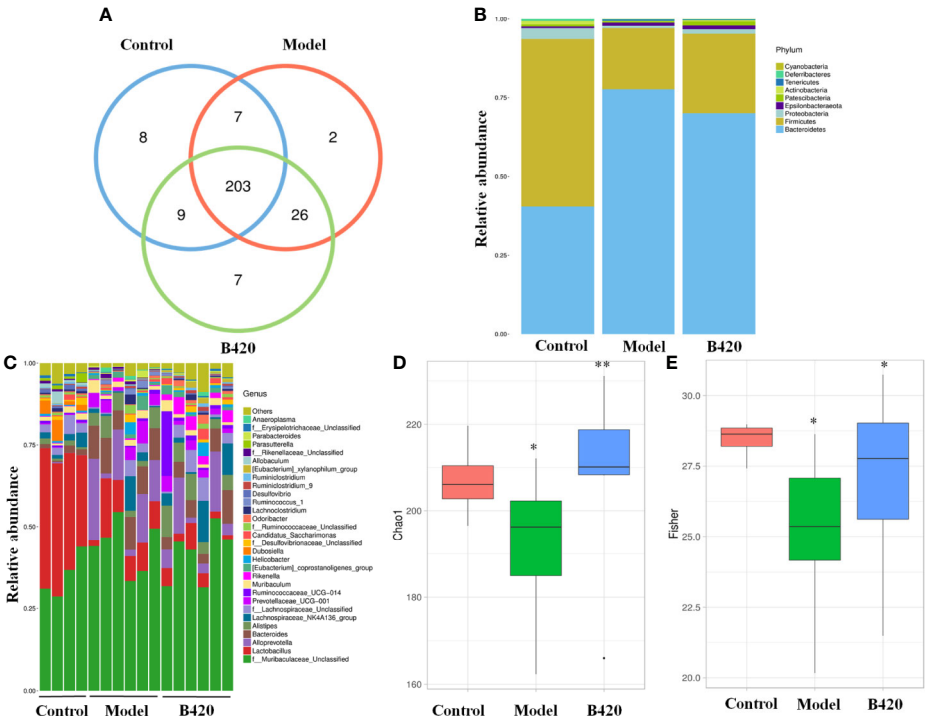

FIGURE 2 | B420 altered the composition and diversity of gut microbiota in EAH mice. Total fecal bacteria from each mouse were detected by 16S rRNA sequencing. **(A)**Venn diagram. **(B)** Relative abundance of bacterial taxa at the phylum level between groups. **(C)** Relative abundance of bacterial taxa at the genus level in each mouse. **(D, E)** Chao1 and Fisher diversity index were shown. In **(A–E)**, Control: n=4, Model and B420: n=6 (*p < 0.05, **p < 0.01).

compared to the control group and B420 treatment restored the two indices, which suggested that B420 exerted stronger positive effects on alpha diversity of the gut microbiota (**Figures 2D, E**).

A principal component analysis (PCA) based on weighted UniFrac distances revealed a different structure between the three groups (**Figure 3A**). To further quantify the differences in species diversity between groups, ANOSIM was projected and the results indicated the differences between groups were significant (**Figures 3B, C**). Furthermore, different abundant species among the three groups were examined by LDA EffectSize analysis. Results showed that the relative abundance of Alloprevotella and Prevotellaceae which were reported to be associated with rheumatoid arthritis (39), were higher in model group. Meanwhile, the potential pathogenic bacteria, such as Bacteroides and Ruminococcus, were also significantly increased in model group. The abundance of beneficial bacteria including Alistipes and Rikenella was significantly increased in B420 group. Importantly, clostridiales, associated with the production of SCFA, was abundant in the B420 group (**Figure 3D**).

Taken together, these results proved that EAH mice had a major alteration in the gut microbiota composition, whereas B420 at least partly altered the gut microbiota dysbiosis, which indicated the significance of probiotic supplement targeting gut–liver axis in maintaining immunological balance of the liver.

B420 Increased the Level of Fecal SCFAs in EAH Mice

SCFAs are major end-products of gut microbial fermentation and are implicated in the regulation of immune system and intestinal epithelial cells (40–42). In our study, the SCFA levels in the feces of AIH patients and controls were detected. The results showed that most abundant SCFAs in feces were acetic acids with less of butyric acids and propionic acids. Importantly, we found that there was a significant decrease of butyric acids as well as propionic acid, isovaleric acid and valeric acid in feces of AIH patients compared to controls (**Figure 4A**). Moreover, cecal feces from mice of the three groups were also collected and analyzed for the presence of SCFAs. Notably, the model group had significantly decreased concentration of butyric acids compared to the control group while B420 treatment increased the concentration of butyric acids (**Figure 4B**). Besides, we investigated the effect of butyrate on liver injury in Con A-mediated autoimmune hepatitis model (**Supplementary Figure 3A**). The results showed that butyrate could alleviate liver inflammation and decrease the transaminase levels (**Supplementary Figures 3B, C**). The butyrate group had lower expressions of RIP3 in the liver tissues compared to the Con A group as well as the expressions of IL-6 and IL-1β (**Supplementary Figures 3D, E**). These results suggest that the protective effects of probiotics therapy on autoimmune diseases might be partly due to alterations in microbial-derived metabolites.

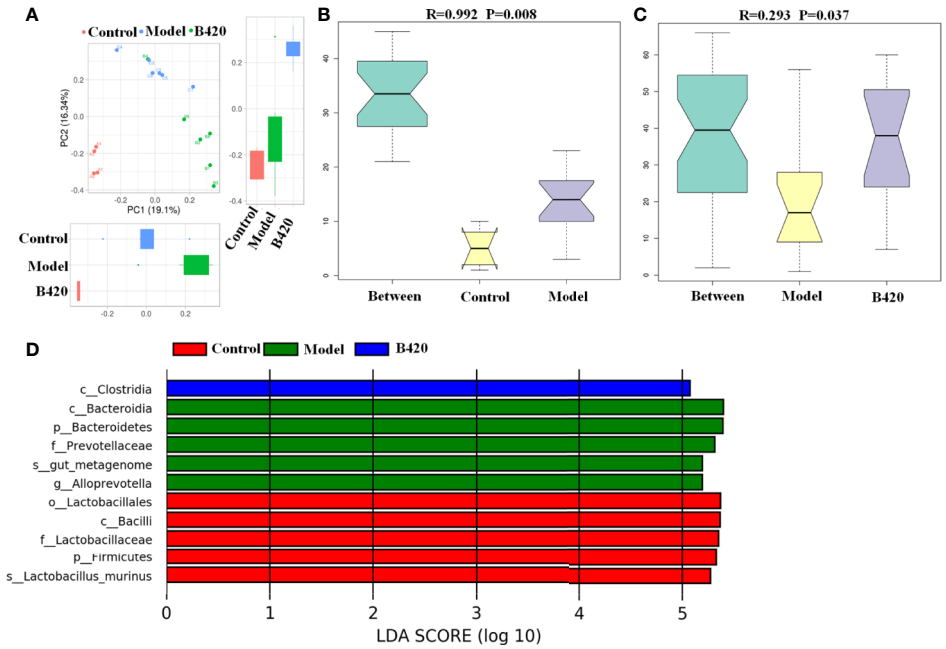

FIGURE 3 | B420 altered the beta diversity of gut microbiota and the key bacterial alterations in mice. Total fecal bacteria from each mouse were detected by 16S rRNA sequencing. **(A)** Beta diversity. **(B, C)** Unweighted Unifrac ANOSIM analysis between **(B)** Control and Model group, **(C)** Model and B420 group. **(D)** Different abundant species at the phylum, class, order, family, and genus level generated by LEfSe analysis was shown. In **(A–D)**, Control: n=4, Model and B420: n=6.

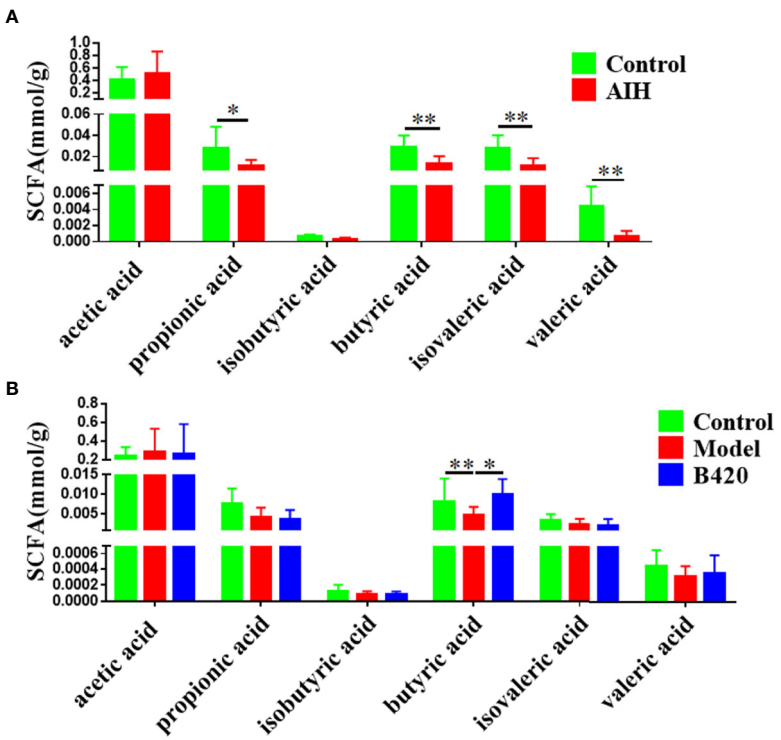

FIGURE 4 | B420 increased the cecal butyric acid in EAH mice. **(A)** Alterations in the SCFA levels in the fecal contents of AIH patients. **(B)** Effect of B420 on SCFA in the cecal contents. In **(A)**, Control: n=6, AIH =14. In **(B)**, n=6 in each group. The data were presented as means ± SD (Student's t-test, *p < 0.05, **p < 0.01).

B420 Alleviated the Damage of Intestinal Barrier Function in EAH Mice

Intestinal barrier is essential for the maintenance of homeostasis in health and disease (43). A wealth of studies had shown that the intestinal barrier, part of the gut–liver axis, played a role in the pathophysiology of autoimmune diseases (44–47). Here, we explored whether the probiotic approach alleviated autoimmune liver injury through targeting impaired intestinal barrier function.

Figure 5A showed the H&E staining of small intestines from the three groups. There was a damage of normal intestinal structure in mice of model group compared to that of control group. However, the B420 group showed lesser intestinal tract lesions compared to the model group. Further, the ratio of villus height to crypt depth was calculated to evaluate intestinal morphological alteration. The ratio in model group was significantly decreased compared to that in the control group, and B420 treatment restored the ratio back to that in the control group (**Figure 5B**). Next, the intestinal permeability and the integrity of the gut barrier were examined. The FITC-dextron and LPS tests showed that the mice in the model group had increased intestinal permeability and higher serum LPS levels compared to those in the control group, and it's worth noting that B420 treatment significantly reduced intestinal permeability and alleviated endotoxemia (**Figure 5C**).

To further assess the integrity of intestinal barrier in these mice, we detected the structural proteins including zonula occludens-1 (ZO-1) and Occludin. Immunostaining of tight junction proteins suggested that the model group had reduced expressions of ZO-1 and Occludin in the small intestinal and B420 treatment increased the expressions of the structural proteins (**Figures 5D, E**). Remarkably, both the mRNA and protein expressions of ZO-1 and Occludin were significantly decreased in mice of model group compared to that in control group and B420 treatment upregulated the expressions of the structural proteins (**Figures 5F, G**). In addition, there was a significant increase in the expressions of TNF-α, IL-6 and IL-1β (barrier-disrupting cytokines) in the gut mucosa of the model group compared to that in the control group and B420 treatment significantly decreased the expression of TNF-α (**Figure 5H**).

Collectively, we addressed that there was intestinal barrier dysfunction accompanied by elevated levels of endotoxin in EAH model and early application of B420 could improve the intestinal

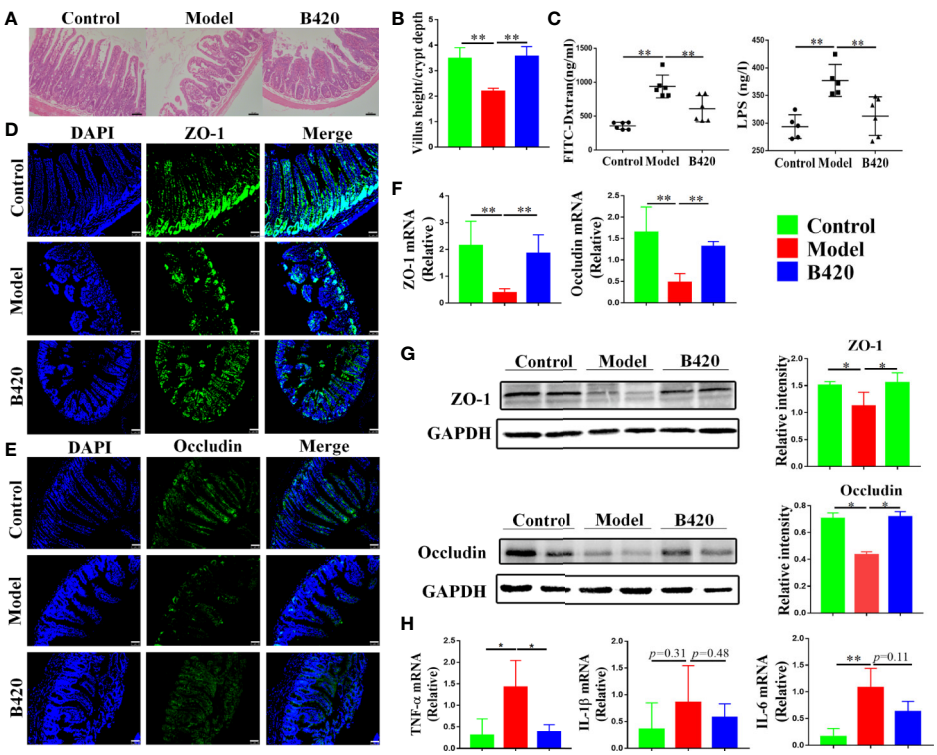

FIGURE 5 | B420 alleviated the damage of intestinal barrier function in EAH mice. **(A)** H&E images of ileum tissues were shown. **(B)** Five crypts per section were evaluated, then microscopically assessed villus height, crypt depth and calculated the ratio of villus height and crypt depth. **(C)** Intestinal permeability was detected using the *in vivo* FITC-dextran assay and serum LPS testes. The FITC-dextran and LPS levels in serum is shown. **(D, E)** The membrane localization of **(D)** ZO-1 and **(E)** Occludin was assessed by immunostaining and visualized by fluorescence microscopy, nuclei were stained with DAPI (blue staining) and the green regions indicated ZO-1 and Occludin. **(F)** Total RNA was extracted from the ileum tissues for real-time PCR analysis. The relative expressions of ZO-1 and Occludin were shown. **(G)** Protein levels of ZO-1 and Occuludin in the ileum tissues from each group were detected by western blotting and the relative intensity was quantified. **(H)** The relative expressions of TNF-α, IL-6, and IL-1β in ileum tissues were analyzed. FITC-dextran, fluorescein isothiocyanate conjugated-dextran. In **(A–G)**, n = 6 in each group. Scale bar: 50μm. The data were presented as means ± SD (Student's t-test, *p < 0.05, **p < 0.01).

barrier, which indicated a promising prospect of novel therapeutic strategies including probiotics and stabilization of tight junctions in AIH.

B420 Inhibited the RIP3-MLKL Signaling Pathway of Liver Macrophages in EAH Mice

RIP3 has been increasingly recognized as a key inflammatory signal adapter, which mediates inflammation through necroptosis as well as non-necroptosis function (30, 48). Our previous studies have found that RIP3 signaling was involved in macrophage/monocyte activation in the liver tissues of AIH patients and is correlated with the levels of serum hepatic enzyme (29). Then, we examined the activation of RIP3 and MLKL (the direct downstream effector of RIP3) in liver macrophages of the mice. With anti-F4/80 Ab to identify Kupffer cells, RIP3 and MLKL were stained in the cells. As shown in **Figures 6A, B**, the majority of F4/80+ macrophages in the liver tissues of EAH mice expressed both RIP3 and MLKL. In contrast, the co-localization of F4/80+ macrophages and RIP3 or MLKL was rarely observed in the liver tissues of controls. Compared to the model group, the B420 group had a lower expression of RIP3 or MLKL in F4/80+ macrophages. Subsequently, the liver tissue of model group showed significantly increased expressions of both RIP3 and MLKL compared to the control group and the B420 group had lower expressions of RIP3 and MLKL (**Figure 6C**). The protein levels of RIP3 and MLKL were then detected by western blotting. As expected, our results showed that the protein levels of RIP3 and MLKL were significantly decreased in model group and the B420 group had a lower expression of RIP3, but the difference of MLKL between the model group and B420 group was not statistically significant (**Figure 6D**).

Thus, the downregulation of RIP3 signaling of liver macrophages might be a critical mechanism involved in immunoregulation and hepatoprotective effects of probiotics application in AIH.

B420 Regulated Pro-Inflammation Cytokines and Chemokines in Liver as Well as Th17 Cells in Liver and Spleen

To further explore the protective mechanism of B420 supplement in AIH, we also evaluated the expressions of liver inflammatory cytokines and chemokines. We found that the cytokine-secretion phenotype in the liver tissue of EAH mice was skewed towards M1-type macrophages leading to a highly inflammatory cytokine milieu enriched for TNF-α, IL-6 and IL-1β and B420 treatment significantly decreased the expressions of TNF-α and IL-6 (**Figure 7A**). Besides, the expressions of chemokine ligand 2 (CCL2) and chemokine receptor type 2 (CCR2) also increased in model group compared to that in the control group and B420 significantly inhibited the expression of CCL2 (**Figure 7B**).

It is generally accepted that cytokine imbalance driven by increased pro-inflammation cytokine production of local innate immune responses favors CD4+ T cells responses and Th17 cells

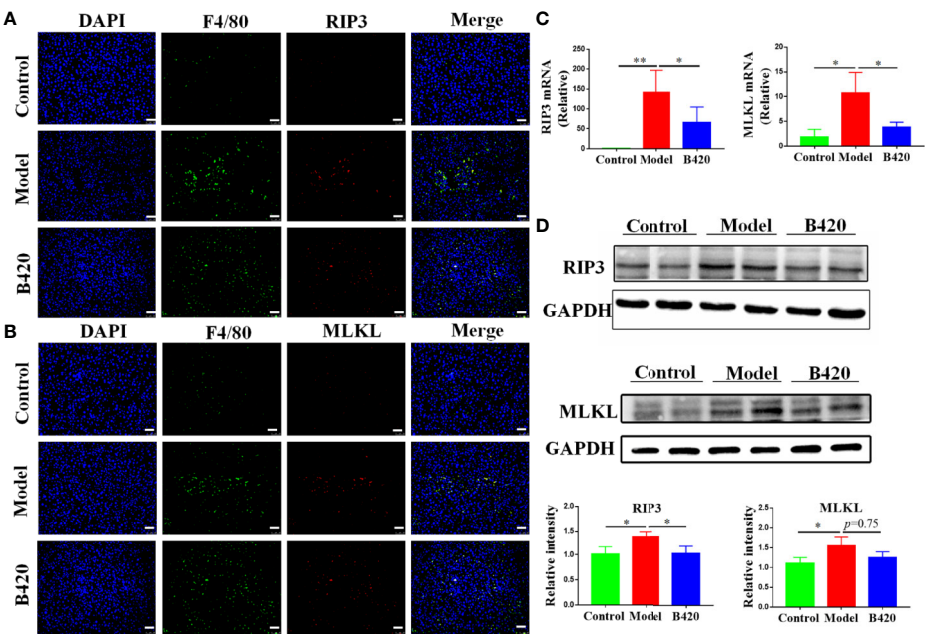

FIGURE 6 | B420 inhibited the RIP3 signaling pathway of liver macrophages in EAH mice **(A)** Representative fluorescence images of liver tissues co-stained with F4/80 and RIP3. F4/80 (green), RIP3(red), DAPI (blue). **(B)** Representative fluorescence images of liver tissues co-stained with F4/80 and MLKL. F4/80 (green), MLKL (red), DAPI (blue). **(C)** Total RNA was extracted from the liver tissues for real-time PCR analysis. The relative expressions of RIP3 and MLKL were shown. **(D)** Protein levels of RIP3 and MLKL in the liver tissues from each group were detected by western blotting and the relative intensity was measured. In **(A–D)**, n=6 in each group. Scale bar: 50μm. The data were presented as means ± SD (Student's t-test, *p < 0.05, **p < 0.01).

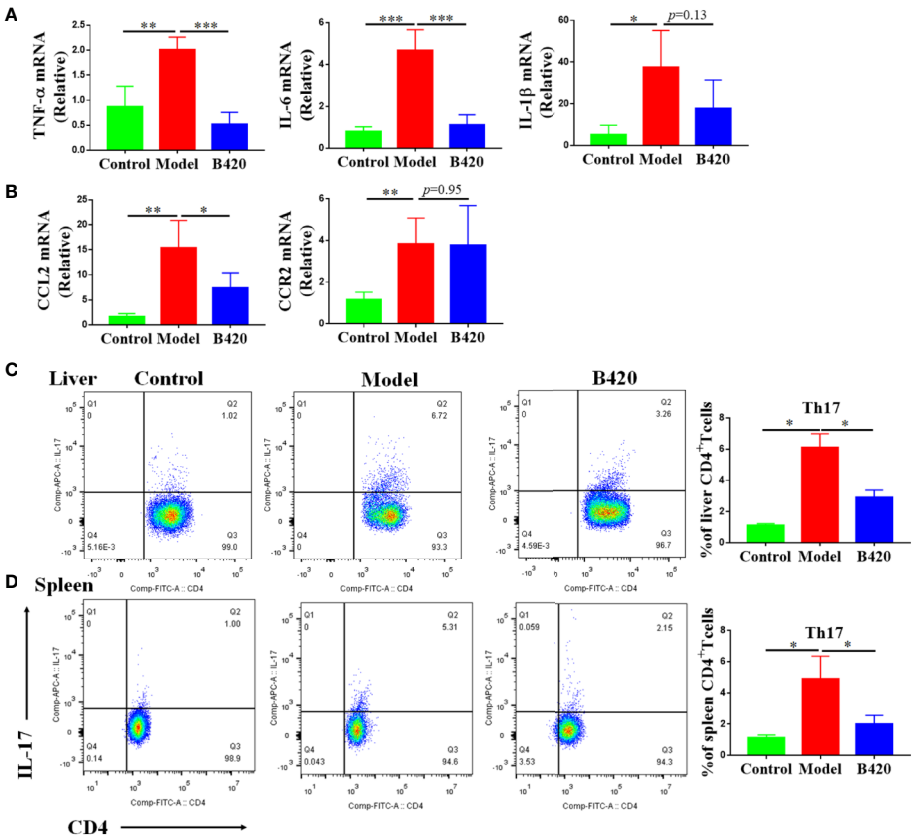

FIGURE 7 | B420 regulated pro-inflammation cytokines and chemokines in liver as well as Th17 cells in liver and spleen. Total RNA was extracted from the liver tissues for real-time PCR analysis and mononuclear lymphocytes were isolated from liver and spleen, counted and stained with cell markers to identify Th17 cells. **(A)** The relative expressions of TNF-α, IL-6 and IL-1β in liver tissues were analyzed by real-time PCR. **(B)** The relative expressions of CCL2 and CCR2 mRNA in liver tissues were detected. **(C)** Typical CD4+IL-17+ Th17 cells flow cytometric plots and the percentage of Th17 cells out of the liver CD4+population were calculated. **(D)** Representative flow cytometry plots and the percentage of Th17 cells out of the spleen CD4+population were calculated. In **(A–D)**, n=6 in each group. The data were presented as means ± SD (Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001).

are effector cells that intensify inflammation and tissue injury (32, 49). Therefore, we measured the percentage of Th17 cells in the CD4 population of liver and spleen *via* flow cytometry. The results showed that the model group had significantly increased percentage of Th17 cells in both liver and spleen compared to the control group. Interestingly, B420 treatment counteracted this change (**Figures 7C, D**). All these data illustrated that B420 might regulate the local cytokine milieu thus affect the adaptive immune response, which contributed to improve the liver immune homeostasis and liver injury in AIH.

Protective Effects of B420-s in LPS-Induced Barrier Injury of Caco-2 Monolayers and Activation of RAW264.7 Cells

Our data indicated that B420 attenuated liver injury through improving intestinal barrier and regulated liver immune homeostasis in EAH mice. However, the link between intestinal barrier integrity and liver immune homeostasis is not clear. To investigate the effects of B420 on intestinal barrier function, we used a vitro model in which Caco-2 epithelial cell monolayers were treated with LPS as it has been demonstrated that LPS caused intestinal barrier dysfunction (50). The western blot results showed that the expressions of ZO-1 and Occludin were dramatically decreased in the LPS group compared to the control group. Whereas, the expressions of ZO-1 and Occludin were significantly increased in groups exposed to 1:50 B420-s or 1:20 B420-s compared to that in the LPS group (**Figure 8A**).

Next, the effects of B420-s on mRNA expressions of various proinflammatory cytokines in RAW264.7 cells were determined by quantitative real-time PCR under LPS-stimulated conditions. As shown in **Figure 8B**, the expressions of TNF-α, IL-6 and IL-1β were significantly increased in LPS-stimulated group compared to that in the control group. In the case of exposure to 1:50 B420-s, the expressions of TNF-α and IL-6 were significantly inhibited, but the expression of IL-1β was not inhibited unless the concentration of B420-s increased to 1:20. Exposure to LPS also increased expressions of CCL2 and CCR2 to a level significantly higher than that in the control group. However, when macrophages were exposed to different

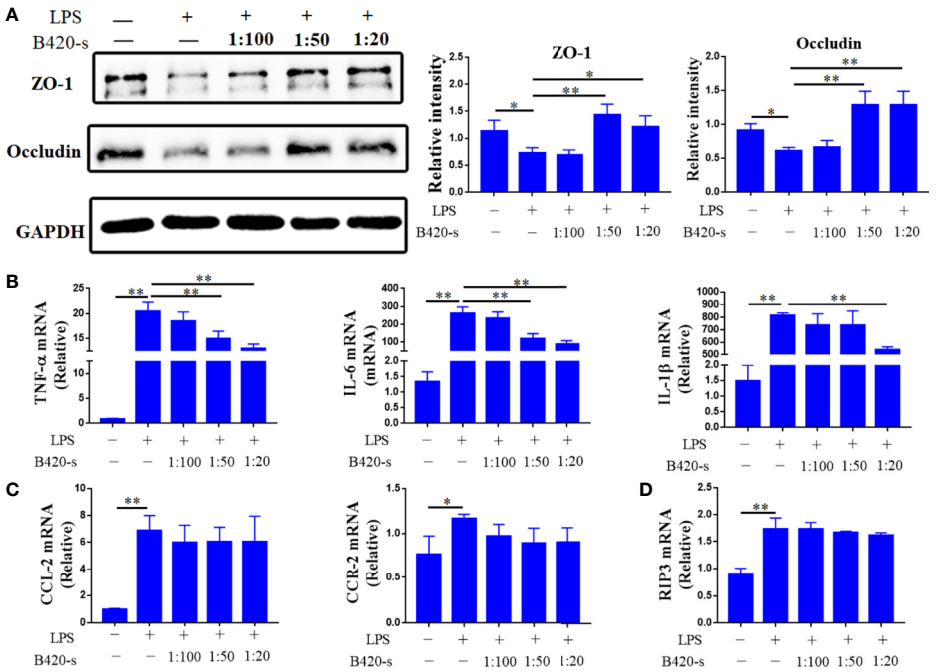

FIGURE 8 | Protective effects of B420-s in LPS-induced barrier injury of Caco-2 monolayers and activation of RAW264.7 cells. The cells were treated with LPS (3 mg/ml) for 12 h in the absence or presence of pre-treatment with different concentrations of B420-s (1:100–1:20). **(A)** The relative protein expressions of ZO-1 and Occludin in Caco-2 cells were analyzed by western blotting. **(B–D)** TNF-α, IL-6 and IL-1β **(B)**, CCL2 and CCR2 **(C)**, RIP3 **(D)** mRNA levels in RAW24.7 cells were detected by quantitative real-time PCR. The data were presented as means ± SD of three independent experiments (Student's t-test, *p < 0.05, **p < 0.01).

concentrations of B420-s combined with LPS, the expressions of CCL2 and CCR2 were not significantly inhibited and RIP3 wasn't inhibited either (**Figures 8C, D**). Further, we used inactive B420-s to stimulate the Caco2 cells and Raw264.7 cells and the results showed that inactivate B420-s had no protective effect on LPS-induced barrier injury and activation of macrophages, which indicated that the direct immunoregulatory and protection of intestinal barrier effects of B420 (**Supplementary Figures 4A–D**). Together, the results of *in vitro* experiments indicated that the immunoregulatory effect of B420 in liver might be partly due to the protection of intestinal barrier.

DISCUSSION

The incidence of AIH is rising and has become an important cause of cirrhosis but the pathogenesis has not been completely explained (1). Recently, environmental factors, especially intestinal dysbiosis and impaired gut barrier function are considered to be associated with the development of AIH (38, 51). The restoration of an altered gut microbiota using probiotics is considered a potential strategy for the prevention and treatment of autoimmune diseases (52, 53). It has been proven that probiotics have beneficial integral functions in many chronic diseases, including inflammatory bowel disease, diabetes and obesity (54, 55). However, few trials have been performed using probiotics in AIH. One of the first colonizers of the human gut, Bifidobacterium, has been well studied for its effect on modulation of intestinal barrier function and SCFA metabolites (56), as well as for its critical role in controlling the immunoregulatory response (57). In our study, we try to explore whether probiotics supplement can alleviate liver injury and its underlying effect on gut–liver axis in EAH model.

It is commonly accepted that gut dysbiosis is associated with AIH and influenced diseases activity (6). However, the effect of therapy targeting intestinal microbiota in AIH remains obscure. The genus Bifidobacterium is one of the most well studied and widely applied probiotic bacteria, especially in the modulation of gut microbiota (58). As reported by Andrea et al., *Bifidobacterium* strains could restore the gut microbial balance in coeliac children as well as re-establishment of the physiological F/B ratio (59). In our study, although inter-animal variance existed in B420 group, we found that B420 significantly increased the alpha diversity of the gut microbiota and altered the composition of EAH mice characterized with the reduction of Bacteroides and Ruminococcus and increasing of *Lactobacillus*, Alistipes and Rikenella at the genus levels. Additionally, B420 treatment increased the abundance of Clostridia which is associated with butyric acid production. Along this line, our data revealed that early intervention with B420 can alter the diversity and composition of microbiota in EAH mice, even though there is an inter-animal variance within the groups. However, further works are essential to get better results.

An accumulating body of evidence demonstrates that a high dietary fiber intake is related to a lower risk of autoimmune diseases (22, 60). This protective effect of dietary fiber might be attributable to the immune-regulation properties of beneficial

microbial metabolic products, such as SCFAs (61). Moreover, SCFAs induce multiple signaling pathways partly through their binding to G-protein coupled receptors (GPRs), particularly GPR41 and GPR43 (62). Studies have implicated a significant role for these GPRs in regulation of health and disease. SCFAs have been shown to have anti-inflammatory and antimicrobial effects, alter gut integrity and regulation of chemotaxis and phagocytosis (63–65). These findings highlight the role of SCFAs as a major signaling molecule that maintains the gut and immune homeostasis targeting metabolite sensing mechanisms such as GPRs. En-De Hu demonstrated that high-fiber diet and sodium butyrate can attenuate the development of AIH through regulation of immune regulatory cells and intestinal barrier function (22). Data from our study showed the concentration of SCFA, especially butyric acid, decreased in feces of AIH patients and B420 obviously increased the concentration of butyrate in cecal feces of EAH mice. However, the concentrations of isovaleric and valeric acid, which were significantly decreased in feces of AIH patients, were not decreased in EAH mouse model. The difficulty of animal models to fully replicate the pathophysiology of human diseases, and differences in the gut microbiota and dietary patterns between mice and humans may contribute to these differences. The underlying mechanisms of the SCFAs and liver immune homeostasis needs further study.

In the past two decades, the immunoregulatory effects of probiotic strains on innate and adaptive immune cells have been evaluated (66). Innate immune cells, for instance macrophages and dendritic cells, recognize microbes and respond to pathogen associated molecular patterns when the bacteria or metabolites are translocated across the intestinal barrier (67, 68). The activated macrophages secrete cytokines and chemokines, affect T-cell proliferation and differentiation and induce adaptive immune responses. Studies have reported that gut microbiota play an important role in shaping the Treg/Th17 axis in adaptive immune response (69). Alba et al. demonstrated the efficacy of Bifidobacterium for the treatment of patients with cirrhosis through inducing a morphologic, phenotypic and functional transition towards an anti-inflammatory profile (70). Rui Yu et al. investigated two *Bifidobacterium. adolescentis* strains for specific immunoregulatory effects, including protection of the Treg/Th17 axis of the cellular immune response system (71). Further studies show that cell surface polysaccharides of Bifidobacterium bifidum can induce the generation of Foxp3+ regulatory T cells through a partially Toll-like receptor 2-mediated mechanism (72). However, A recent study showed an association between high adhesion to epithelial cells of Bifidobacterium and Th17 cell induction, and a subsequent study identified *B. adolescentis* L2-32 as the first human-source commensal inducing Th17 cells (73, 74). Therefore, investigations of the immunoregulatory properties and molecular mechanisms that are critical for the specific function of these strains are of great importance.

In recent years, great importance has been attached to the role of intestinal bacteria in the pathogenesis of autoimmune diseases. It's reported that translocation of intestinal pathobiont drives autoimmunity in mice and human and early but not late antibiotic treatment prevented chronic liver inflammation and autoantibodies (75, 76). Generally, patients with active diseases requires prednisone and immunosuppressive agents to control inflammation in the liver. In our study, early intervention with B420 could ameliorate liver injury of EAH mice and the mechanism involving regulating RIP3 signaling pathway of liver macrophage and cytokines profiles, thus impaired the differentiation of Th17 cells. Of note is that the inhibition effect of B420 on RIP3 signaling was blunted *in vitro* studies, indicating the key determinant factor of B420 on liver immune homeostasis is attributed to the regulation of intestinal barrier function. To our knowledge, our study is the first to focus on the effect of probiotics on regulating gut–liver axis in AIH. The novel finding of our study is that B420 can strengthen intestinal barrier function and further mitigate translocation of bacteria and their metabolites such as LPS, which have been implicated in inhibiting liver inflammation, thus significantly alleviate hepatitis caused by autoimmune factors. The dysregulation of RIP3 signaling is considered a crucial event inducing inflammation through necroptosis (30). Previous studies have already shown that LPS induced programmed cell death and thereby increased the expressions of its target genes, such as RIP3 and MLKL (77, 78). Recent studies demonstrated that some harmful pathogen also activated RIP3 signaling pathway including *Staphylococcus aureus*, *Chlamydia muridarum* and influenza H7N9 virus (79–81). In our study, mice in EAH group showed elevated serum LPS level and increased abundance of pathogenic bacteria including Bacteroides and Ruminococcus. Importantly, the activation of RIP3 signaling was inhibited by B420 treatment. Taken together, these findings demonstrate probiotics can alter the microbial composition of EAH mice and thus facilitate the maintenance of liver immune homeostasis. However, the molecular mechanisms by which probiotics regulate the immune response of the liver need to be further studied.

In summary, our findings showed that early intervention with B420 in EAH mice has beneficial functions and the underlying mechanisms involving modulating the gut microbiota composition and intestinal barrier function, inhibiting the RIP3 signaling pathway of liver macrophages thus decreasing the proportion of Th17 cells were deciphered. Our research shed light on the therapeutic and research potentials for the application of probiotics in AIH. However, the limitation of our study is the lack of evidence from clinical patients and the underlying mechanism targeting intestinal microbiota and intestinal barrier in AIH needs further study. Nevertheless, the encouraging results seen in the EAH model will surely promote further clinical trial development. Furthermore, application of probiotics might be novel options for treatment of AIH.

DATA AVAILABILITY STATEMENT

The 16S rRNA gene sequencing data has been uploaded to SRA—The BioProject ID is PRJNA657497.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Tianjin Medical University General Hospital. This study didn't use client owned animals (noncommercially available animals e.g. pets or livestock). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Authorization of the Ethical Committee of Tianjin Medical University General Hospital.

AUTHOR CONTRIBUTIONS

LZ and BW designed the study. HZ, ML, XL, and WZ performed the experiments. YL, YR, JZ, LG, and XC analyzed the results. HZ and LZ wrote the paper. HZ, ML, and XL contributed equally to this work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.569104/full#supplementary-material

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How Retroviruses and Retrotransposons in Our Genome May Contribute to Autoimmunity in Rheumatological Conditions

Tomas Mustelin * *and Kennedy C. Ukadike*

Division of Rheumatology, Department of Medicine, University of Washington, Seattle, WA, United States

More than 200 human disorders include various manifestations of autoimmunity. The molecular events that lead to these diseases are still incompletely understood and their causes remain largely unknown. Numerous potential triggers of autoimmunity have been proposed over the years, but very few of them have been conclusively confirmed or firmly refuted. Viruses have topped the lists of suspects for decades, and it seems that many viruses, including those of the Herpesviridae family, indeed can influence disease initiation and/or promote exacerbations by a number of mechanisms that include prolonged anti-viral immunity, immune subverting factors, and mechanisms, and perhaps "molecular mimicry". However, no specific virus has yet been established as being truly causative. Here, we discuss a different, but perhaps mechanistically related possibility, namely that retrotransposons or retroviruses that infected us in the past and left a lasting copy of themselves in our genome still can provoke an escalating immune response that leads to autoimmune disease. Many of these loci still encode for retroviral proteins that have retained some, or all, of their original functions. Importantly, these endogenous proviruses cannot be eliminated by the immune system the way it can eliminate exogenous viruses. Hence, if not properly controlled, they may drive a frustrated and escalating chronic, or episodic, immune response to the point of a frank autoimmune disorder. Here, we discuss the evidence and the proposed mechanisms, and assess the therapeutic options that emerge from the current understanding of this field.

Keywords: autoimmunity, retrotransposons, retroelements, nucleic acid sensors, reverse transcriptase, type I interferon, endogenous retroviruses

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***Correspondence:**
Tomas Mustelin
tomas2@uw.edu

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RELEVANT MOLECULAR CONCEPTS OF HUMAN AUTOIMMUNE DISEASES

An important feature of clinical autoimmunity is that patients tend to fall into a discrete number of reasonably well delineated and named disease entities (i.e. diagnoses), rather than spanning the full spectrum of autoimmunity against random antigens. Granted, there is variability and heterogeneity within most such disease entities; and some diagnoses may in fact represent more than one distinct

disease or a series of mechanistically different molecular "endotypes" of the disease. Nevertheless, patients that deviate radically from the typical disease profiles are rare. This pattern of many distinct diseases does not readily mesh with the commonly accepted notion that autoimmunity starts with a simple stochastic loss-of-tolerance blunder by a T cell. Rather, it seems that autoimmune diseases must be the result of distinct and unique pathophysiological processes that evolve over an extended period of time into a specific disease. The two concepts are of course not mutually exclusive, but they shape our thinking in different ways: while the former focuses autoimmunity research on T or B cell antigen receptor repertoire and mechanisms of central and peripheral tolerance, the notion that autoimmunity may arise from specific biological processes broadens the search for disease triggers and attempts to understand the escalation towards disease. The therapeutic ramifications of these two views are also distinct: a T or B cell-centric view calls for immunosuppressive or tolerizing approaches, while the concept of specific biological processes resulting in autoimmunity will look for specific modulation of such processes without the need for suppressing the normal function of the immune system. In this review, we follow the notion that autoimmune disease can have causes other than "stochastic mistakes of adaptive immunity". We accept that T and B cells are critically important for autoimmunity, but we are not convinced that they initiate it.

Although individual autoimmune diseases can be clinically quite different from each other and often are associated with polymorphisms in different genes, and may respond to different targeted therapies, it is also clear that some diseases likely have overlapping pathogenic mechanisms; these mechanistically "related" diseases share cardinal features and symptoms and can co-occur in individual patients (*e.g.*, SLE and secondary Sjögren's syndrome). An example of a group of such "related" autoimmune disease are those characterized by elevated type I interferons (IFNs) (1–4), including large portions of systemic lupus erythematosus (SLE) (1, 5, 6), dermatomyositis (DM) (7), primary Sjögren's syndrome (pSS) (8–10), and several others. Type I IFNs are a hallmark of anti-viral immunity, with which these diseases appear to share other features as well, including autoimmunity against a similar set of proteins involved in nucleic acid processing, as well as the nucleic acids themselves. As in autoimmune diseases, viral infections are often accompanied by fever, headache, loss of appetite, malaise, fatigue, arthralgias, and sometimes skin rash. During viral infections, these responses are transient, while in SLE they become chronic with an unpredictable and often episodic course.

We recently reviewed the currently known and proposed sources of pathogenic nucleic acids and how they can act to drive SLE-like autoimmunity (11). Briefly, the offending nucleic acid could be either DNA or RNA, or both. Pathogenic cytosolic DNA may leak out from the nucleus following extensive damage to chromosomal DNA or mitotic catastrophes (not very likely in autoimmunity). DNA can escape from defective mitochondria, or DNA can be synthesized by reverse-transcription of various species of RNA (particularly from retroelements). Extracellular DNA may spill out from cells dying by a variety of programmed cell death mechanisms, or from commensal gut microbes, and then be internalized and sensed by immune cells. Pathogenic RNA can be (mis)generated and sensed intracellularly or end up in the extracellular space from which it can be internalized, for example as part of immune complexes, to be sensed by endosomal toll-like receptors (TLRs) in immune cells. In this review, we focus on RNA transcripts derived from endogenous retroviruses and retrotransposons and on the extrachromosomal DNA synthesized by reverse transcription of these RNA species. We also discuss the potential contributions of proteins generated by translation of these RNA transcripts, which may form more or less complete virions.

An important concept to keep in mind when contemplating how aberrant DNA or RNA drive autoimmunity is that a multitude of ancient and powerful mechanisms exist within our cells to effectively prevent the expression of potentially problematic sequences in our genome and to effectively degrade and remove aberrant DNA or RNA. These mechanisms are reviewed in section *Defense Mechanisms Against Retroviruses and Retrotransposons: Our Original Immunity*. Their importance to our health is perhaps best illustrated by the serious diseases that arise from mutations in the genes for several of these pathways, including Aicardi-Goutières Syndrome (AGS), which is characterized by constitutively elevated type I interferons and SLE-like autoimmunity. It presents at birth as a suspected neonatal viral infection, which is a medical emergency, but no exogenous virus can be found and the disease continues unabated. Over the years, AGS patients develop severe neurological deficits, perhaps due to direct neurotoxicity of type I IFNs. In regular polygenic SLE, however, it remains unclear if these defense mechanisms are weakened or simply overcome by an abundance of aberrant RNA or DNA. There are many potential variants of these scenarios. We have proposed that the clinical heterogeneity of SLE may be due, in part, to heterogeneity in which pathogenic nucleic acid molecules are present and which sensors and pathways they trigger in individual patients (11). Elucidation of these events may result in the recognition of distinct "endotypes" of SLE, each with its specific therapeutic opportunities.

ENDOGENOUS RETROVIRUSES AND RETROELEMENTS IN OUR GENOME

"By DNA sequence, we are more retroviral than human" is a provocative way of pointing out that a considerably larger portion of our human genome consists of sequences that once were RNA genomes of free and infectious retroviruses that were reverse transcribed into DNA and then pasted into our genome; they are more abundant (8%) than all the exons of our protein-coding "traditional" genes combined (about 1%) (12). Since most, if not all, genomes of eukaryotic and prokaryotic organisms on our planet share this feature of abundant inserted retroviral sequences, it is very likely that the 8% of our genome that is readily recognizable today as retroviral in origin

is, in fact, only the tip of the iceberg. Most such sequences are not positively selected for (but likely the opposite) and over evolutionary time lose their integrity by random mutations, deletions, recombinations, and other mechanisms. For these reasons, sequences older than 100 million years become increasingly difficult to recognize with acceptable confidence.

Since reverse-transcribed retroviral sequences are present in all kingdoms of life, it appears that this influx of genomic material started at the very dawn of cellular life (13–16). In fact, it is quite likely that it was instrumental to the evolution of larger and more diverse genomes: each newly incorporated reverse-transcribed retroviral genome adds ~ 9,500 base pairs to the genome (**Figure 1**), including three major protein-coding genes, *gag*, *pol*, and *env*, plus mRNA splicing sites, to generate transcripts that are translated into at least five proteins, each of which can be proteolytically processed into additional functional units. In addition, each retroviral integration brings two identical long terminal repeats (LTRs), one on each end of the insert, which contain clusters of highly efficient transcription factor binding sites to control transcription of the insert, as well as adjacent regions. In fact, it has been estimated that more than 300,000 regulatory regions (including promoters and enhancers) in our genome are, or contain remnants of, ancient LTRs from otherwise long-lost retroviral inserts. It is also clear that many "traditional" genes are descendants of ancient retroviral *gag*, *pol*, or *env* genes that were co-opted for new uses (17). For example, the RNaseH and integrase domains of the retroviral *pol* gene served as starting material for fundamental building blocks of our immune system (18). It has also been suggested that mRNA splicing was originally a retroviral invention.

The 8% of our human genome that consists of recognizable proviruses (12) (*i.e.* the cDNA of retrovirus RNA genomes, or parts of them), are collectively termed the human endogenous retroviruses (HERVs). Strictly speaking, this term is not entirely accurate since the majority of these sequences were incorporated long before our hominin ancestors became *Homo sapiens*. Hence, the term HERVs should be viewed as the complement of retroviral sequences in their current state, which for essentially

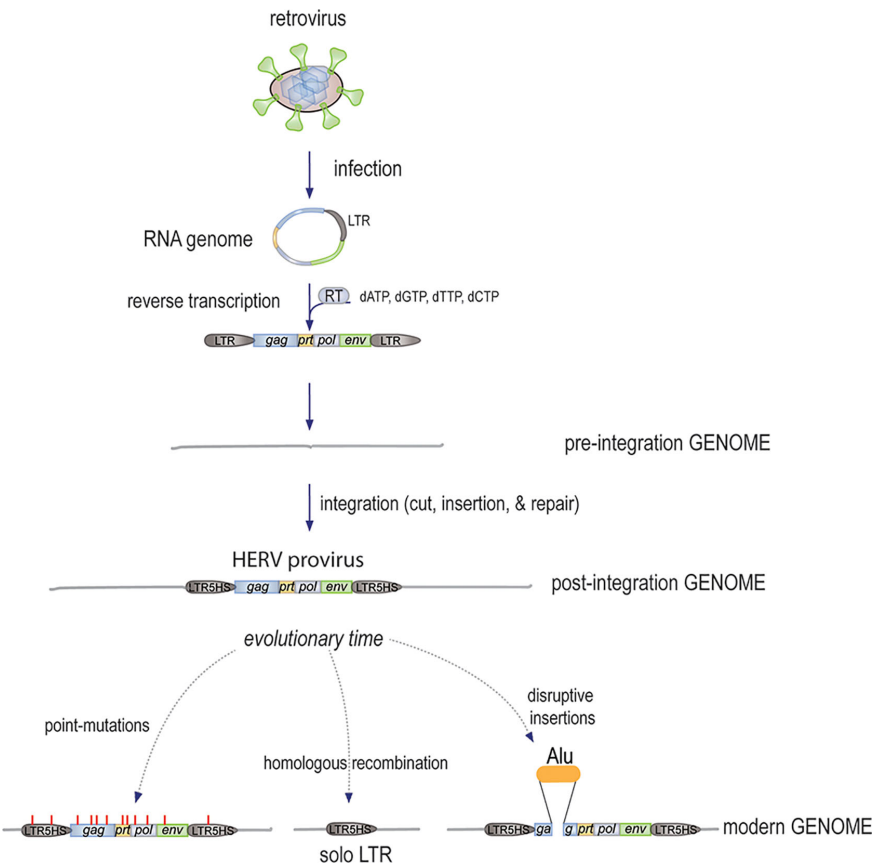

FIGURE 1 | The mechanism by which new HERVs were generated. An infectious free retrovirus infects a host germline cell, releases its circular RNA genome, which remains protected by the associated *gag*-derived nucleocapsid and other core proteins, while the *pol*-encoded RT synthesizes the first strand of linear cDNA starting with the LTR and ending after the second copy of the LTR, followed by second strand synthesis. The resulting dsDNA of approximately 9,500 bp is then inserted into the genome by the endonuclease activity of the Pol protein. The ends are then finalized by the DNA repair machinery with a few added nucleotides. Over evolutionary times, further changes to the HERVs included the accumulation of point-mutations (some introducing stop-codons), deletions by homologous recombination, and disruption by additional insertions.

all of them is different from what they looked like when they were free and contagious retroviruses that infected our ancestors and in the process reverse-transcribed their RNA genome and inserted it into the germline of their host. Although they were subsequently inherited in a Mendelian fashion by all descendants of the original host, there was little evolutionary pressure to maintain them in their intact form; more likely the opposite. Chimpanzees and gorillas have remarkably similar sets of retroviral loci (19–21), except for different mutations and the dozen or so new integrations that occurred in each species since our last shared ancestor lived approximately 6 million years ago.

In addition to the *bona fide* HERVs, an even larger portion of our genome, over 30%, consists of copies of non-LTR retrotransposons (12). Collectively, endogenous retroviruses and retrotransposons are referred to as retroelements. The non-LTR retrotransposons are classified as either "autonomous" or "non-autonomous" depending on whether they contain all the required components necessary for retrotransposition within themselves, or not. The most abundant class of autonomous retrotransposons are the long interspersed nuclear elements-1 (LINE-1 or L1 for short) (22). The biology and potential relevance of L1 in autoimmunity is discussed in section *Non-LTR Retrotransposons—LINE-1 and Alu Elements*.

The non-autonomous retrotransposons include the short interspersed nuclear elements (SINE), such as the Alu (23) elements and SINE-R, VTR, Alu (SVA) elements (24), which all depend on the L1 reverse transcriptase (RT) for their retrotransposition cycle. The Alu sequence itself appears to be a contracted form of the 7SL RNA (25), which is a component of the signal recognition particle. Alu elements have been extraordinarily successful in replicating within our genome: there are approximately 1 million of them in our genome, many of them within introns, where they may modulate gene transcription and mRNA processing.

The Many Families of Retroviral Sequences in Our Genome

The tens of thousands of retroviral sequences that exist in our genome belong to more than a dozen distinct families, which originally were distinct free retroviruses that infected our ancestors during different, but often overlapping, epochs of prehistorical times. Most of these sequences have accumulated numerous mutations and deletions, and some have been disrupted by insertions of other retrotransposons, e.g. Alu elements. The older sequences have more such alterations and have lost their ability to encode full-length retroviral proteins, but the more recently incorporated ones are more complete and still retain the capacity to encode fully functional proteins and to produce viral particles. However, it seems that none of the HERVs are fully infectious anymore.

A basic nomenclature divides Retroviridae into four classes: gammaretroviruses (class I), betaretroviruses (class II), spumaretroviruses (class III), and lentiviruses (class IV). The first three classes are represented in our genome. They are further divided based on the specific tRNA they use for priming of reverse transcription. In essence, the retroviral RT that generates a DNA copy of the circular viral RNA genome uses a cellular tRNA complementary to a short motif in the viral LTR for priming of the reaction. The youngest of the Class II HERVs, for example, used lysine-tRNA for priming and are therefore classified as group K (for lysine), hence named HERV-K. The shortcomings of this classification, *e.g.* its lack of further taxonomic considerations, prompted other classification principles to be proposed. Unfortunately, these efforts to bring order only resulted in several parallel nomenclatures and, as a result, many loci have non-conforming and confusing names, as well as several synonymic designations. A more precise way to add specificity is to mention which chromosome the locus is on and exactly which nucleotide positions it occupies in the human reference genome, *e.g.*, HERV-K119 occupies nucleotides 58,721,242–58,730,698 of chromosome 12.

In this review, we focus only on those families that have been proposed to be of potential relevance in human autoimmunity: primarily the "Human MMTV-like 2" (HML-2) subgroup of the Class II HERV-K (26) and the Class I provirus HERV-E (27). We would postulate, however, that individual HERVs that may be detrimental to our health could belong to any family. At the same time, we find it more likely that the most recently incorporated HERVs, which have retained much of their original features and still can produce virions (albeit all with reduced infectivity), are more likely to cause immune disorders resembling chronic viral infections than the older HERVs, which often are incomplete, and have been "domesticated" by frame-shifts, point-mutations, and stop codons. We accept, of course, that older HERVs may have acquired new properties by stochastic mutations and thereby gained the ability to drive unique pathologies unrelated to the mechanisms of typical antiviral immunity.

HERV-K (HML-2)—The Youngest and Most Intact HERV Family

Although the now (presumably) extinct free retrovirus that gave rise to the HERV-K (HML-2) provirus family (26) repeatedly infected our ancestors for tens of millions of years, we will probably never know what kind of disease it caused at that time. What we can conclude using computational tools from the ~120 genomic loci still present today is that HERV-K(HML-2) first entered our ancestral early hominin germline genome over 30 million years ago (28) and then continued to insert again and again into our germline genome until very recently in evolutionary time (29). Many other retroviruses stopped incorporating into our germline much earlier. Obviously, the potentially vast numbers of infections that did not result in germline insertions are invisible to us today, even if they likely were important for the life-cycle and spread of the virus. Hence, the HERV-K provirus loci in our genome represent a vast underestimate of the number of times the free virus infected and perhaps profoundly affected our ancestors. The insertional polymorphisms (*i.e.* only some people have some of them) (30–32) and the polymorphic deletions (33) observed in human populations today reveal that infections probably continued until times when modern humans were more numerous and

had spread out over larger geographical areas in the last 50–70,000 years. Exogenous HML-2 appears to have infected gorillas relatively recently as well (34).

The age of a HERV-K locus (*i.e.* the time since germline integration) can be estimated from the fact that the single LTR in the circular retroviral RNA genome is reverse-transcribed twice, resulting in two identical LTR copies, one in each end of the resulting genomic provirus (**Figure 1**). Since there is no evolutionary pressure to maintain these sequences, they are assumed to be subject to stochastic mutations at the standard background rate of approximately 0.5 x 10^{-9} per base-pair per year. This "molecular clock" obviously can only be applied to HERV-K loci that have retained both LTRs. Based on this logic, an alignment of the seven youngest HERV-K proviruses was used to deduce *in silico* what the sequence of the original infectious retroviruses most likely was. Albeit not necessarily 100% correct, the resulting sequence gave rise to a fully infectious retrovirus, termed HERV-K Phoenix (35), which has been studied for its tropism, cellular receptors, maturation, ultrastructure by electron microscopy, and ability to reverse transcribe and insert its cDNA into the genome of host cells (35).

The most recent human insertions of the HERV-K (HML-2) subfamily, *e.g.*, HERV-K113 at chromosome 19p12 (36), are also intact enough to produce virions (37), albeit with poor infectivity. Other full-length HERV-Ks with intact open reading-frames are HERV-K108a (at 7p22.1), HERV-K115 (8p23.1), HERV-K118 (at 11q22.1), and HERV-K119 (at 12q14.1). Another seemingly intact HERV-K provirus is located at Xp21.33 in approximately 2% of people, most of whom are of African ancestry (31). HERV-K113 and HERV-K115 are also insertionally polymorphic and exist in 15%–30% of modern humans.

To the extent that we know, these youngest loci are transcriptionally silenced in healthy individuals by extensive DNA methylation and other epigenetic mechanisms, as one would assume for potentially dangerous loci. A consequence of this is that they probably remain silent during the development of T and B cell antigen receptor repertoires in early life, resulting in weak immunological tolerance against the proteins that they can encode. If this indeed is true, aberrant expression of these proteins would likely provoke both cellular and humoral immunity (38). There is supporting evidence for this assumption: increased transcription in malignancies of the breast (39) and prostate (40), and in HIV infected individuals (38, 41–45), leads to both (auto)antibodies against HERV-K proteins and HERV-K-specific T cells. Increased levels of HERV-K transcripts have also been detected in rheumatoid arthritis (RA) blood and synovial tissue (46, 47). The resulting immune response is discussed in more detail in section *Autoantibodies Against Retroviral Proteins in Autoimmunity*.

From the perspective of autoimmune diseases like RA and SLE, which are strongly female-biased, it is interesting to note that the 5' LTR of intact HERV-K loci contain many binding motifs for estrogen- and progesterone-regulated transcription factors. Indeed, these hormones can upregulate transcription many-fold (48). We have replicated this finding (unpublished). HERV-K transcription is also increased by cigarette smoking (49, 50), another risk factor for RA (51, 52).

HERV-E and Other HERVs of Potential Significance

A body of literature describes findings of increased expression of HERV-E in autoimmune disease, particularly SLE (53), as well as the presence of autoantibodies against HERV-E proteins, such as p30 encoded by its *gag* gene (54, 55). HERV-E derived Env protein can be detected in psoriatic skin (56). Compared to healthy individuals, HERV-E mRNA is reportedly increased in T cells from SLE patients, its LTR is hypomethylated, and further expression can be induced by demethylating agents and UV irradiation (57, 58). It has been proposed that autoantibodies against p30 cross-react with class I HLA (55).

A detailed survey of transcripts derived from over 8,000 retroviral sequences in our genome by Akiko Iwasaki and her team (59) found that a large portion of HERV loci are transcribed in transformed cell lines and many also in cells from patients with SLE. Compared to healthy controls, a number of transcripts were more, and a few less, abundant in SLE patients. The overexpressed loci included several HERV-K, HERV-E, HERV-W, and ERV3 loci. The use of computational tools, such as ERVmap designed by these authors (59), or others (60), have begun to uncover the full complexity of this topic. Case in point: the number of spliced and processed mRNAs derived from all the HERVs theoretically rival those of the traditional genes in numbers. If one also includes all retrotransposon transcripts, which sometimes are derived from in intronic or 5' and 3' UTRs of protein-coding genes, the overlap and complexity becomes truly challenging. An important question is which of all these retroviral and retrotransposon transcripts are, in fact, translated into polypeptides that may have consequences for health and disease? Do retroelement transcripts matter if they are not translated? What are the consequences of the production of various retroelement-encoded polypeptides? How might the numerous single nucleotide polymorphisms and other types of polymorphisms within HERVs and retrotransposons affect human health?

Non-LTR Retrotransposons—LINE-1 and Alu Elements

The L1 element (61) appears to represent a remnant of an ancient retrovirus that retained, or later acquired, a degree of autonomy through the conservation of a primordial RT, which endows it with the ability to transpose without having to leave the host cell. This mechanism has been extraordinarily successful over evolutionary time and L1 sequences now occupy 17% of our genome (12, 62–64). While most of the ~500,000 L1 copies are mutated and truncated, some ~180 copies are seemingly intact and a handful of them remain fully active today (65), *i.e.*, they continue to retrotranspose by the L1 "copy-and-paste" mechanism (**Figure 2**), occasionally disrupting genes or regulatory regions by novel insertions (66).

Full-length L1 is a 6-kb sequence with two open reading frames (ORF1 and ORF2) that encode for two proteins: the 40-kDa RNA binding ORF1p protein, and the 149-kDa ORF2p, which has both reverse transcriptase (RT) and endonuclease

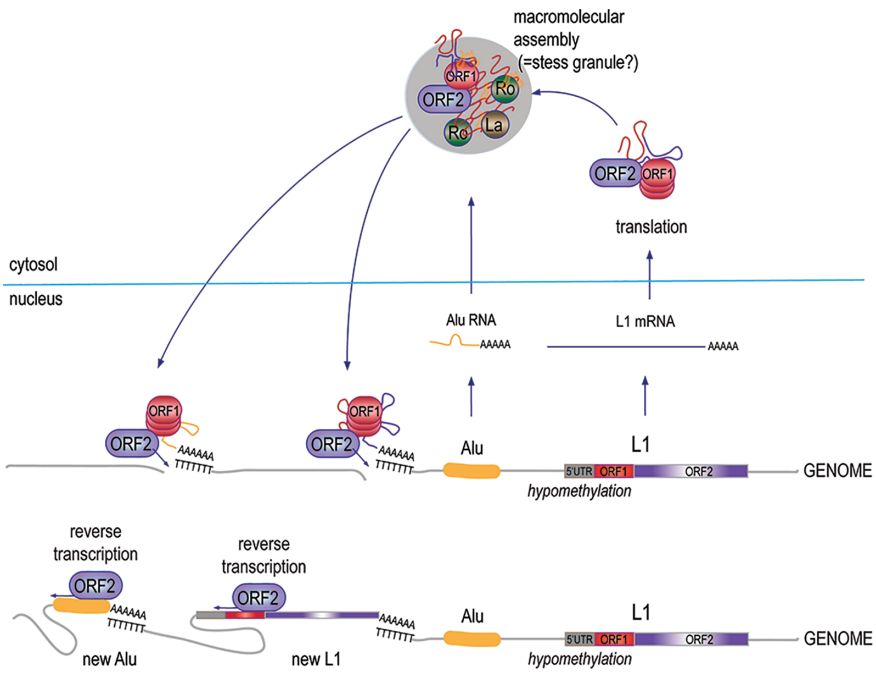

FIGURE 2 | The biology and replication cycle of the L1 element. Approximately, 500,000 copies of the L1 element, most of them truncated and mutated, exist in the human genome across all chromosomes, both within introns of protein-coding genes and in intergenic regions. Transcripts of the few 6-kb full-length L1 loci that retain intact open-reading frames are translated into the p40/ORF1p and p149/ORF2p proteins, which assemble in approximately a 20:1 stoichiometry into complexes with high affinity for RNA, particularly L1 mRNA, as well as Alu RNA. The complexes also include at least 10 other proteins, including Ro60 and La. Under permissive conditions, the RT activity of ORF2p makes a DNA copy of the associated RNA and inserts it into the genome, resulting in a new L1 element, a new Alu element, or a new pseudogene of an accidentally captured other mRNA.

activities. Retrotransposition occurs through a "copy-and-past" mechanism, where the primary transcript is captured by ORF1p, and is then reverse-transcribed by the RT domain of ORF2p primed by a nick made by the endonuclease domain of ORF2p in a genomic poly-T sequence, where the 3' poly-A of the L1 RNA can anneal. The DNA repair machinery then patches up the 5' end of the new insert. From time to time, ORF1p and ORF2p grab the wrong RNA, resulting in the reverse transcription and genomic insertion of an Alu element (67) or a spliced mRNA giving rise to an intron-less pseudogene (63). Even if most pseudogenes are inactive, this mechanism may have created genomic diversity and new material for natural selection to work with. Similarly, ORF2p-mediated reverse-transcription and insertion of a primary (un-spliced) mRNA would result in gene duplication; this may be how gene families were created over evolutionary time.

The RT of L1 ORF2p shows clear, but relatively distant, homology with the RT of the HERVs, suggesting that they all originate from a common ancestral RT. The L1 RT is closer in homology to the RT of hepatitis B virus, a "para-retrovirus", that uses reverse transcription to generate a circular DNA that is not integrated into the host genome. Insects, like *Drosophila*, have retrotransposons (68). Yeast also have retrotransposons similar to L1, called Ty3 (69), as do baculoviruses (70), and prokaryotes in the form of reverse-transcribing bacteriophage (71). Retrotransposons in plants (72) have been called "engines of evolution" (73). All of these examples attest to the truly old roots of these sequence elements.

There is evidence that L1 loci are transcriptionally active in SLE patients (74–76). This appears to correlate with a global decrease in DNA methylation, which is well documented in SLE (77, 78) and likely relates to the decreased expression of DNA methylases DNMT1 and DNMT3a (79, 80). Demethylating agents like 5-aza-2'deoxycytidine (81) also cause a dramatic upregulation of L1 and Alu element transcription in lymphocytes (82). In addition, transfer of 5-aza-2'deoxycytidine-treated T cells into healthy mice results in an SLE-like disease (83). The drugs that can induce "drug-induced lupus", notably hydralazine and procainamide, are also demethylating agents (84). Other known triggers of lupus flares, like UV light, oxidative stress, inflammation and exogenous viruses also induce genomic hypomethylation (85, 86). L1 transcription can also be stimulated by female hormones, which further supports the notable female predilection of SLE and RA.

Among the many reasons to suspect that L1 plays a role in autoimmune disease is the observation that ORF1p resides mostly in macromolecular assemblies (**Figure 2**) that have been proposed to be stress granules (87) together with L1 RNA

and other RNA-binding proteins (88–91), such as Ro60, La (92), and U1 small nuclear ribonucleoprotein of 70 kDa (88), all well-established autoantigens in SLE and related diseases. The protein complexes also contain ORF2p and perhaps newly synthesized DNA made by its RT activity. If released from broken or dying cells, such protein complexes containing two apparently immunogenic proteins as well as both RNA and DNA would likely be of great interest to the immune system.

DEFENSE MECHANISMS AGAINST RETROVIRUSES AND RETROTRANSPOSONS: OUR ORIGINAL IMMUNITY

In support of the notion that incoming integrating retroviral genomes and autonomously retrotransposing sequences have posed serious threats to the integrity of host genomes since the early days of cellular life (despite also contributing positively to evolution), an elaborate set of defense mechanisms against retroviruses and retroelements are present in all cells (93–98). Many of these mechanisms were discovered in the course of HIV research as "restriction factors". It also appears that many of these mechanisms continue to be critically important for human health. Conversely, many prevalent diseases, including cancer and autoimmunity, may be related to incomplete function of these mechanisms (61, 99).

All Roads Lead to Type I IFNs

Type I IFNs are a central hub of antiviral immunity (100). Therefore, it is not surprising that many of the defense mechanisms that cells use against retroviruses and retrotransposons also center on the induction of type I IFNs. Nevertheless, one should keep in mind that many defense mechanisms also have direct anti-viral functions and that many induce other pathways too. Interferons often play an amplifying role and increase the expression of these defenses in a positive feedback loop.

The main threat of a virus is its RNA or DNA genome, which will hijack the cellular biosynthetic machinery for its own replication and virion production, with detrimental and often lethal consequences for the host cell. Even more alarming, retroviruses will reverse transcribe their RNA genome and insert the resulting DNA into the host genome as a permanent provirus. To counteract these ancient foes, evolution has produced several cellular mechanisms for the detection of non-self RNA and DNA (11). Five principal pathways operate in the cytosol and on the cytosolic surface of intracellular organelles: the DNA-sensor "cyclic GMP-AMP synthase" (cGAS) (101), the RNA sensors "retinoic acid-inducible gene I" (RIG-I) (102) and "melanoma differentiation-associated gene 5" (MDA5) (102–104), and the two kinases "protein kinase RNA-activated" (PKR) (105, 106) and DNA-activated protein kinase (DNA-PK). A sixth pathway responds to extracellular DNA or RNA brought into the cell by receptor-mediated endocytosis and is initiated by Toll-like receptors (TLRs) 3, 7, 8, and 9 in the endosome. A mechanism for cross-talk of the extracellular and intracellular sensing pathways consists of the transporter protein SIDT2 (107), which channels dsRNA through the endosome membrane into the cytosol, where it can trigger MDA5. There are several additional recently discovered DNA and RNA sensors, such as DDX1, 21, 36 and 41, IFI16, and Aim2 (108). All these pathways primarily promote type I IFN production through activation of IRF3 and related transcription factors. Some also activate signaling pathways that lead to the production of other cytokines. The resulting type I IFNs are secreted, bind to the type I IFN receptor, and signal through the JAK/STAT pathways to upregulate the expression of proteins with direct anti-viral activity, including nucleases, helicases, chaperones, and many of the sensors and their adapters and signaling proteins (100). Another important effect is the upregulation of MHC molecules to facilitate the recognition of the virally infected cell by cytotoxic T cells.

Most patients with SLE (or related diseases) have elevated levels of type I IFNs (3, 4, 109), which is best detected as the high expression of IFN-stimulated genes (ISGs), now referred to as the "IFN signature" and seen in 70-90% of SLE patient populations world-wide (5, 110–113), as well as in patients with pSS (10, 113), systemic sclerosis (114, 115), polymyositis (PM) and DM (7, 116), and in a small subset of rheumatoid arthritis (RA) (117, 118). The elevated IFNs include not only IFNα and IFNβ, but also the less known IFNε, IFNκ, and IFNω, as well as type II IFN (IFNγ) (10), and type III IFNs (IFNγ1, IFNγ2, and IFNγ3) (119), which collectively appear to play an important role in pathogenesis (2, 120, 121). Type I and III IFNs are functionally overlapping (all genes induced by type III IFNs are also induced by type I IFNs), but IFNγ is instrumental in a distinct aspect of the immune system, namely the activation of T helper 1 cells, cytotoxic T cells, natural killer (NK) cells, and other elements of a general immune response. Nevertheless, some 900 of the 1,300 ISGs induced by IFNγ are also induced by type I IFNs, which induces a total of over 1,500 ISGs, suggesting much overlap in downstream consequences.

Type I IFNs have a spectrum of effects on the immune system and beyond, particularly upregulating numerous aspects of anti-viral defense. They stimulate emergency myelopoiesis (122), monocyte differentiation into myeloid dendritic cells (123, 124), antigen presentation, cytotoxic T cell differentiation (125), and B cell differentiation into plasma cells (126). These hallmarks of anti-viral immunity also characterize SLE and other autoimmune conditions.

DNases, RNases, and Aicardi-Goutières Syndrome

To neutralize dangerous DNA or RNA, cells express a number of DNases and RNases, the function of which also prevent untimely triggering of DNA and RNA sensors. Remarkable insights into the dynamic biology behind these processes was gleaned from studies of the monogenic disease known as Aicardi-Goutières syndrome (AGS) (127–132), in which loss-of-function mutations in any one of a number of enzymes lead to constitutively high production of type I interferons (IFNs), neurological deficits due

to IFN toxicity, and autoimmunity that resembles SLE very much. Loss of the cytosolic DNase *TREX1* (99, 129, 133, 134) causes the accumulation of non-chromosomal DNA made by L1 ORF2p (135, 136), while mutations in any of the three subunits of *RNASEH2* (129, 132) cause the accumulation of DNA : RNA heteroduplexes made by ongoing reverse transcription (132). Another AGS gene, *SAMHD1* (137, 138), directly counteracts reverse transcription by dephosphorylating the required deoxynucleotide triphosphates. Together, these defects show that IFN-driving aberrant DNA apparently results from reverse transcription of cellular RNAs at a surprisingly high spontaneous rate. The only cellular enzyme capable of this reverse transcription is the ORF2p of L1, which is a highly efficient RT (61, 139, 140). IFN production (141) triggered by L1 can use many cellular RNA templates, including its own mRNA (63, 64) or Alu transcripts, to generate DNA species that drive the interferon production pathway. This mechanism also operates in cellular senescence (142).

In a mouse model of AGS, the *Trex1*$^{-/-}$ mouse (99), the animals develop a systemic inflammation with immune cell infiltrates in many organs and they die early from a severe carditis. These animals can be rescued from death by treatment with the RT inhibitors tenofovir plus nevirapine (143), indicating that reverse transcription is a key step in the pathogenesis of systemic inflammation in this model. However, there is also a published paper refuting these data (144). More importantly, the IFN signature can be reduced substantially in AGS patients by RT inhibitors used for the treatment of HIV (145).

Factors That Reduce Retrovirus Infectivity and Retrotransposition

The fact that the vast majority of HERVs and retrotransposons have been rendered largely inactive and/or harmless (to the best of our knowledge) attests to the power of the spectrum of defensive mechanisms employed by cells both acutely and over evolutionary time. The default acute mechanism employed by cells to silence unwanted or dangerous genes is the modification of deoxycytosine in DNA by methylation. This modification also facilitates the addition of suppressive histone H3 K4-dimethyl marks to keep these loci transcriptionally silent. In this context, it is interesting to note that many of the drugs notorious for causing "drug-induced lupus", such as hydralazine and procainamide, are demethylating agents. Experimentally, 5-aza-deoxycytosine can also be used to reduce the methylation of the genome. This also causes an increase in the expression of L1 and many HERVs. Ultraviolet light (UVB) also reduces genomic methylation, likely with the same de-repression of retroelement transcription. UVB is also a well-recognized trigger of lupus flares.

A good example of retrotransposon control is seen with the large number of interrupted retrotranspositions of L1 in which the reverse transcription was terminated before it reached the 5' end. As a result of this, many L1 copies lack portions of ORF1 or the 5'UTR regulatory region and cannot retrotranspose. Many of the mutations seen in HERVs and L1 may have been deliberately introduced by the APOBEC family (146) of IFN-inducible cytidine deaminases, which recognize viral or retroviral sequences and rapidly introduce mutations into them (147). This mechanism has been shown to be effective at reducing the virulence of new retroviruses (148, 149) and the ability of retrotransposons to replicate (150).

Another example is Moloney leukemia virus 10 (MOV10), an ATP-dependent helicase that unwinds L1 RNA during reverse transcription to reduce the retrotransposition (151–153) process in a somewhat unclear manner (95). It also participates in the defense against retroviruses (154). MOV10 is located in the macromolecular complex of RNA-binding proteins that also includes L1 ORF1p and ORF2p, as well as SLE autoantigens Ro60 and La (89–91).

There are numerous additional cellular mechanisms to counteract each step of the retrovirus life cycle and the retrotransposition of repetitive elements (155). Many of these mechanism also serve as defenses against other types of viruses and many of them were uncovered in the course of HIV research (98). It is presently not known if any of these mechanisms are compromised in patients with SLE or other autoimmune conditions.

RNA Interference, Argonaute, Piwi, and Other Nucleic Acid-Based Defenses

Many prokaryotes employ an interesting defense mechanism in which short pieces of the genetic material of past pathogens are kept in a region of the genome to serve as recognition modules for the defense against reinfection. This mechanism (known as CRISPR/Cas9) has an RNA-based counterpart in eukaryotes, including humans, in the form of RNA interference mechanisms that utilize retrotransposon-derived miRNAs, piRNAs, and potentially antisense transcripts from HERVs. Particularly, the piRNA/Piwi pathway appears to be important for protecting the integrity of the germline genome (156). One of the effectors of these still incompletely understood pathways is Z-DNA binding protein-1 (ZBP-1) (157), also known as DAI, which binds dsRNA or DNA that adopt the Z-conformation. ZBP-1 then activates both the IRF3 pathway for type I IFN production and the RIP3 kinase pathway that triggers cell death by necroptosis (158). The relevance of ZBP-1 in Crohn's disease (159) and other inflammatory conditions (158) was recently demonstrated.

MOLECULAR MECHANISMS BY WHICH ENDOGENOUS RETROVIRUSES AND RETROTRANSPOSONS COULD CAUSE IMMUNE PATHOLOGY LEADING TO AUTOIMMUNITY

A number of mechanisms have been proposed over the years for how HERVs or L1 could cause diseases like cancer and autoimmunity. In cancer, it is thought that some combination of active retrotransposition catalyzed by L1 ORF2p (160), genomic recombinations caused by highly similar repetitive sequences (*e.g.*, LTRs), or promoter/enhancer effects of HERV LTRs or L1 5' UTRs can create gross chromosomal abnormalities, tumor suppressor disruptions, and loss of

normal transcriptional control. In autoimmunity, other aspects of HERV and L1 biology are probably more relevant.

It should be stated upfront that despite the many genetic, experimental, and supportive correlative findings, it is still possible that none of the myriad of HERV and L1 sequences that constitute at least 25% of our genome (close to 40% if one includes Alu elements and other SINEs) have any role at all in autoimmunity because they have been sufficiently "domesticated" and have lost their immunogenicity and potential to raise an anti-viral response or to skew biological processes in any meaningful way. That said, there are many plausible aspects to the general hypothesis that HERVs and/or L1 can promote or even trigger autoimmunity (17, 161, 162). We believe that the vast majority of retroelements are harmless, some even beneficial (17), but that a select few are dangerous and participate in the pathogenesis of common autoimmune diseases like SLE or RA by mechanisms that are discussed below.

Autoantibodies Against Retroviral Proteins in Autoimmunity

In the 1990s, a number of researchers made the surprising discovery that serum immunoglobulins from patients with RA, SLE, or other autoimmune diseases, reacted with Human Immunodeficiency Virus (HIV) proteins, *e.g.*, p24 of the HIV capsid (163–166), even if these patients had never encountered the virus. Such HIV-reactive antibodies were found in exceedingly few healthy subjects, but reportedly in up to 60% of RA patients. A likely answer to this conundrum was provided by the subsequent discovery (167) that endogenous retroviruses in the human genome, particularly HERV-K (168), are transcriptionally activated in some RA patients (46, 169). This raised the possibility that HIV-reactive antibodies in patients are, in fact, antibodies against HERV proteins that have a sufficient degree of sequence homology with HIV proteins. Indeed, two papers (170, 171) reported that 16% of RA patients have antibodies against an epitope in the HERV-K envelope protein (amino acids 19-37). It should be noted that the percentage of positive patients in the earlier papers was higher, presumably because the tested antigens were full-length proteins in their native state, while later papers mainly used selected peptides and therefore may have missed many autoantibodies.

We have replicated the detection of elevated IgG autoantibodies against HERV-K Env proteins (not peptides) in RA patients (submitted for publication). These antibodies were also present in pediatric patients with juvenile idiopathic arthritis (JIA), and they were higher in smokers than in non-smokers. Anti-Env antibodies were also detected in some control (i.e. non-RA) individuals, in SLE patients (172), and in patients with breast cancer (173, 174) and other hormonally driven cancers. Several HERV-K loci are reportedly transcriptionally active in these cancers (175–177), perhaps through the action of sex hormones on the HERV-K LTRs (48). Notably, RA is a female-biased disease with a 4:1 female-to-male ratio. Autoantibodies have also been reported against proteins of HERV-E, particularly the Gag protein of HERV-E clone 4-1 (178). We would not be surprised if it was found that patients have autoantibodies against additional HERV proteins.

Could Autoantibodies Against Retroviral Proteins Be Directly Pathogenic?

The presence of anti-HERV autoantibodies in patients with autoimmune diseases prompted many researchers to wonder if HERVs play a role in autoimmunity. One proposed mechanism to connect these autoantibodies to autoimmune disease was the hypothesis of "molecular mimicry", postulating that the relevant epitopes for these anti-HERV autoantibodies may be sufficiently similar to amino acid motifs in self-proteins to cross-react with such *bona fide* autoantigens and result in autoimmunity. In our view, this hypothesis (which has also been proposed for exogenous viruses) seems rather unlikely and it is not supported by patient-based data. While a few instances of three to four identical amino acid residues can be found in HERV proteins and proteins like collagen or IgG, these were not shown to be epitopes for autoantibodies. This hypothesis also assumes that retroelement proteins are not "self", in contrast to proteins encoded by traditional genes, and that humoral or cellular immunity only against the latter could be pathogenic. In addition, central tolerance against the relevant epitopes in traditional self-proteins should automatically also prevent the same sequence from being immunogenic when present in a different class of self-proteins, *e.g.*, HERVs.

Another simple hypothesis focuses on the plasma membrane location of Env proteins. When expressed, these transmembrane glycoproteins cluster into microdomains together with intracellular Gag to form virions that eventually bud off to leave the host cell. During this time, autoantibodies against Env would be predicted to bind the exposed Env with potential consequences like complement fixation or antibody-dependent cellular cytotoxicity (**Figure 3**). In both cases, the cells expressing Env can be killed under circumstances that would be pro-inflammatory. In this scenario, inflammation would follow the same pattern as in antiviral immunity, except that the offending virus is a HERV and the response would be that of autoimmunity. Since the HERV in question is irrevocably fixed in the host genome, it cannot be eradicated by the immune response and any cell that subsequently expresses Env would be treated as a virally re-infected cell by a recall immune response. This could well result in a pattern akin to what we see in clinically relevant autoimmune disorders.

Could Protein Citrullination be Linked to Retroviral Proteins?

Among the autoimmune diseases, RA is unique in that post-translational deimination of arginine residues, also known as citrullination, plays an important role in creating autoantigens. While a low amount of citrullination is a part of normal physiology, much elevated levels of citrullination of proteins that perhaps never are citrullinated at all in healthy individuals can be induced by a process termed "lethal hypercitrullination" or "leukotoxic hypercitrullination" (179, 180). This reaction is induced by any agent that creates pores in the plasma membrane that are large enough to allow Ca^{2+} to rush into cells, such as the membrane-attack complex of activated complement, polymerized perforin from cytotoxic T cells or NK cells, or

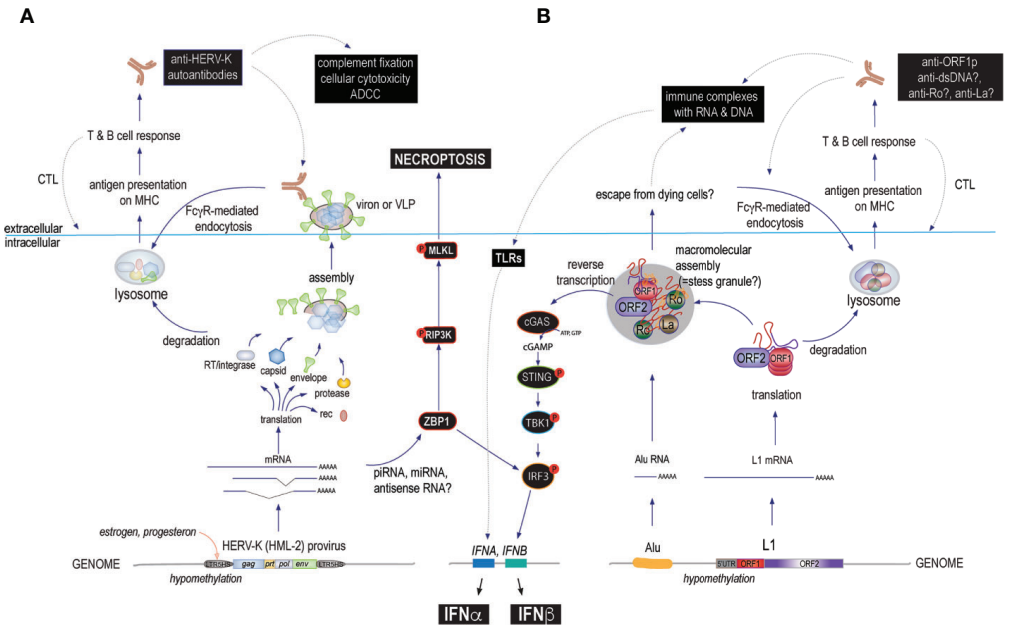

FIGURE 3 | The main proposed mechanisms by which HERVs and L1s may cause immune responses that could escalate to autoimmune disease if they become chronic or recurring. **(A)** starting at the bottom (intracellularly): environmental or internal factors first reduce the suppressive DNA methylation of 5' LTRs allowing transcription factors such as those regulated by female hormones to actively transcribe one or several HERV loci. The resulting transcripts are spliced and processed and some of them are translated into proteins, while others can associate with antisense RNA, small interfering RNAs of the miRNA or piRNA classes, or form internal loop structures that are recognized by ZBP-1 (or other sensors or RNase-based enzymes), which can signal through IRF3 to IFN production or, alternatively, *via* the RIP3 kinase pathway to cell death by necroptosis. HERV proteins can assemble into more or less complete virions that may remain exposed on the cell surface or even bud off as mature virions. These proteins can be degraded and processed for antigen-presentation on class I or II MHC molecules to activate T cells and B cells to generate both cellular and humoral immunity. Both arms target cells expressing the relevant HERV for immune attack. **(B)** L1 may drive a similar immune response, except that the ORF2p RT can generate intracellular DNA to directly trigger cGAS or other DNA sensors resulting in type I IFN production (primarily IFNβ). Another unique feature of L1 biology is the assembly of the two L1 proteins with other RNA binding proteins, many of which are well-known autoantigens, into aggregates that also contain L1 RNA and other RNA species, such as Alu element RNA or processed mRNAs. These bodies may also contain DNA newly synthesized by the ORF2p RT and hence will even more resemble virions if they are released from dying cells. This would result in an immune response and autoantibodies against several of their components, such as Ro60, in addition to the L1 proteins themselves. Lastly, such autoantibodies would further promote the uptake of L1 protein/RNA/DNA complexes by FcγR-expressing immune cells, including plasmacytoid dendritic cells and further stimulate type I IFN production (primarily IFNα) through TLR pathways. In all these scenarios, the repeated boosting of anti-HERV or anti-L1 immune responses would lead to increasingly powerful immune-mediated destruction of the cells that express them. Disease-relevant pathways are indicated with black boxes.

certain bacterial toxins like α-toxin from *Staphylococcus aureus*. Among immune cells, neutrophils are the most prone to undergo a strong hypercitrullination reaction (180). Hence, if the neutrophils express Env in an RA patient with anti-Env autoantibodies, these cells could well be killed by complement or by Env-specific cytotoxic T cells. This hypothetical model of neutrophil killing and hypercitrullination linked to anti-HERV immunity is under investigation in our laboratory.

Autoantibodies Against Retrotransposon Proteins and Associated Proteins

We recently reported that SLE patients also frequently have autoantibodies against the L1-encoded ORF1p protein (181), which is physically associated with Ro, La, snRNP70, and other well-known SLE autoantigens (87, 88, 91, 182) together with RNA in macromolecular assemblies (which may be stress-granules). Indeed, SLE autoantibodies recognized several other proteins in purified ORF1p-containing complexes (181). Furthermore, anti-ORF1p titers correlated with SLE disease activity, lupus nephritis, anti-dsDNA levels, and complement consumption (181). They were also present in pediatric lupus patients with newly diagnosed disease (our unpublished observation). In contrast, RA patients were negative for anti-ORF1p antibodies with only sporadic exceptions.

At this time, we do not know whether anti-ORF1p autoantibodies are pathogenic *per se*. Because ORF1p is intracellular, and hence out of reach for extracellular antibodies, we are inclined to believe that they do not contribute directly to pathology in SLE and merely reflect the expression of immunogenic ORF1p. However, it is likely that they form immune complexes with extracellular ORF1p, which could escape from dying or broken cells (**Figure 3**). If so, intact ORF1p likely would have bound RNA and exist in a complex with Ro60, La, and other proteins that it associates with in the cells. Such immune complexes could induce type I IFNs upon internalization by plasmacytoid dendritic cells and could promote antibody responses against all the proteins and nucleic acids in these immune complexes. In support of this

possibility, isolated anti-Ro60 immune complexes from SLE patients were shown to contain RNA from both Alu elements and L1 (182).

Direct Pathogenic Effects of Proteins Encoded by HERVs and Retrotransposons

After the initial discovery of autoantibodies reactive against HERV-encoded polypeptides in the 1990s, researchers began to search for the corresponding mRNAs and proteins in a variety of cells and tissues, such as RA synovium (183, 184), initially using degenerate primers and little insight into the shear multitude of retroviral and retrotransposon sequences in our genome. With the sequencing of the human genome (12), the complexity of the issue became more obvious. To this day, there are relatively few commercially available reagents to detect HERV or L1 proteins and only a limited number of computational tools to analyze retroelement transcription profiles in RNA-Seq data sets. Furthermore, it is now clear that large numbers of retroelement loci are transcribed even in healthy individuals (59), including many that probably do not encode for proteins or only for short peptides if translated at all.

The presence of autoantibodies against HERV and L1 proteins suggests that they are at least moderately immunogenic. An important unanswered question is to what extent retroelement-encoded polypeptides are expressed in the thymus and bone marrow during T and B cell antigen receptor repertoire selection and the formation of central tolerance. From the acute use of extensive DNA methylation to silence the transcription of unwanted and potentially dangerous sequences, we project that the youngest HERVs are least likely there and that tolerance against many of them is weak or absent.

In agreement with this notion, a recent paper (185) demonstrated that pancreatic islets in non-obese diabetic (NOD) mice (but not control mice) release microvesicles, which contain endogenous retroviral Gag and Env proteins, probably in the form of complete or near-complete virions. The NOD mice developed antibodies against these proteins, as well as specific T cells, which caused diabetes when adoptively transferred. Elimination of Gag prevented diseases. These data show that abnormal activation and expression of endogenous retroviruses can trigger an anti-retroviral immune response and autoimmunity (185). This scenario is also depicted on the left side of **Figure 3**.

Besides acting as antigens for the host immune response, the proteins and peptides encoded by HERVs and L1 have other properties that could be relevant (17). For example, retroviral Env and Gag proteins can mediate cell fusion events, while mature and processed Env of HERV-K, which form transmembrane trimers, binds to heparan sulfate-containing surface proteins (186). Hence, aberrant expression of these proteins may cause pathological fusion and adhesion events that could prove problematic. In addition, certain Env portions may have immunomodulatory effects.

The "superantigen" encoded by the *env* gene of HERV-K18, which was incorporated into the human genome 7.8–14.4 million years ago and has accrued a number of amino acid substitutions in its *env* gene, made a big splash in the field when its T cell activating properties and presence in type 1 diabetes patients was first reported in *Cell* in 1997 (187). The proposition was that this protein causes a polyclonal T cell activation, which then leads to autoimmunity. Subsequent papers were not in full agreement (188) and despite subsequent papers finding this "superantigen" expressed in JIA patients (189) and that it is inducible by Herpes viruses (190), the interest in HERV-K18 slowly waned. Nevertheless, the lesson from this specific case is that random mutations may not only reduce the ability of a retroviral component to cause pathology, but may also, by chance, give them new and dangerous properties. Certain sequences in the Env protein also appear to have immunosuppressive properties. How such motifs might act and whether they were important for the life-cycle of free infectious HERV-Ks are not known.

The L1-encoded ORF1p and ORF2p proteins have been detected in samples from patients with SLE or pSS (74) by immunoblotting and immunohistochemistry. ORF1p was present in kidney biopsies from lupus nephritis patients and in salivary gland from pSS patients. This staining coincided with IFNβ in glandular cells and with IFNα in the infiltrating immune cells. As activation of L1 elements in autoimmune patients (74–76) appears to involve demethylation of the 5' UTR (74, 76, 78), these authors also analyzed the methylation of CpG sites in this regulatory region and found it to be reduced in patients with elevated L1 expression. L1 ORF2p is also present in the ductal cells of salivary gland biopsies from patients with pSS (191).

We have detected ORF1p protein in neutrophils of juvenile and adult SLE patients, including in low-density granulocytes from these patients (submitted for publication). These findings are compatible with the emerging role of the neutrophil as a cell type of interest in SLE pathogenesis.

Could Transcripts From Proviruses and Retroelements Be Pathogenic?

While primary transcripts from endogenous retroviral or retrotransposon loci are synthesized by the same machinery that transcribes and processes other genes and therefore should be indistinguishable from any other cellular transcripts, two recent papers published in *Nature* (158, 159) showed that they have a propensity to form double-stranded structures, perhaps through transcription of the complimentary strand as well. They found that such dsRNAs are recognized by the host defense protein ZBP-1, which binds both DNA and RNA in their Z-conformation. This binding activates ZBP-1 to trigger type I interferon production through IRF3, as well as activation of the RIP3 kinase pathway leading to cell death by necroptosis. Necroptosis, in turn, is a very immunogenic process and leads to autoimmune disease (158, 159).

Another possibility is that some species of retroelement RNA may have features or motifs that somehow resemble viral RNA and therefore are recognized by the RNA-sensors RIG-I or MDA5 (192), a challenging task given the abundance of cellular RNA species. Antisense sequences (transcripts from the other DNA strand) could provide the answer. The delicate

balance between the recognition of self- versus foreign RNA is well illustrated by the *IFIH1* A946T allele, which encodes a variant of MDA5 that enhances anti-viral immunity, but increases the risk of autoimmunity (193, 194).

It is curious that some retroelement RNAs like Alu transcripts need to be edited by adenine deimination catalyzed by ADAR1 to remain harmless (195). In the absence of this editing (*e.g.*, loss of ADAR1), these RNAs form double-stranded hairpin loops that trigger the RNA sensors leading to AGS (196). There is also evidence that some 30% of SLE patients have constitutively activated RNA sensors, detectable as an aggregation of the downstream mitochondrial antiviral signaling (MAVS) adaptor protein (197). It is not known what RNA species was responsible for this activation in the patients.

Reverse Transcriptases, DNA Sensors, and Type I Interferons

A mechanism with clear potential for pathogenicity is the conversion of retroelement RNA into extrachromosomal DNA by reverse transcription. If not rapidly degraded by the DNase TREX1 (or other DNases), such aberrant DNA will trigger DNA sensors like cGAS, which in turn drive the production of type I IFNs. This is apparently what happens constitutively in patients with loss-of-function mutations in *TREX1*. It also appears to occur in at least a subset of SLE patients: the second messenger 2'3'-cyclic-guanosine-adenosine-monophosphate (cGAMP), which is synthesized exclusively by cGAS upon DNA binding, was detected by mass spectrometry in 7 of 30 SLE patients (198). While it may seem that this represents a small portion of SLE patients, it is important to recognize that the data represent a single snap-shot in time for each patient and that cGAMP is a short-lived second messenger present in minute quantities. Thus, it may well be that cGAMP is periodically elevated in many more SLE patients than reported.

While the DNA species that triggers cGAS in SLE patients remains unknown, there are only two likely possibilities, as we have discussed before (11): mitochondrial DNA or DNA synthesized by a cellular RT. There are only three types of RTs in our genome: telomerase (*TERT*), the RTs encoded by the *pol* genes of HERVs, and ORF2p encoded by L1. Telomerase only synthesizes TTAGGG repeats in the ends of our diploid chromosomes using the *TERC* RNA template (199, 200), while retroviral RTs supposedly only convert the RNA genome of an incoming retrovirus to a DNA provirus that is inserted into the genome during acute infection. Hence, L1 ORF2p is the most likely to produce DNA that can trigger type I IFN production through cGAS activation in SLE patients. ORF2p has robust RT activity (139, 140, 201), which is key for retrotransposition (202), and is sensitive to some clinically used RT inhibitors (203, 204).

Possible Roles of Genomic Alterations Resulting From Retrotransposition in Autoimmunity

Lastly, a unique potential mechanism by which retrotransposons, and perhaps also HERVs, could impact human health is by retrotransposition, *i.e.* by inserting a brand-new reverse-transcribed copy into a new genomic location. This can occur early in embryogenesis (205) when the genome is broadly hypomethylated and extensively transcribed, including retroelements of all classes. During this time, RT activity is high, extrachromosomal DNA is readily detectable, and L1 elements are capable of active retrotransposition (206). In fact, more than a hundred novel genetic diseases have been found to be caused by L1 retrotranspositions into vital genes (207), disrupting their regulation or function. It is also clear that active L1 retrotransposition occurs in certain neurons during development of the central nervous system (208) and that this creates somatic mosaicism of unclear neurological relevance (209). It is also possible that similar L1 retrotranspositions could occur in immune cells to generate T cells with abnormal behavior, leading to autoimmunity. One could imagine that the disruption of the gene for an important negative regulator of immunity, for example in a hematopoietic stem cell, could result in populations of overly reactive T cells. While this is an interesting possibility, there is no evidence of it at this time other than in cancer.

A different type of genomic alterations also occurs in malignant cells, namely the recombination of retroelement loci that have a high degree of sequence identity but located in different chromosomes. The large number of single LTRs ("solo-LTRs") throughout our genome appear to be the result of such recombinations. Again, there is no evidence for this type of genomic alterations in human autoimmunity, but it might be worthwhile to search for them.

Malignancy-Related Autoimmunity

An immune response against a tumor involves the recognition of tumor-specific epitopes, which consist of point-mutations in common self-proteins, aberrantly spliced or modified proteins, or proteins that normally are only expressed during early embryonic development (*i.e.* carcinoembryonic antigens). Particularly when the immune response against the tumor is strong and succeeds in eliminating the malignant cells, there is an obvious risk of epitope spreading and further escalation into autoimmunity. There are numerous examples of such "paraneoplastic" syndromes and collateral damage, for example vitiligo (i.e. the killing of normal melanocytes) in patients with melanoma after a successfully boosted anti-tumor immune response.

Carcinogenesis typically involves a broad genomic demethylation and de-repression of many genes, including carcinoembryonic antigens, as well as numerous HERVs and L1s. It therefore seems very likely that proteins encoded by these retroelements serve as tumor-specific antigens. This notion is supported by the presence of anti-HERV-K autoantibodies and HERV-K-specific T cells in breast cancer (173, 174). If this response is successful and eradicates the (pre)malignant cells, the immunological memory of these antigens would readily serve to re-engage the immune system if the same proteins were expressed again in a non-malignant cell type. There is currently no clear evidence that patients with autoimmune

disease would have fought off a malignancy prior to developing their autoimmunity, but this possibility should be explored if possible.

TESTING THE HYPOTHESES

Resolving the question of whether HERVs and/or L1 retrotransposons contribute to human autoimmune diseases is a monumental challenge. Ultimately, only significant effects of therapeutic interventions in double-blinded, placebo-controlled human clinical trials will be able to conclusively prove causalities that have been inferred from molecular mechanistic experiments, correlative patient observations, and, perhaps, animal models. Disproving a proposed mechanism is even harder.

Regarding animal models, due to the approximately 80–100 million years of evolutionary distance between mice and humans, there are fundamental differences between our repertoires of retroviruses and retrotransposons. Most of the human HERVs are more recent, and do not exist in the mouse genome. Instead, mice still have many fully competent retroviruses, such as mouse mammary tumor virus (MMTV), which is distantly related to the HERV-K family. Disease models based on MMTV would be difficult to interpret. It is very interesting to note, however, that the autoimmunity-prone strains of mice (e.g. MRL, or NZB) have larger sets of active endogenous retroviruses than other strains. Mice also have numerous L1 insertions, albeit not quite as many as humans.

It will be important to construct a set of plausible molecular hypotheses of pathogenesis and to test them for supportive patient-based evidence, or, conversely, lack thereof. Since many diseases like SLE are clinically heterogeneous, one cannot assume that the molecular mechanisms that underpin them will be the same in every patient. Instead, it might be more productive to start with the assumption that each disease contains two or more "endotypes", *i.e.* patients with a clinically similar disease, but with distinct molecular underpinnings. This concept is well accepted in oncology and is making inroads in respiratory diseases, where asthma is now understood to contain "eosinophilic asthma", "Th2-high asthma", "epithelial asthma", and "allergen/IgE asthma", each of which respond well to different targeted therapies. These endotypes of asthma are not possible to distinguish clinically, but require laboratory measurements of eosinophils, cytokines, or IgE to classify and treat.

To advance, and eventually elucidate, the role of HERVs and retrotransposons in human autoimmunity, some major tasks that should be undertaken include:

- A comprehensive cataloguing of the expression of HERVs and retrotransposon transcripts that are differentially expressed in cells and tissues from a range of autoimmune and other diseases, with an emphasis of disease-to-disease comparisons, and a filtering of the data sets by the capacity of transcripts to be translated.
- A broader and more detailed characterization of which proteins encoded by HERVs and L1 that become targets of autoantibodies in patients with different autoimmune diseases.
- The use of high-sensitivity proteomics to determine when and where such proteins are present, including an analysis of whether they are expressed in the thymus.
- The identification of T cell epitopes on retroelement proteins and characterization of the relevant T cells.
- Identification of the exact nature and source of nucleic acid species that drive type I IFNs in patients with type I IFN gene signature.
- An evaluation of the roster of anti-retroelement defense mechanisms to identify disease-related deficiencies or abnormalities in patients.
- A thoughtful selection of testable drug targets based on plausible mechanisms, followed by the development of therapeutic molecules that will enable clinical trials with relevant pharmacodynamic measures (biomarkers) to ensure that the drugs have the desired biological impact before asking if they have therapeutic effects.

THERAPEUTIC IMPLICATIONS

Currently used therapeutic regimens for autoimmune conditions consist of more or less broadly immunosuppressive drugs, which often provide unsatisfactory efficacy and/or unacceptable safety concerns. The development of more efficacious drugs with more precision and therefore, hopefully, improved safety profiles is very challenging as long as our understanding of the molecular mechanisms that initiate and propagate these diseases is so incomplete. We anticipate that scientific discoveries and breakthroughs in the coming years will open up new avenues for the development of better new drugs (210). If some of the hypotheses discussed in this review have any merit, what might new drug targets look like?

An already tested opportunity is the direct inhibition of type I IFNs, which appear to be instrumental in the pathogenesis of SLE and related diseases (111, 211). These drugs have been both encouraging and disappointing. Anifrolumab, an antibody that blocks the type I IFN receptor (IFNAR1) used by all type I IFNs, met with a statistically significant efficacy in phase 2 clinical trials in SLE (212) and met its primary endpoint in one of two phase 3 trials. In contrast, two different antibodies that block IFNα alone (213), sifalimumab and rontalizumab, were efficacious only in a small subset of SLE patients. Together, these trials reveal that type I IFNs beyond the 13 isotypes of IFNα are important, at least in some patients. Furthermore, although anifrolumab was clinically more efficacious, and neutralized the IFN signature by over 90% in the treated patient population, it did not benefit all the included patients.

Inhibitors of protein kinases that mediate the signaling from IFNAR, such as the JAK-family kinase TYK2, or signaling pathways that lead to type I IFN production, like TBK1, or the

necroptosis-inducing RIP3K, could also prove therapeutic even if these pathways are in broader use. Another possibility is to intervene in the biology that produces the nucleic acid species that initiate type I IFN production. Because anti-viral immune responses that include production of type I IFNs also include many additional pathways (although many of them amplified by IFNs), such therapeutics may be more efficacious than IFNAR blockade. Drugs that stimulate DNases or RNases, augment their function, or prevent their negative regulation, or that inhibit the enzymes that produce the offending DNA or RNA, e.g. reverse transcription, could be therapeutic. Indeed, RT inhibitors are efficacious in IFN-driven systemic inflammation observed in the *Trex1*$^{-/-}$ (a DNase) mouse model of AGS (143) and reduce the IFN signature in patients with AGS (145). RT inhibitors also eliminated all symptoms of the pSS-like diffuse salivary and lacrimal gland inflammation in HIV patients in a small clinical trial (214). It will be interesting to see if RT inhibitors would also benefit SLE patients.

The DNA- or RNA-sensors that are activated by aberrant or excessive nucleic acid species could also be targeted by inhibitors. Indeed, c-GAS inhibitors are under development in a growing number of companies, which is also true for drugs targeting RIG-I or MDA5. An obvious risk with these drugs is that they can compromise anti-viral immunity. This is also true for antibodies that block type I IFNs yet appears to be manageable.

As we learn more about the immunogenicity of retroelement proteins and how various retroelement transcripts act to trigger ZBP-1 or RNA sensors, additional drug targets likely emerge. However, it currently seems that most of the transcriptional regulation and transcript processing of retroelements is mediated by the same cellular factors that regulate and process our traditional protein-coding genes. Retroviral LTRs, for example, use a host of transcription factors that regulate numerous other genes as well. Nevertheless, it is possible that RNA-based therapeutic molecules could be designed that more selectively interfere with pathogenic sequences. This space is still totally unexplored.

DIAGNOSTIC AND PROGNOSTIC IMPLICATIONS

The heterogeneity in clinical manifestations of autoimmune diseases (215) often make them challenging to accurately diagnose and properly treat, and to determine their prognosis with any precision. This challenge has prompted the development of various high-sensitivity and -specificity clinical and laboratory classification criteria and disease activity indices to help in the management of patients with these diseases. However, while these methods are reasonably objective and helpful in clinical trials, there is wide variability in their applicability owing to the often-seen discordance between laboratory evidence of immunologic activity and actual physical evidence of clinical disease activity.

This discordance between laboratory and clinical disease activity leaves plenty of room for improved or adjunctive diagnostic methods that could help in closing that gap, and perhaps also help to improve the prognostication of the diseases relative to treatment. Given the significant correlations of anti-HERV-K Env antibody with RA disease activity (170, 171) (also our unpublished data) and anti-L1 ORF1p antibody with SLE disease activity (181), the potential for their use as diagnostic and prognostic markers is not too far-fetched. By the same token, with regard to potential surrogate markers for innate immune system activity in disease flares (i.e. type I IFN production in SLE), a similar approach could be undertaken whereby IFN signature gene expression is routinely monitored. However, we understand that these principles are still experimental and thus require more investigations for the validation of their actual clinical utility.

AUTHOR CONTRIBUTIONS

TM and KU contributed equally to the writing of this review and share accountability for its content. All authors contributed to the article and approved the submitted version.

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GLOSSARY

Autoantibody	antibody reactive with an antigen encoded by the host's genome, including retroelements.
DNA transposon	genomic sequences that can move to new locations in the genome without a reverse-transcription step.
HERV	human endogenous retrovirus, the genomic sequence (in its current form) of an originally free retrovirus that infected our ancestors and inserted its reverse-transcribed genome into the germline of their host. HERVs are inherited in a Mendelian fashion. The HERVs have accumulated genetic alterations, such as point mutations, small or large deletions, insertions, etc in a time-dependent manner.
LTR	long terminal repeat, the approximately 650-bp region of the retroviral genome that exerts transcriptional control. The LTR contains numerous transcription factor binding sites and the transcriptional initiation site, but is normally silenced by DNA methylation and histone modifications and other epigenetic means. There is a single LTR in the circular RNA genome of a free retrovirus, but during reverse-transcription for genomic insertion, the LTR is reverse-transcribed twice, ending up in the 5' and 3' ends of the insert.
Retroelement	general term for all genomic sequences that can be transcribed into RNA, reverse-transcribed to DNA, and then reinserted into a new location of the genome. Endogenous retroviruses are LTR-retrotransposons, while other retrotransposons are not.
Retrotransposon	essentially a synonym of retroelement, but is often used for non-LTR retroelements.
Provirus	the genomic cDNA for a retrovirus, endogenous or exogenous.
Solo-LTR	as a result of annealing between the two identical LTRs of a retroviral provirus followed by excision repair, only one LTR sequence is left in the genome. An insertionally polymorphic HERV may be present in the human genome as a provirus, a solo-LTR, or be absent. In the latter case, the location is referred to as "pre-integration" sequence.
Reverse transcriptase	the enzyme that synthesizes a DNA strand complementary to a single-stranded RNA template. Upon completion, an RNase domain of the reverse-transcriptase degrades the RNA template, allowing the reverse transcriptase to synthesize a second complementary DNA strand.

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REVIEW
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Epstein-Barr Virus and Multiple Sclerosis

Gunnar Houen[1,2]*, *Nicole Hartwig Trier*[2] *and Jette Lautrup Frederiksen*[2,3]

[1] Institute of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark, [2] Department of Neurology, Rigshospitalet, Glostrup, Denmark, [3] Institute of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark

Multiple sclerosis (MS) is a neurologic disease affecting myelinated nerves in the central nervous system (CNS). The disease often debuts as a clinically isolated syndrome, e.g., optic neuritis (ON), which later develops into relapsing-remitting (RR) MS, with temporal attacks or primary progressive (PP) MS. Characteristic features of MS are inflammatory foci in the CNS and intrathecal synthesis of immunoglobulins (Igs), measured as an IgG index, oligoclonal bands (OCBs), or specific antibody indexes. Major predisposing factors for MS are certain tissue types (e.g., HLA DRB1*15:01), vitamin D deficiency, smoking, obesity, and infection with Epstein-Barr virus (EBV). Many of the clinical signs of MS described above can be explained by chronic/recurrent EBV infection and current models of EBV involvement suggest that RRMS may be caused by repeated entry of EBV-transformed B cells to the CNS in connection with attacks, while PPMS may be caused by more chronic activity of EBV-transformed B cells in the CNS. In line with the model of EBV's role in MS, new treatments based on monoclonal antibodies (MAbs) targeting B cells have shown good efficacy in clinical trials both for RRMS and PPMS, while MAbs inhibiting B cell mobilization and entry to the CNS have shown efficacy in RRMS. Thus, these agents, which are now first line therapy in many patients, may be hypothesized to function by counteracting a chronic EBV infection.

Keywords: Epstein-Barr virus, multiple sclerosis, immune evasion, central nervous system, chronic infection, relapsing-remitting

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***Correspondence:**
Gunnar Houen
gunnarh@bmb.sdu.dk;
gunnar.houen@regionh.dk

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INTRODUCTION

Multiple sclerosis (MS) is a disease affecting the central nervous system (CNS), with inflammation and demyelination of nerves, eventually resulting in nerve damage and disabilities. MS can take different courses, most often in the form of relapsing-remitting (RR) cycles of disease activity or more rarely as a primary-progressive (PP) disease. RR MS can progress over many years and may eventually develop into a secondary-progressive (SP) disease (1–3).

Initial symptoms of MS are often recorded as solitary symptoms, i.e., a clinically isolated syndrome in the form of optic neuritis (ON) or other neurological disturbances isolated in time and space (1–4). Diagnosis of MS relies on the so-called McDonald criteria, latest updated in 2017 (5). These criteria include detection of active inflammatory foci in the CNS as seen by positron emission tomography (PET) and magnetic resonance imaging (MRI) and intrathecal production of

immunoglobulins (Igs), measured as an elevated cerebrospinal fluid (CSF)/serum IgG index, as a free light chain index or as the occurrence of oligoclonal bands (OCBs) of IgG in CSF (6–10). Each oligoclonal band is a result of intrathecal antibody (Ab) synthesis by single B cell clones and therefore, specific CSF/serum Ab indices (AIs) may also be elevated, e.g., Abs to various viruses, corresponding to the specificity of some of the OCBs (11–15). Accordingly, the OCB Abs show evidence of antigen (Ag) exposure, somatic hypermutation and affinity maturation (16–19).

Differential diagnoses for MS are neuromyelitis optica (NMO) and major oligodendrocyte glycoprotein (MOG) Ab-associated demyelinating disease, but other diseases may also mimic some aspects of MS, including acute disseminated encephalomyelopathy (ADEM), CNS neoplasms and various other diseases with the potential to affect the CNS (20–22).

Therapy of MS was previously mainly empirical and relied on several low molecular weight (LMW) drugs, including glatiramer acetate, teriflunomide, dimethyl fumarate, fingolimod, cladribine and others, however, biological drugs have been introduced for treatment of RRMS, including beta-interferon and several therapeutic monoclonal Abs (MAbs) (23–25). Especially the array of MAbs approved for MS treatment has expanded and currently range from Natalizumab, an integrin α4β1/α4β7 MAb, Alemtuzumab, a CD52 Mab, to MAbs targeting the B cell surface marker CD20 (Rituximab, Ocrelizumab) (25–28). Most interestingly, the latter have been found to have an effect also on PPMS (27, 28).

MS ETIOLOGY AND EPIDEMIOLOGY

No consensus about MS etiology exists at present and theories range from idiopathic loss of self-tolerance, over molecular mimicry to chronic virus infections. However, it is generally accepted that MS involves a combination of genetic predisposing factors and environmental influences (29–34). MS has a female preponderance, which most likely is due to genetic factors and incidence is highest after puberty, which may be ascribed to either genetic or environmental factors or both.

Genetic factors influencing development of MS are in particular major histocompatibility class II (MHC II) alleles, of which some increase susceptibility (e.g., human leukocyte antigen (HLA) DRB1*15:01), while others decrease susceptibility. Likewise, some MHC I alleles also appear to be protective (e.g., HLA A*02.01), while others increase susceptibility. Overall, more than 100 genes have been found to have an influence on development of MS, of which most are involved in immune system functioning and in particular lymphocyte and Ab functioning (1–3, 29–40).

Environmental factors with an impact on MS incidence include sunlight exposure/vitamin D (vitD) deficiency, dietary and other compounds, smoking and some virus infections [e.g., Epstein-Barr Virus (EBV)] (30).

MS is most prevalent on the Northern hemisphere, a finding which can most likely be related to the intensity of sun light, which may in turn be explained by levels of vitD synthesis. Actually, vitD concentrations have been found to be correlated with MS incidence/prevalence (39, 41–43).

Smoking increases the risk of MS, but some other uses of tobacco may actually reduce the risk of MS (30, 44–46). Other environmental compound exposures have been found to have an effect om MS susceptibility (30) and recently, propionic acid and the composition of the intestinal microbiota has been reported to influence or be influenced by MS (47–49).

Obesity, especially in adolescence has been reported to have an effect on MS susceptibility, but it is unclear whether this may be attributed to genetically determined factors or environmental/socio-economical influences or a combination of different effects, e.g., a low-grade neuro-inflammatory effect or a vitD-sequestering effect (50–53).

Virus infections have for long been suspected to be involved in MS development (29–32, 54–56). Most investigations have focused on EBV, which remains the most likely candidate for a causative virus, but other viruses may also play a role as discussed below.

EPSTEIN-BARR VIRUS (EBV)

EBV is a member of the Human Herpes Virus (HHV) family, which also includes Herpes Simplex Virus (HSV) 1 and 2, Varicella Zoster Virus (VZV), Cytomegalovirus (CMV), HHV 6 and 7, and Kaposi Sarcoma Virus (KSV) (57–59). EBV is an enveloped virus with a 120 kB double-stranded DNA genome, coding for about 85 proteins and a number of non-coding RNAs (60–65).

EBV is transmitted to new victims with saliva and infects pharyngeal epithelial cells. When released from the epithelial cells, EBV infects B cells in the associated underlying tissue, where it may be propagated or enter a state of latency, depending on the B cell environment and the state of the host immune response (66–70). Initially, in the absence of an adaptive immune response, B cells are induced to lytic production of virus. Upon entry to the cell, EBV uncoats in the cytoplasm and transfers its DNA to the nucleus, where an ordered sequence of viral gene

Abbreviations: Ab, antibody; ADEM, acute disseminated encephalomyelitis; Ag, antigen; AI, antibody index; AuAb, autoantibody; AuAg, autoantigen; B, B cell; B', EBV-infected B cell; BBB, blood-brain barrier; BKV, B. K. Virus; CD, cluster of differentiation; CIS, clinically isolated syndrome; CMV, Cytomegalovirus; CNS, central nervous system; CSF, cerebrospinal fluid; D, dedritic cell; Di, Diphteria; EBV, Epstein-Barr Virus; f, female; FLC, free light chains; HERV, Human Endogenous Retrovirus; Hib, Hemophilus influenzae B; HHV, Human Herpes Virus; HLA, human leukocyte antigen; HPV, Human Papilloma Virus; HSV, Herpes Simplex Virus; Ig, immunoglobulin; IM, infectious mononucleosis; JCV, John Cunningham virus; KSV, Kaposi Sarcoma Virus; L, ligand; LMW, low molecular weight; M, macrophage; m, male; MAb, monoclonal antibody; MIG, microglia cell; MMR, Measles-Mumps-Rubella; MOG, major oligodendrocyte glycoprotein; MRI, magnetic resonance imaging; MS, multiple sclerosis; MuV, Mumps virus; NMO, neuromyelitis optica; OCB, oligoclonal bands; ODC, oligodendrocyte; ON, optic neuritis; PCR, polymerase chain reaction; PD, programmed death; Pe, pertussis; PET, positron emission tomography; Pol, polio; PP, primary-progressive; RR, relapsing-remitting; RuV, Rubella Virus; SP, secondary-progressive; T, T cell; t, time; Te, tetanus; VitD, vitamin D; VZV, Varicella Zoster Virus.

expression then takes place. First, immediate early genes are expressed, coding for transcription factors and other proteins involved in control of the host cell, next early genes are expressed, coding for proteins involved in viral DNA replication, followed by late genes, coding for capsid proteins and other proteins involved in mature virus production [e.g., envelope (glyco)proteins)]. Finally, virions are released from the cell by a process resembling the reverse of endocytosis. At later stages, when an adaptive immune response has been established, EBV may enter a latent state, where only few or no viral genes are expressed, but the viral genome may still be replicated along with cellular DNA. This state is called "deep" latency, where from the virus may be reactivated in response to B cell activation (66, 71–80).

As a counter-measure to host immune responses, EBV has evolved a multitude of immune evasion mechanisms, counteracting both host cell intracellular anti-viral processes and host extracellular innate and adaptive immune responses. Cellular anti-viral pathways are many and EBV devotes a large part of its genome to control of cellular anti-viral apoptosis mechanisms and to immune evasion (81–86).

The adaptive immune response to EBV involves both Ab-dependent processes and cytotoxic T cells, and EBV has evolved mechanisms to evade these as described above, e.g., by down-regulating MHC I to avoid recognition by cytotoxic T cells. Therefore, control of EBV relies to a large extent on natural killer cell surveillance of infected cells with too little MHC I on the surface, which is in turn counter-balanced by EBV by upregulation of non-classical MHC molecules (87–102).

Despite the many evasion mechanisms of EBV, the host immune system eventually forces EBV into latency, where a minimal number of EBV genes are expressed as described above. However, T cell immunity eventually wanes with time, allowing EBV to reactivate under certain conditions with lytic production of virions, thus re-invigorating the immune response, again forcing the virus into latency, a cyclic process which may go on for the rest of a person's life with smaller or larger intervals, depending on the person's immune system profile.

Decreased capacity for immune control of EBV may, in some cases manifest itself as a tendency to develop EBV-related diseases, including infectious mononucleosis (IM), various cancers, MS, and other relapsing-remitting autoimmune diseases (e.g., systemic autoimmune diseases) (103–112).

EBV AND MS

In MS, much evidence indicates a role for EBV and specifically that EBV-infected B cells have entered the CNS at some point of disease development (**Table 1**). As described above, some of the major characteristics of MS are the presence of an elevated IgG index and OCBs in the CNS, representing various B cell clones synthesizing Abs in the CNS (6–8). The elevated IgG index and the OCBs cannot reflect simple diffusion of Abs from serum to CSF, since the IgG index is calculated relative to the albumin ratio and the OCBs test is only regarded as positive, when the OCBs are absent from serum. Similarly, intrathecal presence of elevated free light chains represent synthesis of Abs in the CNS (9, 10). Intrathecal synthesis of Abs is also reflected in elevated specific antibody indexes (AIs), representing intrathecal synthesis of Abs to Measles Virus (MeV) antigens (Ags), Mumps Virus (MuV) Ags, HZV Ags, Rubella Virus (RuV) Ags, and other pathogen Ags (11–16). EBV AIs are also elevated, however, not necessarily to the same extent as other AIs, despite the presence of high levels of Abs to EBV in serum of MS patients (15, 124). Interestingly, there is a high degree of correlation between Ab concentrations in serum and in CSF for most or all of the virus Abs described above (15). Since the elevated CSF levels are not caused by diffusion from serum to CSF and since there is a highly significant correlation between serum and CSF Ab levels, the only likely explanation is that there

TABLE 1 | Evidence for Epstein-Barr virus (EBV) involvement in multiple sclerosis (MS).

MS trait/characteristic	EBV relation	References
Elevated IgG index	CNS entry of EBV-infected B cells and differentiation to plasma cells	(6)
OCBs in CSF	CNS entry of EBV-infected B cells and differentiation to plasma cells	(6, 8)
Elevated FLCs	CNS entry of EBV-infected B cells and differentiation to plasma cells	(9, 10)
Elevated specific AIs	CNS entry of EBV-infected B cells and differentiation to plasma cells	(11–19)
CNS inflammatory foci	T cell attack on CNS EBV-infected B cells	(1, 2, 5)
Demyelination in CNS	Inflammatory damage to oligodendrocytes and stimulation of macrophages and microglia cells	(1–3)
AuAbs to myelin AuAgs	Inflammation-induced stimulation of (EBV-infected) B cells and damage to oligodendrocytes	(113–116)
Therapy with CD20 MAbs	Killing of EBV-infected B cells, prevention of CNS entry	(27, 28)
Therapy with integrin MAbs	Prevention of CNS entry of EBV-infected B cells	(117, 118)
Therapy with EBV-specific T cells	Killing of EBV-infected B cells, prevention of CNS entry	(119, 120)
Female preponderance	Reduced EBV control (immune suppression due to menstruation (blood loss, healing, hormonal factors)	(1–3, 30)
Incidence increases after puberty	Increased exposure to EBV, reduced capacity for EBV control due to thymus involution	(3)
HLA DRB1 predisposes	Increased entry and/or decreased immune control of EBV	(1–3, 29–40)
IM predisposes	Increased load of EBV-transformed B cells	(30, 54–56, 121–123)
VitD deficiency predisposes	Reduced EBV control (immune suppression due to vitD deficiency of leukocytes, (e.g., T cells, NK cells)	(39, 41–43)
Smoking predisposes	Reduced EBV control (immune suppression by smoke) and/or increased frequency of EBV reactivation	(30, 44–46)
Obesity predisposes	Reduced EBV control due to immune suppression	(50–53)

Ab, Antibody; Ag, antigen; AI, antibody index; AuAb, autoantibody; AuAg, autoantigen; CD, cluster of differentiation; CNS, central nervous system; EBV, Epstein-Barr virus; FLC, free light chains; HLA, human leukocyte antigen; IM, infectious mononucleosis; MAb, monoclonal antibody; NK, natural killer; VitD, vitamin D.

has been or is a continuous influx of Ab-producing B cells from blood to CSF, most likely in the form of B cell blasts which have differentiated to plasma cells concomitantly in the periphery and in the CNS.

Many studies have revealed increased amounts and increased frequencies of EBV Abs in MS, however, such studies are hampered by the nearly ubiquitous presence of EBV in adults. Moreover, the results seem to depend somewhat on the EBV Ags used and the assay methodology.

Seroconversion from negative to positive for EBV Abs generally increases with age. It has a major incidence peak early in childhood and shows a second peak, especially for females, around puberty, co-incident with the approximate age of IM and co-incident with the female predominance in MS (3, 103, 104, 106, 125–128). EBV infection correlates with pediatric MS and essentially all children with MS are found to be positive for EBV Abs, whereas the positivity rate is considerably lower in healthy children (54, 129–132). When using an array of Ags and methods, all adult MS patients are also found to be positive for EBV Abs and it appears that MS development generally depends on prior EBV infection (54–56, 121, 122, 130, 133–137). Furthermore, prior IM has been found to increase the risk of MS by more than 2-fold by itself and more in combination with other predisposing parameters (30, 54–56, 121–123, 138, 139).

In contrast to the Ab-based studies, polymerase chain reaction (PCR)-based investigations on EBV DNA and RNA in blood, CSF and saliva have generally shown no or only minor differences between MS patients and controls (140–142). These results may depend on the patient cohorts and the methods employed, but they do indicate that the role of EBV in MS reflects a predominantly latent infection (as in most infected persons) with occasional reactivation and transient lytic virus production. However, sequencing-based studies have indicated an association between the presence of EBV variants and MS (143, 144).

In situ hybridization and PCR studies on brain material from MS patients have in some cases indicated the presence of EBV DNA in lesions, but other studies have yielded negative results (145–148). Immuno-histochemical studies are few, but one study has demonstrated the presence of EBV Ags in post-mortem brain tissue of MS patients (149).

Other viruses, including RuV, MuV, MeV, CMV, HHV6, VZV, John Cunningham Virus (JCV), and Human Endogenous Retrovirus W (HERV-W) have also been suggested to play a role in MS, either by themselves or in combination with EBV infection (30, 54, 150–154). This may simply reflect a viral Ag-induced reactivation and stimulation of EBV-infected B cells with specificity for the virus(es) in question (i.e., a secondary role for these viruses), or it may reflect a more active role of the viruses. The virus Ab profile varies much between individual patients, thus favoring a primary role of EBV and a secondary role of other viruses (15). Interestingly, CMV seropositivity appears to afford some protection against MS development (30, 135). CMV is evolutionarily related to EBV, so it may be a likely possibility that CMV may exhibit some cross-reactivity with and protection against EBV (59).

As described above, EBV control relies to a large extent on T cells and NK cells. It could therefore be hypothesized that MS patients have a deficiency in the cellular immune control of EBV and possibly also other viruses. CD8 T cell infiltration of MS brain lesions has been demonstrated in several studies but defective T cell control of EBV has also been reported in MS patients (155–157). This could indicate an imbalance in the T cell control of EBV in MS patients, and one study has actually found increased programmed death (PD) 1 on CD8 T cells with resulting decreased cytolytic activity against EBV-infected B cells (158), while PD1 has also been reported to be increased on regulatory T cells (159).

DISCUSSION

MS has traditionally been regarded as an autoimmune disease. However, the occurrence of autoantibodies (AuAbs) in MS (e.g., myelin basic protein (MBP) and major oligodendrocyte glycoprotein (MOG) Abs) is limited to only some patients and the pathogenic role of AuAbs remains debatable, while the search for autoantigens (AuAgs) in MS continues (113–116, 160–173). For this reason, models of MS etiology have for long revolved around T cells as major contributors. The role of T cells has been suggested to involve idiopathic loss of self tolerance with expansion of self-reactive T cell clones, defective regulatory T cells, infections in combination with (T cell) molecular mimicry and epitope spreading, bystander T cell activation, exhaustion of infection-related T cells, or combinations/imbalances of these (1–3, 30, 54, 173–182). Even though EBV-infected B cells appear to play a major role in MS, is an important role for T cells not excluded. EBV-infected memory B cells will be sensitive to stimulation by both their cognate Ags and specific CD4-positive T helper cells and will be a target for CD8-positive cytotoxic T cells. Both stimulation by T helper cells and attack by cytotoxic T cells will contribute to inflammation around EBV-infected B cells. Thus, a major role for T cells in MS is likely, in agreement with the predominance of T cells in MS lesions (1, 2, 173–182).

Thus, exhaustion of cytotoxic T cells and/or NK cells would seem to be highly relevant in relation to EBV involvement in MS as indicated above. This view has gained momentum from the relatively big success of B cell-targeted therapies in MS and CD20 MAbs are now the choice of treatment in many newly diagnosed MS patients (27, 28). These drugs can be hypothesized to work either by elimination of self-reactive B cell clones or elimination of EBV-infected (memory) B cells. As the frequencies of AuAbs in MS are variable and as CD20 is not expressed on differentiated Ab-producing "plasma" B cells, the first possibility can be regarded as more hypothetical (although a contribution of this to therapeutic outcome remains a possibility). Consequently, the second possibility, elimination of EBV-infected memory B cells, appears to be the most likely mechanism for the therapeutic effects of CD20 MAbs. The results described above indicate that EBV-transformed B cells proliferate or have proliferated in the periphery and entered the CNS at some point of disease evolution in connection with

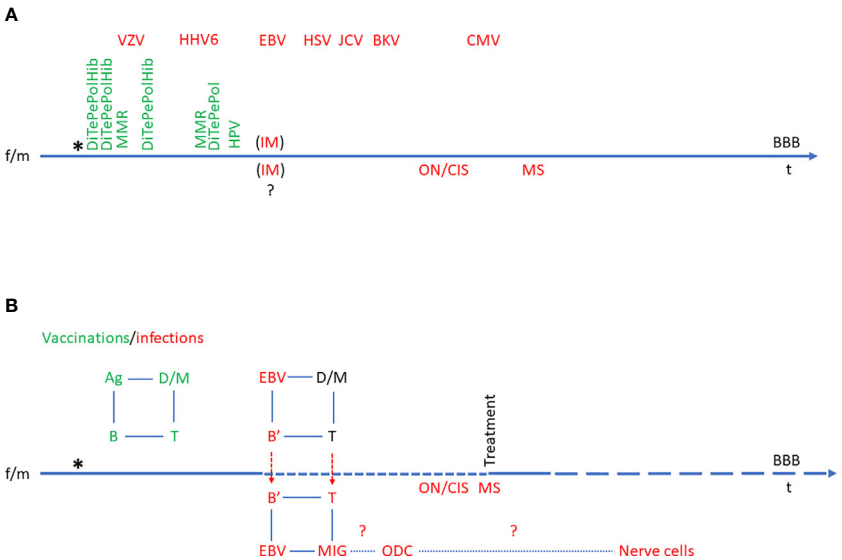

FIGURE 1 | Model of Epstein-Barr virus (EBV)'s role in multiple sclerosis (MS). The time line also represents the blood-brain-barrier (BBB) and events across the BBB. The birth of a child subsequent to the mixing of a female (f) and a male (m) set of genes is indicated by an asterisk (*). **(A)** Time course of normal immune system development with vaccinations (green) and infections (red). The order and time course of vaccinations is defined by vaccination regimens. The order of infections is individual and variable, so the sequence indicated is hypothetical. In some individuals, EBV infection may manifest itself as IM, and it is not known to which extent infectious mononucleosis (IM) affects the CNS at the time of primary infection, but it is known to increase the risk of ON/CIS and eventually MS. **(B)** Schematic presentation of etiological immunological reactions in multiple sclerosis in relation to vaccinations and infections. The normal immunological feed-back loop is indicated in green (e.g., vaccination-induced Ag uptake by dendritic cells (D) and macrophages (M), which interact with T cells, which in turn interact with B cells and vice versa). In the case of EBV infection, the immunological feed-back loop is re-programmed to the advantage of EBV, resulting in chronic infection of B cells (B'). These may enter the CNS (particularly in the case of IM) and be followed by T cells. This results in inflammation in the CNS with the feed-back loop also involving microglia cells (MIG) and at some point also oligodendrocytes (ODC) and eventually, nerve cells. Ag, antigen; B, B cell; B', EBV-infected B cell; BBB, blood-brain barrier; BKV, B. K. Virus infection; CIS, clinically isolated syndrome; CMV, Cytomegalovirus infection; D, dendritic cell; DiTePePolHib, Diphtheria-Tetanus-Pertussis-Polio-Hemophilus influenzae B vaccine; EBV, Epstein-Barr virus infection; f, female; HHV6, Human Herpes Virus 6 infection; HPV, Human Papilloma Virus vaccine; HSV, Herpes Simplex Virus infection; JCV, John Cunningham Virus infection; IM, infectious mononucleosis; m, male; M, macrophage; MIG, microglia cell; MMR, Measles-Mumps-Rubella vaccine; MS, multiple sclerosis; ODC, oligodendrocyte; ON, optic neuritis; t, time; T, T cell; VZV, Varicella Zoster Virus infection.

relapses (RRMS) or have entered the CNS at some point in disease evolution (SPMS and PPMS) (**Figure 1**). CD20-targeted MAbs are administered intravenously and are not expected to enter the CNS to any major degree (in line with the occurrence of CNS OCBs and elevated IgG index not deriving from diffusion from the blood stream). Therefore, the efficacy of these drugs must derive from an effect on CD20-positive B cells in the periphery, both in RRMS and PPMS, indicating that the import of EBV-transformed B cell to the CNS is a continuous process.

Other treatments with an effect in MS can also be related to a role of EBV. Natalizumab inhibits lymphocyte mobilization and entry to the CNS by targeting integrin α4β1/α4β7 (117, 183). Integrins may be used by EBV as entry receptors (118) and Natalizumab might therefore both inhibit entry of EBV to integrin-expressing cells and may also inhibit mobilization and entry of EBV-infected B cells and EBV-directed T cells to the CNS by a general inhibition of lymphocyte trafficking.

Some other low molecular weight MS drugs have also been reported to have an effect on EBV, in particular Teriflunomide, which has been reported to inhibit EBV lytic replication and to influence the immune response to EBV (118, 184). Similarly, the role of vitD in MS can be regarded as a general immune-stimulatory effect as can other environmental factors (e.g., propionic acid, which has been found to reactivate EBV (thus re-invigorating an EBV-targeted immune response) (119). Smoking can theoretically affect the disease course both by reducing immunity and by reactivating EBV, two effects that may partly oppose each other, thus possibly explaining the apparently protective role of some uses of tobacco (54).

In line with the role of EBV, small trials of MS therapy with autologous *in vitro*-expanded EBV-specific T cells have shown a beneficial effect in some patients (119, 185). The theory of EBV involvement in MS was proposed early by Pender et al. and it has been made likely that MS patients have a deficient T cell control of EBV-infected cells (54, 120, 155, 186–197). The theory of EBV involvement in MS has subsequently been elaborated and substantiated by many studies as described above and summarized in **Table 1**. Several models have been proposed based on the accumulated evidence for the role of EBV in MS (198–201). **Figure 1** represents an attempt to visualize much of this evidence.

In conclusion, the infectious, transforming, anti-apoptotic and immune-evasion properties of EBV makes it a highly likely candidate for an etiologic agent in MS. However, much remains to be investigated in future studies. For example, MS shows characteristics of an indolent neoplastic disease

(metastasis, clonal expansion, overlap with lymphoma, etc.). Thus, the role of the transforming properties of EBV in MS should deserve attention. If the pathogenic role of EBV-specific T cell exhaustion can be confirmed, treatment of MS with immune check point inhibitors (e.g., PD1 and/or PD1 ligand (PD1L) MAbs), known to be effective in several forms of cancer may become a possibility.

AUTHOR CONTRIBUTIONS

GH made the first manuscript draft. All authors contributed to the article and approved the submitted version.

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Intestinal Microbes in Autoimmune and Inflammatory Disease

Wan-Jung H. Wu[1,2], *Daniel F. Zegarra-Ruiz*[2] *and Gretchen E. Diehl*[2*]

[1] Immunology Graduate Program, Baylor College of Medicine, Houston, TX, United States, [2] Immunology Program of the Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, NY, United States

Autoimmune diseases and chronic inflammatory disorders are characterized by dysregulated immune responses resulting in excessive and uncontrolled tissue inflammation. Multiple factors including genetic variation, environmental stimuli, and infection are all thought to contribute to continued inflammation and pathology. Current evidence supports the microbiota as one such factor with emerging data linking commensal organisms to the onset and progression of disease. In this review, we will discuss links between the microbiota and specific diseases as well as highlight common pathways that link intestinal microbes with multiple autoimmune and inflammatory diseases.

Keywords: multiple sclerosis, autoimmunity, inflammation, inflammatory bowel disease, type 1 diabetes, rheumathoid arthritis, systemic lupus erythematosus, intestinal microbiota

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***Correspondence:**
Gretchen E. Diehl
diehlg1@mskcc.org

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INTRODUCTION

Increases in autoimmune and inflammatory diseases are a major health problem currently affecting over 200 million people worldwide and represent a leading cause of death for women under 65 (1, 2). Better understanding of factors that affect disease progression and initiation will lead to new ways to address these important health issues.

In the human body, the microbiota dynamically interacts with the host at all barrier sites with the largest load of microbes residing within the intestine (3). Commensals coevolved with humans and provide multiple benefits including facilitating nutrition and xenobiotic metabolism, enhancing barrier function, inhibiting pathogens, and modulating immunity (3). Alteration in the microbiota composition is linked to dysregulated immunity and is associated with inflammatory and autoimmune diseases (4–9).

While individual studies find a number of disease-associated changes, how these changes relate to disease initiation or amplification are still being elucidated. Importantly, understanding host regulation by intestinal microbes or of microbial physiology have led to greater understanding of a number of diseases. For example, microbial factors such as metabolites can play an important role in modulating intestinal and systemic inflammation and a subset of metabolites are linked to multiple diseases (10). Short-chain fatty acids (SCFAs), which are converted from dietary fiber and as the main energy source for colonocytes, directly support intestinal epithelial health (11). SCFAs also promote differentiation of regulatory T cells (Tregs) supporting an anti-inflammatory environment within the gut and at distal sites (12–15). While many types of microbes can generate SCFA, the main producers are Firmicutes and Bacteroidetes and increased proportion of these organisms is associated with human health (16). Dietary factors such as fiber can also shape the microbial community by modifying the metabolic landscape resulting in microbial compositional changes that can modulate diseases (17, 18). Common

associations with metabolites highlight how common metabolic pathways utilized by distinct microbes could modulate disease. They also give clues to common pathways that could be manipulated to treat these diseases.

Additionally, in many of these diseases, increased microbes or microbial products can be found in the blood indicating that changes to the intestinal barrier may be a common feature (19–21). However, whether these changes are causative or a consequence of disease development remains to be seen (22).

On the host side, several pathways associated with microbiota-regulated immune responses are linked to autoimmune and inflammatory diseases (23–25). Mutations in HLA-DR, toll like receptors (TLRs), inflammasome, and autophagy components are associated with multiple diseases where they lead to dysregulated immune responses and increased inflammation (26–29).

In this review, we will discuss association of the microbiota with pathways involved in the pathogenesis of inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), and type I diabetes (T1D) to highlight commonalities between diseases as well as point out disease specific associations.

INFLAMMATORY BOWEL DISEASE

IBD is characterized by dysregulated immune responses against the microbiota leading to chronic inflammation in the gastrointestinal (GI) tract. The major forms of IBD are ulcerative colitis (UC), which is limited to the colon, and Crohn's disease (CD), which can affect tissue throughout the GI tract (30). In IBD patients, there are reductions in potentially anti-inflammatory microbes such as Bacteroidetes, *Lachnospiraceae* (16), and *Faecalibacterium prausnitzii* (31, 32) alongside increases in potentially inflammatory microbes such as Proteobacteria and *Ruminococcus gnavus* (30, 33–39). Further, increased mucosa-associated bacteria (16, 40) results in greater contact between gut microbes and immune system and leads to anti-bacterial immunity associated with IBD pathogenesis (41–45).

In humans, over 240 genetic loci are associated with risk for IBD (23, 46–49). Gene mutations in pathways related to interactions with the microbiota highlight common mechanisms for disease development (23). Mutations are found in genes associated with microbial recognition including nucleotide-binding oligomerization domain-containing protein 2 (NOD2); anti-inflammatory mechanisms including IL-10 and IL-10 receptor (50–53) and barrier repair including IL-22 (54, 55). Many have found these pathways are induced by the microbiota and the microbiota is important for barrier repair in mouse models of disease (56–62). However, microbes also drive pathology and rederivation to germfree is protective in T cell dependent models (59, 63).

In addition, diet and dietary metabolites are critical factors in IBD pathogenesis (64). In IBD patients, specific bacteria, such as butyrate producers *Faecalibacterium prausnitzii and Roseburia hominis* are decreased (32, 65). The crucial roles of diet and dietary metabolites are shown in multiple mouse models where high fiber diets or direct administration of SCFA are beneficial while loss of the SCFA receptor, Gpr43, is pathogenic (12–15). Tryptophan metabolites can also mitigate colitis severity. These are ligands for the aryl hydrocarbon receptor (AhR), which activates IL-22 and IL-10 production and is negatively associated with colitis (66–69). A tryptophan-free diet exacerbates pathology in colitis models (70), whereas *Lactobacillus bulgaricus*, an AhR-activating bacterium, ameliorates pathology (66, 71). Secondary bile acids are additional metabolites with both pro- and anti-inflammatory functions that can promote differentiation of Tregs or Th17 cells within the intestine and in peripheral sites (72, 73). Bile acids can also regulate intestinal bacterial growth by enhancing biofilm formation thereby increasing colonization by pathogens such as vancomycin-resistant *Enterococcus* in mice (74). These studies together highlight the complex interaction between the host, diet, and intestinal microbes that can underlie alterations in disease pathology.

As evidence supports the potential for the microbiota in maintaining intestinal homeostasis and preventing inflammation, there is great interest in utilizing microbes as treatment for IBD patients. The administration of probiotics shows success in animal models (75, 76) and some patients (77, 78). However, broad scale benefits are yet to emerge (78). This may be due to the genetic complexity or other environmental factors associated with IBD. Another alternative is fecal microbiota transplants (FMT), which are utilized successfully to treat *C. difficile* infection (79). Several trials demonstrate success in some UC patients (80, 81). FMT increases microbiota diversity in responders and non-responders (80), demonstrating that increased diversity alone is not sufficient for benefit. Interestingly, recent work found expanded intestinal bacteriophages in patients who did not respond after FMT with bacteriophages exacerbating colitis in animal models (82). More work needs to be done to understand how FMT can shape the recipient's microbial community to define if this method can broadly ameliorate diseases.

Together, work in IBD demonstrates myriad ways the microbiota interacts with the host to regulate local inflammation and suggests a number of microbiota-related pathways to target for treating this disease. Understanding affected pathways in IBD have also improved understanding of how microbes impact other inflammatory and autoimmune diseases and will lead to a broader understanding of how to utilize the microbiome to improve patient outcomes.

SYSTEMIC LUPUS ERYTHEMATOSUS

SLE patients suffer from production of autoantibodies and proinflammatory cytokines that cause disease in multiple organs including skin, blood, and kidneys with many environmental influences, including the gut microbiota (83). SLE patients exhibit intestinal and oral dysbiosis. As with other autoimmune diseases, studies find decreased bacterial diversity correlated with disease activity (6). Oral and gut microbiota from SLE patients are enriched in the family *Lactobacillaceae*, with *Bifidobacteria* and Clostridiales decreased in the intestine (84–86).

Further, antibodies and T cells from SLE patients recognize bacterial antigens from the oral, intestinal, and skin microbiota including *Propionibacterium propionicum* and *Bacteroides thetaiotaomicron* (6, 84, 85, 87). Molecular mimicry is a possible link between the microbiota and SLE. One of the most common autoantibodies associated with SLE targets the broadly expressed

RNA binding protein Ro60 (88). Antibodies against Ro60 are commonly found before SLE symptoms develop (89). Some human commensals produce proteins similar to human Ro60 and, although these bacteria are found in both healthy donors and lupus patients, only lupus patients have antibodies and T cells reactive with human Ro60 and microbial Ro60 orthologs (87). In addition, in SLE patients, disease severity correlated with *R. gnavus* enrichment. Further, SLE patients with severe disease had IgG antibodies that recognized cell wall lipoglycans from a subset of *R. gnavus* strains. Importantly, auto-DNA antibodies from SLE patients with lupus nephritis were cross-reactive with *R. gnavus* lipoglycans (6).

Multiple spontaneous and inducible mouse lupus models have given great insight into how microbiota changes regulate pathology. Intercross of mouse strains NZW with BXSB results in spontaneous lupus-related antiphospholipid syndrome and liver damage, predominately in male mice, due to an extra copy of the TLR7 gene. In these mice, *Enterococcus gallinarum* translocates to the liver and triggers autoimmune responses. Depletion of this pathobiont with vancomycin suppressed bacterial translocation, autoreactive T cells, and autoantibodies. Monocolonization of germfree mice with *E. gallinarum* increased gut permeability, plasmacytoid dendritic cells (pDCs) and Th17 cells in the intestine lamina propria and mesenteric lymph nodes, exacerbating disease and mortality (90).

Bacterial metabolites also modulate SLE as seen with *Lactobacillus* which, as discussed above can modulate intestinal inflammation by producing AhR-activating ligands (66, 71). In mouse models, and in contrast with IBD, a high protein diet with a high tryptophan content is associated with increased pathology by promoting anti-double stranded DNA autoantibody production and increased T follicular helper (Tfh) cells (91). A metabolic screening from feces of lupus prone mice homozygous for the NZM2410 lupus susceptibility quantitative trait loci (Sle1, Sle2, and Sle3) showed increased intestinal tryptophan-derived bacterial metabolites with enriched fecal *Lactobacillus* (91).

In contrast, and similar to IBD, a high fiber diet is associated with improved outcomes in mouse lupus models (86). In a TLR7-dependent model, there was outgrowth of *Lactobacillus reuteri*, which then translocated to the mesenteric lymph node, spleen, and liver. Translocation led to increased pDC production of type I interferon (IFN-I), exacerbating disease pathogenesis and mortality. Treatment with SCFAs or a high fiber diet suppressed *L. reuteri* outgrowth and translocation, reducing excess IFN-I and ameliorating disease (86). This example shows both direct and indirect effects of gut commensals on disease progression.

Together, these results demonstrate that the gut microbiota can modulate lupus pathogenesis by molecular mimicry, changes in bacterial translocation, metabolites, or microbe-microbe competition. Each can result in a dysregulated immune response in distal tissues including Th17 cell and pDC recruitment and activation of IFN-I pathways that together amplify disease.

RHEUMATOID ARTHRITIS

RA is a chronic synovial inflammation characterized by immune infiltration in the joints due to lost tolerance including B and T cell responses against self-proteins with a citrulline residue leading to cartilage degradation and bone erosion (92). In a subset of RA patients, bacterial DNA and peptidoglycan–polysaccharide complexes are found in the synovium (93). RA patients exhibit oral dysbiosis, characterized by enrichment of *Porphyromonas gingivalis* and *Lactobacillus salivarius* and intestinal dysbiosis with increased Gram-positive bacteria (94, 95). These changes in the oral and gut microbiota are linked to clinical variations in RA (93). Increased abundance of *Lactobacillus* correlated with increased total IgG titers, while other oral microbes such as *Prevotella* spp. correlated with rheumatoid factor (95). *Prevotella copri* is enriched in fecal samples of patients and individuals at risk for RA. A subset of RA patients has *P. copri*-specific Th1 and Th17 cells along with IgG and IgA antibodies which correlates with increased proinflammatory cytokine levels and more severe disease (95–97). Interestingly, RA therapies partially restores the microbiota to more closely resemble one found in healthy controls (95).

In mice, TLR2 and TLR4 engagement modulates autoimmune arthritis (98). IL-1 receptor antagonist–knockout (*Il1rn*$^{-/-}$) mice spontaneously develop autoimmune arthritis due to uncontrolled IL-1 signaling (99). Disease progression is delayed in germfree *Il1rn*$^{-/-}$ mice (98). A single injection of a TLR2 agonist or monocolonization with *Lactobacillus bifidus* was sufficient to restore pathogenesis (98). However, as with other diseases, there are complex interactions between these pathways. *Il1rn*$^{-/-}$ mice lacking TLR2 exhibited exacerbated disease with increased bone destruction mediated by Th1 cells, suggesting a dual role for TLR2 in disease (98). BALB/c ZAP-70(W163C)-mutant (SKG) mice spontaneously develop chronic arthritis due to a naturally occurring mutation of the ZAP-70 gene, a signal transduction molecule downstream of the T cell receptor (100, 101). Germfree SKG mice do not develop disease (100). Conventionalization with altered Schaedler flora (ASF), a defined community of eight bacteria including *Lactobacillus* species, was sufficient to induce arthritis, supporting the role of gut microbes in pathogenesis (100). Further supporting microbiota shifts found in RA as amplifying disease, conventionalization of SKG germfree mice with fecal samples from RA patients elicited more severe arthritis with higher levels of IL-17A as compared to fecal samples from healthy controls (97). Similarly, *P. copri*-monocolonized SKG mice have exacerbated disease with increased Th17 cells (97). Colonization with Segmented filamentous bacteria (SFB), a Th17 cell inducing mouse commensal, exacerbates a K/BxN autoimmune arthritis model (in which KRN T cells recognize glucose-6-phosphate isomerase) by expanding Tfh cells, which promote the production of autoantibodies involved in RA (102). These data show that gut microbes can modulate immune responses involved in RA such as Th1 and Th17 cells recruitment and expansion exacerbating the inflamed tissue environment.

MULTIPLE SCLEROSIS

MS patients suffer from autoimmune responses against the brain and spinal cord due to T cell targeting of oligodendrocytes resulting in demyelination and axonal loss (103). MS patients exhibit intestinal dysbiosis with increases in the Euryarchaeota and Verrucomicrobia phyla. Specifically, *Methanobrevibacter smithii*

TABLE 1 | Summary table for the relationship between bacteria and autoinflammatory and autoimmune diseases.

Bacteria - Family	Bacteria - Species	Disease	Abundance	Human Subjects	Ref.	Mouse Model	Ref.	Effect	Mechanism/Pathway (Metabolite)
Akkermansiaceae	***A. muciniphila***	MS	Enriched	60P and 43HC	(8)				
Bacteroidaceae	***B. ovatus***	T1D	Enriched	8P and 24HC	(114)				
	B. thetaiotaomicron	SLE	Enriched			TLR7 overexpression	(87)	Exacerbates	Molecular mimicry/Autoantibodies
Bifidobacteriaceae	***Bifidobacteria***	SLE	Decreased	40P and 22HC	(85)				
		T1D	Decreased	11P and 22HC	(113)				
Clostridiaceae	***SFB***	RA	Enriched			K/BxN	(102)	Exacerbates	Immune dysregulation/Tfh +Autoantibodies
Enterobacteriaceae	***E. coli***	IBD	Enriched	447P and 221HC	(35)	DSS	(33)	Exacerbates	Immune dysregulation/IL-17
		IBD	Enriched	21P and 7HC	(34)	DSS	(33)	Exacerbates	Immune dysregulation/IL-17
		IBD	Enriched	59P	(33)	Salmonella infection	(56)	Improves	Immune dysregulation/IL-10
Enterococcaceae	***E. gallinarum***	SLE	Enriched	3P and 5HC	(90)	(NZW × BXSB)F1	(90)	Exacerbates	Immune dysregulation/AhR (AhR ligands)
Lachnospiraceae	**Not identified**	IBD	Decreased	129P and 61HC	(16)				
		T1D	Decreased	11P and 22HC	(113)				
	R. gnavus	IBD	Enriched	20P and12 HC	(37)				
		SLE	Enriched	61P and 17HC	(6)				
		T1D	Enriched	415P and 267HC	(9)				
Lactobacillaceae	***Lactobacillus***	SLE	Enriched	20P and 19HC	(84)	Sle1, 2 and 3	(91)	Exacerbates	Immune dysregulation/AhR (Tryptophan-derivatives)
	L. reuteri	SLE	Enriched	12P and 22HC	(86)	TLR7.1 Tg	(86)	Exacerbates	Immune dysregulation/Type I IFN
		MS	Enriched			EAE	(109)	Improves	Immune dysregulation/AhR (Indole-related)
	L. salivarius	RA	Enriched	77P and 80HC	(95)				
	L. bulgaricus	IBD	Enriched			DSS	(71)	Improves	Immune dysregulation/AhR (AhR ligands)
	L. murinus	MS	Enriched			EAE	(110)	Improves	Immune dysregulation/AhR (Indole-related)
Methanobacteriaceae	***M. smithii***	MS	Enriched	60 P and 43 HC	(8)				
Porphyromonadaceae	***P. gingivalis***	RA	Enriched	65 P and 18 HC	(94)				
Prevotellaceae	***P. copri***	RA	Enriched	83 P and 50 HC	(96)	SKG	(97)	Exacerbates	Immune dysregulation/Th17
Ruminococcaceae	***F. prausnitzii***	IBD	Decreased	127 P and 87 HC	(65)				
		IBD	Decreased	26 P	(32)				

P, patient; HC, healthy control.

and *Akkermansia muciniphila* are enriched in the stool of patients and their abundance decreased after treatment (8). In addition, reduced levels are found of bacteria belonging to the Clostridia clusters XIVa and IV and Bacteroidetes, microbes well known to produce SCFA and induce Treg cells (104, 105).

As with other diseases, in the mouse model of MS, experimental autoimmune encephalitis (EAE), pathology is ameliorated in germfree mice with lower levels of IFN-γ and IL-17A and increased Treg cells (106). Interestingly, in the relapsing remitting MS mouse model, in which $CD4^+$ T cells are specific for myelin oligodendrocyte glycoprotein (MOG), transfer of intestinal microbes from MS patients but not from healthy monozygotic twins increased incidence of disease due to decreased T cell IL-10 production (107). *A. muciniphila* also affects T cell differentiation by inducing Th1 differentiation in PBMCs from both healthy donors and MS patients, potentially contributing to the proinflammatory environment in MS (108).

Similar to IBD and in contrast with SLE, in mouse models tryptophan can protect from pathogenesis. Colonization of mice in the EAE model with *Lactobacillus reuteri* through its conversion of tryptophan into AhR agonists, activates IFN-I responses in astrocytes and limits disease severity (109). Another *Lactobacillius* species, *L. murinus* reduces EAE severity by inhibiting Th17 cell differentiation (110). Intestinal colonization by *L. murinus* is suppressed by a high salt diet, which also amplifies disease (110). Together these studies demonstrate a gut/brain axis in which gut microbes and metabolites modulate immune responses including innate and adaptive immunity at distal sites to influence disease onset and severity.

TYPE 1 DIABETES

Immune destruction of pancreatic β-cells by islet-specific autoreactive $CD8^+$ T cells results in lost insulin production and T1D (111). A longitudinal human study analyzing stool samples from the Environmental Determinants of Diabetes in the Young (TEDDY) cohort identified reduced microbial pathways related to fermentation and synthesis of SCFAs as well as decreased microbial diversity as well as reduced *Bifidobacteria* and *Lachnospiraceae* and overabundance of *Blautia*, *Rikenellaceae*, and *Ruminococcus* in patients who progressed to T1D (9, 112). In a similar cohort, children who progress to T1D show changes in the Bacteroidetes/Firmicutes ratio and increased *Bacteroides ovatus* (9, 112–115).

In contrast with other disease models, germfree non-obese diabetic (NOD) mice have increased islet destruction demonstrating that in diabetes, microbes may limit disease severity (116). However, some microbes are likely pathogenic as depletion of Gram-negative gut microbes in neonatal mice results in decreased diabetes incidence with fewer IFN-γ producing T cells (117). Supporting the complicated pro- and anti-inflammatory signals downstream of microbes, in contrast with IBD, loss of MyD88 protects specific pathogen free (SPF) or ASF colonized NOD mice from diabetes however rederivation to germfree, restores disease incidence (116). As in SLE, bacterial translocation can be a factor in T1D pathogenesis. In a model of streptozotocin (STZ)-induced T1D, gut microbial translocation to the pancreatic lymph node led to recognition of bacterial MDP by the intracellular NOD2 receptor resulting in increased number of Th1 and Th17 cells and increased islet destruction (118). Similar to the enrichment seen in children that develop T1D, STZ-treated mice also had increased intestinal *Bacteroides* (114, 118). Gut microbiota also plays a role in sex differences in autoimmune diseases. In SPF NOD mice, female mice have a higher incidence of disease than male mice with no differences between the sexes in germfree mice (119). Cecal microbiome transplants from male to female mice reduced islet inflammation and autoantibody levels due to microbiome changes along with hormonal and metabolic changes downstream of elevated testosterone (119).

In NOD mice, as with IBD and MS, SCFAs, notably butyrate, decreased the incidence and severity of diabetes with reduced frequency of autoimmune $CD8^+$ T cells and B cells and increased Tregs and IL-10 production (120). Treatment with SCFAs increased the abundance of *Bacteroides*, which protected against disease when transplanted to germfree NOD mice (120).

Together, in mouse models, gut microbes and gut microbial metabolites can modulate immune responses involved in T1D including pancreas T cell infiltration as well as shaping the balance between pro- and anti-inflammatory T cell responses, thereby influencing disease onset and severity.

CONCLUSION

In this review, we provided examples of mechanistic ways microbes can alter disease pathology in IBD, SLE, RA, MS, and T1D models with microbes playing a role in pathology of additional autoimmune diseases (121–124). While we focused on bacteria, emerging data suggests potential roles for yeast and enteric viruses in modulating immune responses and autoimmune and inflammatory disease (125–128).

We have highlighted disease specific interactions as well as numerous common links between the microbiota and human disease (**Table 1**). Common associations relate to microbial behaviors such as translocation or microbial metabolites that are shared between multiple microbes. Understanding these common functions and as the host pathways regulated by the microbiota will enable for identification of targetable pathways to treat multiple autoimmune and inflammatory disease.

AUTHOR CONTRIBUTIONS

W-JW and DZ-R performed literature searches and with GD wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Epstein-Barr Virus and Systemic Autoimmune Diseases

Gunnar Houen[1,2*] *and Nicole Hartwig Trier*[2*]

[1] Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark, [2] Department of Neurology, Rigshospitalet, Glostrup, Denmark

Epstein-Barr Virus (EBV) is an extremely successful human herpes virus, which infects essentially all human beings at some time during their life span. EBV infection and the associated immune response results in production of antibodies (seroconversion), which occurs mainly during the first years of life, but may also happen during adolescence or later in life. Infection of adolescents can result in infectious mononucleosis, an acute serious condition characterized by massive lymphocytosis. Transmission of EBV mainly occurs through saliva but can rarely be spread through semen or blood, e.g. through organ transplantations and blood transfusions. EBV transmission through oral secretions results in infection of epithelial cells of the oropharynx. From the epithelial cells EBV can infect B cells, which are the major reservoir for the virus, but other cell types may also become infected. As a result, EBV can shuttle between different cell types, mainly B cells and epithelial cells. Moreover, since the virus can switch between a latent and a lytic life cycle, EBV has the ability to cause chronic relapsing/reactivating infections. Chronic or recurrent EBV infection of epithelial cells has been linked to systemic lupus erythematosus and Sjögren's syndrome, whereas chronic/recurrent infection of B cells has been associated with rheumatoid arthritis, multiple sclerosis and other diseases. Accordingly, since EBV can shuttle between epithelial cells and B cells, the systemic autoimmune diseases often occur as overlapping syndromes with symptoms and characteristic autoantibodies (e.g. antinuclear antibodies and rheumatoid factors) reflecting epithelial and/or B cell infection.

Keywords: antibodies, Epstein-Barr virus, connective tissue disease, systemic autoimmune diseases, human herpes virus

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Linda Ann Spatz,
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****Correspondence:***
Gunnar Houen
gunnarh@bmb.sdu.dk
Nicole Hartwig Trier
nicole.hartwig.trier@regionh.dk

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INTRODUCTION

Epstein-Barr Virus

Epstein-Barr Virus (EBV) is a lymphotropic herpes virus and the causative agent of infectious mononucleosis (IM) (1–4). It was originally discovered in cells isolated from African Burkitt's lymphoma and first later on, was it recognized that EBV is highly prevalent worldwide (5).

EBV is a member of the Human Herpes Viruses (HHVs) family, comprising eight viruses distributed on three subfamilies (Alpha, Beta, Gamma). EBV, which is also called HHV4, belongs to the Gammaherpesviridae, genus Lymphocryptovirus (6, 7). The circular double-stranded genome of EBV is approximately 172 kilobases, with more than hundred genes coding for approximately 85 proteins (**Table 1**) and approximately 50 non-coding RNAs (8–12).

TABLE 1 | Epstein-Barr virus (EBV) proteins and their functions.

Function	Protein
Entry glycoproteins	BLLF1 (gP350), BZLF2 (gP42), BMRF2m, BXLF2 (gH), BKRF2 (gL), BALF4 (gP110), BLRF1 (gN), BHLF1, BDLF2
Lytic replication	BRRF1, BZLF1, BRLF1, BMRF1 (EA/D), BSLF1, BBLF4, BBLF2/3, BALF5, BALF2
Viral DNA synthesis	BORF2, BaRF1, BXLF1, BLLF3, BKRF3, BMLF1/BSLF2
Late gene expression	BGLF4, BGLF3, BcRF1, BFRF2, BDLF4, BVLF1, BDLF3.5
Packaging and translocation of viral DNA	BFLF1, BFRF1A, BBRF1, BGRF1/BDRF1, BALF3, BGLF1, BVRF1
Capsid	BCLF1 (VCAp160), BFRF3 (VCAp18), BORF1, BDLF1, BVRF2, BdRF1 (VCAp40)
Tegument	BNRF1 (VCAp143), BPLF1, BSRF1, BBRF2, BGLF3.5, BGLF2, BTRF1, BLRF2 (VCAp23), BRRF2, BKRF4
Virion assembly and egress	BFLF2, BFRF1, BBRF3 (gM), BXRF1, BOLF1, BBLF1
Latency Stage I:	EBNA1
Stage II:	EBNA5, LMP1, LMP2A, LMP2B
Stage III:	EBNA2, EBNA3, EBNA4, EBNA6
Lytic immune-modulators	BCRF1 (vIL-10), BARF1, LF2, BNLF2a, BDLF3 (gp150), BILF1, BHRF1 (EA/R), BALF1, BGLF5
Uncharacterized proteins	BLLF2, BNLF2b, BWRF1, LF3, LF1, RPMS1, A73, BARF0, BILF2

Several strains of EBV exist. The first EBV variants identified were type 1 (type A) and type 2 (type B). While type 1 (B95-8, GD1, and Akata) is the main EBV type prevalent worldwide, type 2 (AG876 and P3HR-1) is as abundant as type 1 in sub-Saharan Africa (13). The EBV variants have different replicative properties and individuals may become superinfected with two or more strains (14–16).

The structure of EBV is typical of HHVs and related viruses (**Figure 1**) (17). It has an outer lipid envelope, derived from the producing host cell, wherein several viral proteins are embedded in addition to host cell-derived membrane proteins. Many of the viral envelope membrane proteins are glycoproteins (gPs). Currently, 13 gPs have been identified, 12 of which are expressed only during the productive, lytic replication cycle and one of which (BARF1, a decoy viral colony-stimulating factor 1 receptor (vCSF1R)) may be expressed during latency as well. Some of these are listed in **Table 1** (18). Inside the envelope is the viral tegument, in which the capsid is embedded with its enclosed genome and associated proteins.

The life cycle of EBV is characteristic of a large enveloped DNA virus, being composed of primary infection, latency, and lytic reactivation phases. In addition, EBV has an ability to infect several cell types (19). The EBV genome encodes 9 different envelope entry gPs (**Table 1**). The functions of all of these are not completely understood, but the roles of the most important gPs are known in much detail. The tropism of newly released EBV virions is determined by the envelope gPs, which in turn vary somewhat depending on the host cell (20). The major cell types infected by EBV are epithelial cells and B cells. Epithelial cells are the first cell type to be infected, as EBV is transmitted to recipients through saliva. Next, B cells are infected when EBV gains access to the underlying tissue after release from the oropharyngeal epithelium (21–25). EBV virions released from epithelial cells have a preference for B cells and EBV virions released from B cells have a preference for epithelial cells, due to the composition of the envelope gPs (20, 26, 27).

Epithelial cell infection may occur by direct fusion of the viral envelope membrane with the plasma membrane of the target cell. Attachment of the virus to the cell surface primarily occurs *via* gH/gL interaction with Ephrin A2 (EphA2) and αvβ5/αvβ6/αvβ8 integrins and *via* BMRF1, which interacts with β1 integrins, but EBV gP350/220, which interact with complement receptor (CR)2 (CD21) and CR1 (CD35) also plays a role in epithelial cell attachment. The gH/gL interaction with integrins is mediated by a KGD motif on gH, and the interaction between gH/gL and EphA2 occurs through the receptor's ligand binding and fibronectin type III repeats and is mediated by the gP42 binding site on gH. Upon attachment and interaction with integrins or EphA2, a conformational change in gH/gL allows interaction with the trimeric gB, which in turn changes conformation and facilitates viral entry by acting as a fusogen (20, 28–32).

Other EBV proteins may also play a role during infection of epithelial cells, e.g. BMRF2, which can bind integrin αvβ1 and

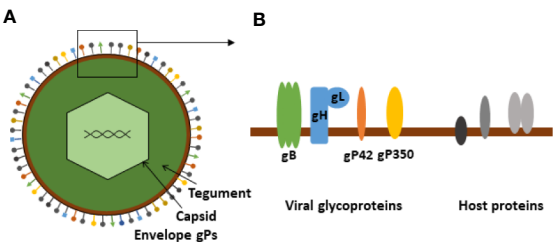

FIGURE 1 | Schematic presentation of Epstein-Barr virus. **(A)** Schematic illustration of the basic EBV structure. **(B)** Enlargement of membrane section showing viral envelope glycoproteins (entry complex) and putative host-derived membrane proteins.

BDLF2, which can bind non-muscle myosin heavy chain IIA. Moreover, gB, itself can bind neuropilin-1 and IgA directed to EBV envelope proteins may enhance infection through the polymeric IgA receptor (28, 32–36).

B cell infection is mediated by gP350/220, which binds CR2 and CR1, together with gP42, leading to the formation of a complex together with major histocompability complex (MHC)-II (37). Upon attachment, the virion is endocytosed and gH/gL can form a fusion complex with gP42-MHC-II, thus inducing a conformational change in gH/gL (similar to what happens upon gH/gL interaction with integrins and/or EphA2). As a result, trimeric gB changes conformation and promotes fusion of the viral membrane with the endosome membrane, thus releasing the virus to the cytoplasm (**Figures 1–3**) (20, 26, 27). The structural and mechanistic basis of B cell entry has been elucidated in much detail by solving the structures of gB, gP42, complexes of gH/gL, gP42/MHC-II (human leukocyte antigen (HLA)-DR1) and of gH/gL/gP42/MHC-II in pre- and post-fusion conformations (20, 38, 39). This has allowed modelling not only of the EBV B cell entry complex, with the involved gPs acting sequentially and in concert, but also of the epithelial cell entry complex. Thus, gH/gL/gB appears to constitute a core entry machinery and gP42 seems to be a primary determinant of EBV tropism, since it participates in and promotes B cell infection but inhibits epithelial cell infection by binding to the EphA2/integrin-binding site(s) on gH/gL (20).

Successful entry and viral take-over of cellular control leads to an ordered sequence of transcription of viral genes, translation of viral mRNAs and finally, replication of the viral DNA and assembly of new virus (**Figure 3**). The virion assembly and egress from the host cell utilizes the host cell exocytosis machinery and involves several viral proteins apart from the structural, tegument and envelope proteins (**Table 1**) but is less understood than the entry process (40, 41). Collectively, EBV utilizes several characteristic major host cell membrane proteins for entry and release and due to the properties of gP42, it has a preference for epithelial cell infection when produced by B cells and vice versa, assuring that some virions will eventually return to salivary gland cells and be able to be transmitted to new individuals.

Infection activates the intracellular antiviral mechanisms and induces an extracellular immune response against EBV antigens, with generation of specific helper T cells, antibodies and cytotoxic T cells and activation of natural killer (NK) and NK T cells (NKT) (42–46). In response to this, EBV has evolved mechanisms for evading the extracellular innate immune system and the host cell's innate antiviral systems together with adaptive immune system evasion mechanisms and the virus devotes a substantial part of its proteins and non-coding RNAs to this (47–51). Together, the innate and adaptive immune evasion mechanisms of EBV assure its persistence in the host. A major aspect of the immune evasion strategy is EBV's ability to enter a latent state with minimal expression of viral genes and minimal presentation of viral peptides to the immune system (19, 52–54). This mainly occurs in (memory) B cells, but latency may also take place in epithelial cells. From the latent state, EBV can occasionally reactivate, e.g. in response to antigen stimulation of memory B cells, resulting in lytic production of virions upon expression of an ordered sequence of viral genes (55–57). This in turn mounts an increased immune response against EBV, neutralizing infected cells and forcing the virus into latency again. Reactivation may also occur upon "waning" of the cellular immunity to the virus and infected individuals through the rest of their lives experience a persistent "battle" with EBV. Depending on the host immune system and environmental factors, some individuals may eventually suffer from EBV-related diseases, either as a result of EBV immune evasion or as a result of EBV infection of other cell types (T cells, NK cells, NKT cells, monocytes/macrophages, and others), which may take place in some instances.

Epstein-Barr Virus Immune Evasion

As a part of the common evolutionary history of humans and EBV, the virus has evolved a multitude of immune evasion mechanisms, including wrapping itself in host cell-derived membranes (envelopment) and the ability to switch between latent and lytic life stages (50, 58). Most of the immune evasion proteins of EBV are expressed during the lytic cycle and some are shown in **Table 1** as "immune modulators". More EBV proteins are presumably involved in immune evasion and many EBV proteins serve two or more functions.

In the latent state, as mentioned above, there is minimal expression of viral genes and minimal presentation of viral peptides to the immune system (19, 52–54). In the "deep" latency state, only EBV nuclear antigen (EBNA)1, which assures maintenance and replication of the EBV genome along with host cell chromosomes, is expressed. In order to avoid presentation of EBNA1-derived peptides on MHC-I, the EBNA1 protein contains a characteristic AG repeat sequence, which interferes with proteasome processing and which interacts with nucleolin to restrain its expression. Moreover, EBNA1 also contains characteristic RG repeat sequences, which may play a role in immune evasion (59–61). Upon switching to lytic cycle with production of viral proteins, EBV downregulates MHC-I and interferes with presentation of viral peptides on MHC-I *via* BDLF3-induced ubiquitination of MHC-I (62). Likewise, in B cells, EBV can also downregulate MHC-II by BDLF3-induced ubiquitination of MHC-II (62) and gP42 can be released in a soluble form, which inhibits interaction between MHC-II and the T cell receptor (63, 64). Other EBV proteins are involved in minimization of MHC-I expression, including BNLF2a, BILF1, BGLF5. The exonuclease BGLF5 degrades cellular mRNAs including those for MHC-I and BILF1 associates with cell surface MHC-I and enhances its degradation, while BNLF2a prevents MHC-I peptide loading by inhibiting the transporter associated with peptide loading (TAP) (65–68). As a means to avoid NK cell recognition, EBV upregulates non-classical MHC during the phase of viral protein synthesis. Lytic production of viral proteins and RNAs as well as replication of viral DNA requires that EBV can prevent cellular apoptosis and EBV has evolved an elaborate set

of proteins for pacifying intracellular virus-sensing apoptosis-inducing mechanisms including downregulation and inhibition of toll-like receptors (47, 49, 50, 62, 68–71).

EBV also produces soluble mediators, which interfere with mobilization of the adaptive immune system. BCRF1 encodes a viral IL10 homologue (vIL10), which dampens inflammation (72–75) and, as mentioned above, BARF1, encodes a decoy vCSF1R, which binds CSF1 and thereby limits mobilization of hemopeoietic stem cells (76, 77).

The viral envelope derived from the host cell (**Figure 2**) offers substantial protection to the enclosed viral particle by mimicking a host extracellular vesicle. In principle, the viral envelope may contain all host-derived membrane proteins relevant for "disguise" and immune evasion (e.g. MHC molecules, complement regulators, Fc receptors, phagocytosis-inhibitory ("don't-eat-me") molecules, etc.). However, to be able to exit from the host cell in a controlled process, and to be able to infect other cells, several viral gPs have to be inserted into the envelope membrane as mentioned above. These proteins are targets for innate immune recognition and antibody (Ab) production, as described in the preceding paragraph, but extensive glycosylation with host-derived glycans affords considerable protection against pattern recognition (scavenger) receptor (including complement) and Ab recognition ("glycan shielding"). Moreover, as described, some of the immune reactions may actually be exploited for viral infection and spreading, f.ex. "hitchhiking" with complement/CRs (e.g. EBV entry in B cells) or with Abs bound to viral envelope gPs/Fc receptors (FcRs) (e.g. cytomegalovirus entry in monocytes/macrophages or EBV entry in B cells with cell surface immunoglobulins (Igs) against EBV envelope gPs) (78–80). Despite the many immune evasion mechanisms of EBV, the normal healthy human immune system is able to eradicate active virus and force it into a quiescent ("immune silent") state (latency). Since EBV appears to be able to evade most or all innate immune system components, the final "victory" of the immune system must rely on cellular immune control of EBV involving a combination of T cells, NK cells and NKT cells, in accordance with all available evidence of EBV immunity. The molecular details of how this results in EBV latency instead of cell killing are not known, but it is firmly established that EBV has evolved mechanisms of latency as an ultimate, opportunistic and effective immune evasion strategy.

Epstein-Barr Virus Epidemiology

A majority of children becomes infected with EBV early in life and seroconversion, the appearance of Abs to EBV peaks around 1–2 years of life, where the majority of infectious cases is non-complicated and may even go unnoticed. A second peak in seroconversion is seen in puberty, due to increased frequency of close social contact with already infected persons. Infection in adolescence is more problematic and may result in IM in many cases, popularly denoted "kissing disease" (1–4). For the majority of infected individuals latent infection does not appear to influence the general health, however, dysregulation of latency or inability to control the lytic infection may lead to development of lymphoproliferative diseases and lymphoma (81).

The course of EBV infection is determined by the virus load and an individuals' immune system state, which in turn is determined by the person's gene composition, other infection history and several environmental factors, which all may influence the immune capacity of a person to various degrees.

Genetic factors influencing EBV control are in principle all genes of the immune system. In practice, T cells, NK cells and NKT cells have turned out to be of utmost importance (42–44, 46). Relatively few studies have addressed genetic factors associated with EBV infection, presumably due to the ubiquitous occurrence of EBV. Consequently, since essentially all persons eventually become infected, genetic associations will only relate to the age of infection. Epidemiological studies have indicated an association of some MHC-II and -I alleles and EBV seropositivity. Moreover, mannan-binding lectin insufficiency has been linked to EBV seropositivity as well (82). Also, some polymorphisms in the (IL) 10 gene and other immune system genes have been linked with EBV seropositivity (83). However, all these studies are hampered by a relative scarcity of seronegative persons.

Besides from genetic factors, environmental factors are known to affect a person's EBV status. Currently identified factors are sunlight/Vitamin D (VitD), smoking and body mass index (BMI) (84, 85). These factors may be assumed to influence the general

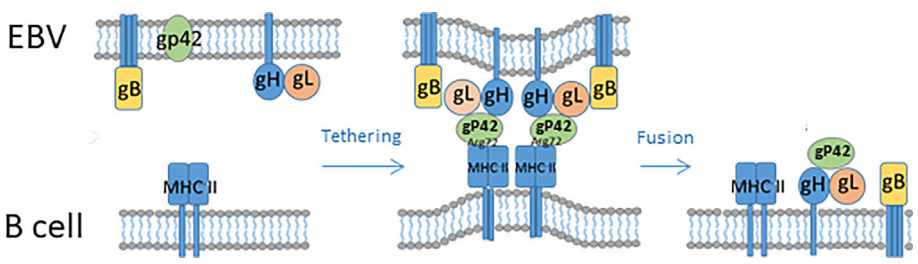

FIGURE 2 | Schematic illustration of EBV fusion with the cellular lipid bilayer of B cells. For gP42 to become active, the protein is cleaved N-terminally. gP42 interacts with gH/gL, and the complex interacts with gB. gP42 interacts with the β1 domain of MHC-II, which ultimately results in membrane fusion.

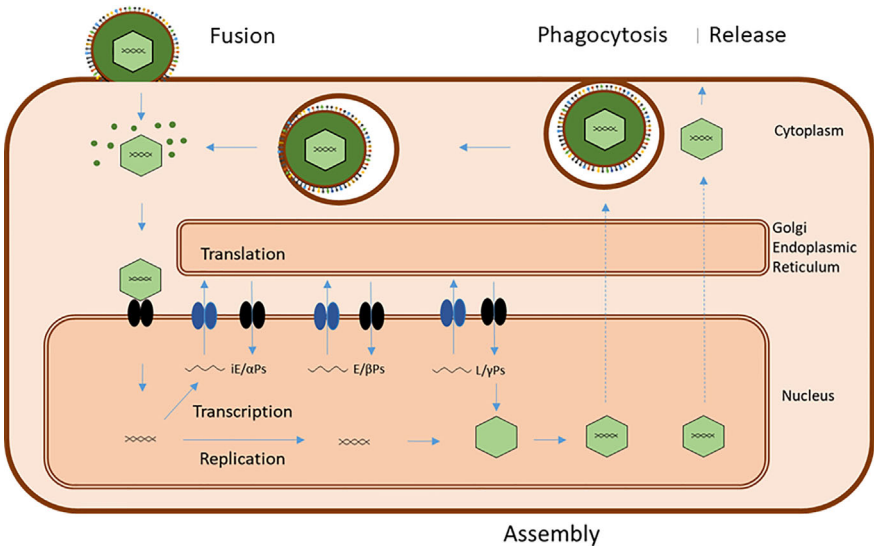

FIGURE 3 | Common basic EBV infection scheme. Viral entry can occur by direct fusion of the viral plasma membrane-derived envelope with the target cell membrane or by endocytosis/phagocytosis of virus followed by fusion of virus envelope and endosome/phagosome membrane. Both processes release virions and viral tegument proteins into the cytoplasm. Released virions are transported to the nuclear membrane and the viral genome introduced into the nucleus together with associated proteins. This initiates transcription of viral genes in a sequence of immediate-early (iE) genes, coding for regulatory alfa-proteins, early genes, coding for catalytic beta-proteins, and late (L) genes, coding for structural gamma proteins. Translation of viral messenger RNAs takes place on ribosomes in the cytoplasm and on the endoplasmic reticulum, and the viral proteins are routed to different locations for subsequent virus assembly. Successful replication of viral genomes and transport of capsid proteins to the nucleus results in assembly of virions, which travel to the plasma membrane by a series of envelopment/fusion events involving intracellular membranes (stippled lines) ending with budding of mature virus with a plasma membrane envelope, containing viral glycoproteins and host-derived membrane proteins. Premature cell death releases a mixture of "naked" virions and diffentially enveloped viruses.

immune status of individuals and thereby affect susceptibility to EBV infection. E.g. sunlight/VitD has been proposed to protect against autoimmunity by increasing the number of CD8+ T cells available to control EBV infection (84). Moreover, obesity has been proposed to impact the cellular immune response to infections and induce a state of chronic immune-mediated inflammation (85), but more studies are required to understand these associations. Finally, prior infections may play a role in shaping an individual's immune repertoire and resulting capacity to combat later infections, as evidenced by the more serious course of EBV infection in adolescence or later in life.

Epstein-Barr Virus Serology – Assays, Antigens

The presence of EBV nucleic acid material in infected persons can be determined by numerous methods, e.g. by direct sequencing, fluorescence in-situ hybridization (FISH) and polymerase chain reaction (PCR) analysis of blood samples for EBV-derived DNA or RNA, while (prior) infection/reactivation may also be demonstrated by PCR analysis of saliva (86–91). In relation to testing of EBV in biopsy tissues, molecular detection of EBV-encoded RNA transcripts by FISH remains the gold standard. Moreover, EBV-encoded RNA hybridization and EBV LMP1 immunostains are used routinely to detect latent EBV in tissues affected by posttransplant lymphoproliferative disorder (PTLD) or in enlarged nodes from IM patients (92). Traditionally, serology is the simplest way to test for EBV infection and even for evaluating acute versus remote infection in healthy individuals. High serological titers serve as a tumor marker for some EBV-related malignancies, but titers are not a dependable tumor marker in immunocompromised hosts. EBV viral load testing by quantitative DNA amplification of blood samples has proven useful for early diagnosis and monitoring patients with PTLD (92).

Acute infection may also be inferred from analysis of IgM to viral antigens, while prior infection may be inferred from the presence of IgG to EBV antigens, and IgA can be used as a measure of epithelial infection load (45). Using three EBV antigens, viral capsid antigen (VCA) IgG, VCA IgM and EBNA1 IgG, it is normally possible to distinguish an acute from a past infection. While the presence of VCA IgM and VCA IgG without EBNA-1 IgG indicates a current acute infection, does the presence of VCA IgG and EBNA1 IgG without VCA IgM typically indicate a past infection (93).

Among the 85 proteins encoded in the EBV genome, several have been used for detection of Abs to EBV including EBNA1, EBNA2, VCAp23, VCAp18, early antigen diffuse (EAD), gP350, BARF1 (**Table 1**) (15, 94–97). IM has previously been associated with the presence of so-called heterophile Abs, however, this test has a rather low specificity and it remains unclear, what the test actually measures (2, 98).

Since induction of Abs follows a pattern of viral Ag production, seropositivity will depend on a person's ability to

control EBV and the balance between latent and lytic EBV infection. Moreover, any assay has a characteristic sensitivity and specificity for EBV detection, and some individuals may be judged false negative or positive. Thus, to fully define the incidence and prevalence of EBV infection in a population, several assays should be used, preferably combining assays for detection of viral nucleic acids, Abs to different viral antigens and the frequency of virus-specific T cells. Optimally, different detection principles may also be used; e.g. for Ab detection: enzyme-linked immunosorbent assay (ELISA) and immunoblotting, for T cell detection: antigen-induced cytokine release and peptide-MHC tetramer assays, and the assays should target different parts of the viral genome or different viral antigens representing both latent and lytic states. This is evidently very labor-intensive but may be realized by using multiplex techniques.

Epstein-Barr Virus and Diseases

Many diseases are known to be associated with EBV infection and prior IM increases the risk of many of these diseases (2, 99). IM itself is a prolonged state of fever, swollen lymph nodes, fatigue, malaise and various other symptoms. Few studies have focused on genetic factors associated with IM. Similar to EBV infection itself, some MHC-I and -II alleles and polymorphisms in the IL10 gene have been associated with IM development (82).

In contrast to the scarcity of information about genetic factors involved in EBV infection itself, several data has been published relating to EBV involvement in diseases and genetic factors associated with these. Several types of cancer, notably B cell lymphomas and nasopharyngeal epithelial carcinomas, affecting the two primary cell types targeted by the virus, are caused by EBV (99–102). This can be ascribed to EBV's ability to evade cellular antiviral mechanisms and control cellular apoptotic pathways and to its capacity for immune evasion (103). However, several other diseases affecting other cell types, which may become infected by EBV are known, including T cell lymphomas, NK cell leukemias and other T cells, NKT cells and NK cell lymphoproliferative diseases (101, 104, 105). Moreover, several systemic autoimmune diseases (SADs) and multiple sclerosis (MS) have been demonstrated to be associated with chronically relapsing EBV infection and inefficient immune control of the virus.

Systemic Autoimmune Diseases

SADs are a group of partly overlapping syndromes, also called connective tissue diseases, since they often are accompanied by inflammation of connective tissues. The SADs include the relatively common rheumatoid arthritis (RA) and the more rare conditions Sjögren's syndrome (SS), systemic lupus erythematosus (SLE), systemic scleroderma (SSc), and others (**Table 2**) (106, 107).

Epstein-Barr Virus and Rheumatoid Arthritis

The clinical characteristics of RA are swollen and painful joints, caused by synovial inflammation eventually resulting in exaggerated connective tissue deposition (pannus formation) and bone erosion, with resulting disability. Moreover, RA is frequently accompanied by systemic complications such as vascular disease, osteoporosis, and others (108–110). Most RA patients have characteristic autoantibodies (AuAbs) including rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPA)s, but many also have anti-nuclear Abs (ANAs) (111, 112). The etiology of RA is commonly ascribed to genetically determined defective self-tolerance, but environmental factors are known to play a dominating role, including EBV infection (113–116). Alleles of many genes are known to contribute to RA, notable HLA-DRB1 alleles containing shared epitope (SE) motives, but many other genes affecting the immune system and in particular lymphocytes have an impact (108, 110, 117). Tumor necrosis factor (TNF) plays an important role in a large proportion, if not most RA patients, and therapeutic Abs targeting TNF have good therapeutic efficacy in many patients (109, 118).

EBV evidently plays an important role in the etiology of RA, although not all evidence indicates an association between RA and EBV (119). Mechanisms behind the role of EBV in RA may include either molecular mimicry in the initiation of RA, bystander activation effects or chronic recurrent infection of joint epithelial cells and synovial B cells. The characteristic ACPAs seen in a major proportion of RA patients have been found to represent Abs to a citrullinated region of EBNA2, an important transcription factor of EBV expressed in lytic phases (120). Presumably, EBNA2 and possibly also other EBV proteins become citrullinated by peptidyl arginine deiminase (PAD) enzymes during the inflammatory process in RA joints (121, 122). RFs have been found to target cryptic epitopes of IgG heavy chains, presumably being released by lysis of EBV-infected B cells (123) and MHC-II molecules with SE motives (certain HLA-DRB1 alleles) have been found to be optimal ligands for EBV gP42, thus favoring EBV infection of B cells with these forms of MHC-II (31). Thus, the major characteristics of RA can be related to chronic EBV infection, and actually, serum EBV

TABLE 2 | Systemic autoimmune diseases (SADs) and their characteristics.

Disease	Genetics	Environmental factors
Mixed connective tissue disease (MCTD)	HLA-DRB1, multiple genes	VitD, smoking, EBV, sunburn, silica dust
Polymyositis – dermatomyositis (PM-DM)	HLA-DRB1, multiple genes	Smoking
Rheumatoid arthritis (RA)	HLA-DRB1, PTPN22, multiple genes	VitD, smoking, EBV
Sjögren's syndrome (SS)	HLA-DRB1, PTPN22, multiple genes	VitD, EBV, inverse correlation with smoking
Systemic lupus erythematosus (SLE)	HLA-DRB1, C', multiple genes	VitD, smoking, EBV, sunburn, silica dust
Systemic sclerosis (SSc) HLA-DRB1, multiple genes Silica dust, solvents		

DNA has been found to correlate with disease activity (124). Furthermore, EBV has been demonstrated to be present in the synovium of RA patients (115, 125, 126).

EBV and Sjögren's Syndrome

SS is a disease resulting in progressive destruction of exocrine salivary and lacrimal gland tissue. The major clinical characteristics are xerostomia and xeropthalmia in addition to fatigue and various other symptoms, which may also affect other organ systems (127, 128). Patients most often have ANAs and characteristic AuAbs are Ro60 and La Abs, but various other AuAbs may also be present. In addition, RFs are present in a majority of patients, whereas ACPAs are usually absent (128).

The etiology of SS has been suggested to involve several environmental and genetic factors, molecular mimicry and bystander activation (129, 130). Genetic factors include certain MHC-II (especially some HLA-DRB1) alleles, some MHC-I alleles and components of the interferon regulatory system (131). Environmental factors include vitD deficiency, smoking, silica dust exposure and virus infections (129). Especially EBV infection has been associated with SS (132, 133). The mechanisms involved in SS are presumably similar to RA and other SADs, but are much less studied. RA and SS often co-exist and SS primarily affects the epithelial tissues targeted by EBV, i.e. salivary and lacrimal glands, making the association with EBV infection particularly attractive.

Epstein-Barr Virus and Systemic Lupus Erythematosus

SLE is a disease, which clinically presents with a heterogenous array of symptoms, often evaluated by the SLE disease activity index (SLEDAI) or similar indexes, including complementemia, DNA Abs, leukopenia, thrombocytemia, fever, fatigue, skin rash, UV sensitivity, mucosal ulcers, alopecia, pleuriris or pericarditis, proteinuria, hematuria, nephritis, myositis, arthritis, vasculitis, headache, stroke, and more rarely, neuropsychiatric symptoms (134–137). The disease may show a relapsing/remitting course, depending on the efficacy of treatments (138, 139).

SLE has been described as an immune complex disease, since it is often associated with decreased levels of complement components (140). Other characteristics are the presence of ANAs, notably DNA Abs, which are included in the SLEDAI, but in many cases AuAbs to a heterogenous panel of AuAgs are present and changes in the AuAb profile may reflect changes in disease activity (141–144).

Genetically predisposing factors are first of all certain HLA-DRB1 alleles, but multiple immune system genes, including other MHC-II alleles and some MHC-I alleles, as well as genes affecting cellular waste removal, have been found to influence disease development (145, 146).

Major environmental factors promoting development of SLE are silica dust exposure, sun burn, smoking, vitD deficiency and EBV infection (147–153). The etiology has been suggested to involve molecular mimicry between EBV EBNA1 and cellular Ro 60, and/or bystander activation (154, 155).

Decreased immune control of chronic EBV infection has been found to be a contributing factor, if not a major cause (152, 156, 157), but other infections may also play a role in SLE development or exacerbation (158, 159). The presence of DNA Abs and other ANAs would seem to be compatible with infection by a DNA virus in combination with inefficient removal of apoptotic and necrotic material.

DISCUSSION

SADs constitute a group of partly overlapping autoimmune disease syndrome and include systemic sclerosis (SSc), mixed connective tissue disease (MCTD) and polymyositis/dermatomyositis (PM/DM) in addition to RA, SS, and SLE (**Table 2**). These diseases share several genetic and environmental factors, in particular the predisposing effect of certain HLA-DRB1 alleles (although not exactly identical alleles), the predisposing effect of EBV infection and of factors, which can be related to EBV infection (e.g. vitD deficiency) (**Table 2**) (106, 107, 160–171).

The evidence for a major etiological role of EBV is particularly strong for RA, where several of the clinical characteristics can be related to EBV as described above (RFs, ACPAs, SE-allele disposition). Current treatments can also be related to EBV infection, e.g. CD20 monoclonal antibodies (MAbs), which presumably diminish the burden of EBV-infected (memory) B cells, and TNF MAbs, which possibly diminish the burden of EBV infection by an anti-inflammatory effect (172–174). The evidence for an etiological role of EBV in SLE is also strong and seems to point to EBV infection of epithelial cells in combination with decreased removal of apoptotic/necrotic cell debris (175). Thus, these two prototype SADs can be seen as the results of a chronic, poorly controlled, relapsing/remitting EBV infection targeting the two major host cells of EBV; B cells in RA and epithelial cells in SLE. In RA, relapses most likely follow re-activation of EBV in (memory) B cells upon Ag stimulation. This results in production of EBV-transformed B cell blasts, which by their very nature will attempt homing to bones and therefore will have a tendency to populate joints, where the concomitant lytic EBV production may also result in EBV infection of synovial epithelial cells. In SLE, B cells will also be involved, thus accounting for the common involvement of joints and other symptoms overlapping with RA, however, the major target cells affected are epithelial cells, thus accounting for the common skin and mucosal pathology, while the defective removal of EBV and cellular debris results in immune complex deposition in affected organs and in particular kidneys, by virtue of their filtrating actions. SS has been studies less intensively than RA and SLE but the relation to EBV is nevertheless even more obvious. In SS, pathological symptoms reminiscent of both RA and SLE are seen. This again reflects the tendency of EBV to "shuttle" between B cells and epithelial cells and in particular the ability of EBV to return to salivary (and lacrimal) gland

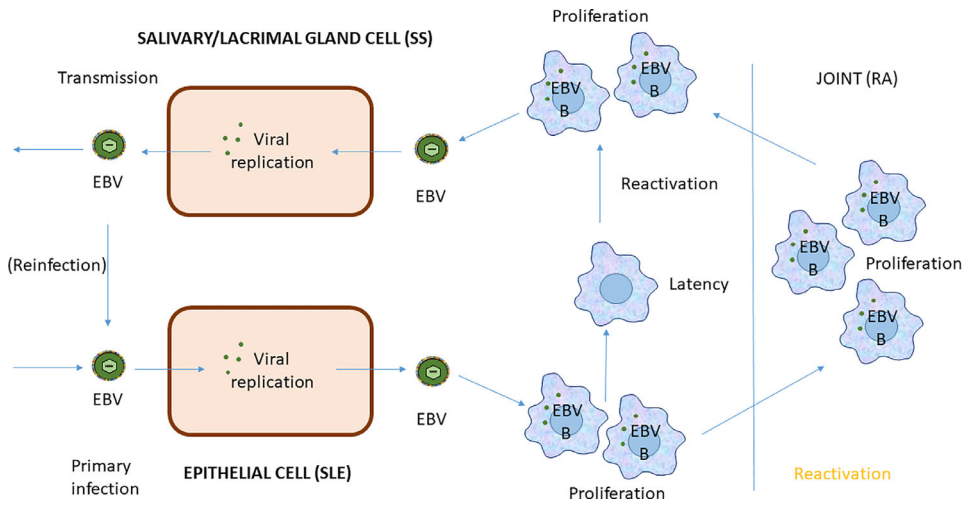

FIGURE 4 | EBV Infection cycle in B cells of systemic autoimmune diseases.

epithelial cells as part of its natural life cycle (**Figure 4**). Thus, SS may in some respects be thought of as SLE effecting the exocrine glands, while SS also has many characteristics in common with RA.

Other autoimmune diseases, especially MS have also been found to depend on EBV infection in several aspects (176). The question therefore arises, how EBV can be involved in these apparently diverse diseases? A common feature seems to be decreased immune control of EBV. T cells are crucial for the control of EBV (and other viruses) and defective/exhausted T cell repertoires are characteristic of SADs (177). This allows for chronic infections with continuous cycles of relapses and remissions. However, while this may explain a common involvement of EBV (or other viruses) in disease etiology, it does not explain the different clinical appearances and the differences in e.g. association with different HLA alleles. A plausible explanation is that the role of EBV does not depend solely on e.g. entry, which in RA seems to be facilitated by SE-containing HLA alleles. Other HLA interactions must also be involved, e.g. presentation of EBV and/or host peptides, interactions with the peptide loading complex, interaction with other EBV or host proteins, etc. In general will the genetic composition of the host determine the fate of EBV in different cell types, including the interactions of EBV attachment and entry proteins with the target cell membrane proteins, the ability of the host cell to undergo apoptosis and the possibility to support lytic production of virus, and the efficiency of adaptive immune control of EBV. Since there are large differences in individual immune systems and in infection histories, one possibility for the different appearances of EBV-related diseases could also be individual mutations in EBV genomes during chronic infections and/or re-infections, and/or different rates of co-infection with other viruses. Patients with SADs are often prone to various infections, possibly due to inherent or acquired immune deficiencies, which predispose to coinfection with other viruses e.g. cytomegalovirus and others, which have been suggested to play a role in SAD development (178–181).

Patients with SADs also have increased tendency to develop cancer, including various forms of lymphoma. This may relate to secondary effects of treatment with immunosuppressive drugs but may also reflect an inherent ability of EBV to cause transformation of B cells and epithelial cells (13, 99–102, 160).

CONCLUSION

EBV has been found to play a role in several, if not all SADs. It remains unclear, whether the role of EBV is primarily in initiation of disease (e.g. by molecular mimicry) or is simply due to the chronic relapsing-remitting nature of EBV infections. Many characteristics of especially RA can be ascribed to EBV infection, but this may also be the case for other SADs. Future studies should focus on interaction of EBV proteins and non-coding RNAs with host molecules and on the role of other viruses in relation to EBV infection.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Putative Pathobionts in HLA-B27-Associated Spondyloarthropathy

Tejpal Gill[1] *and James T. Rosenbaum*[2,3]*

[1] Division of Arthritis and Rheumatic Diseases, Department of Medicine, Oregon Health & Science University, Portland, OR, United States, [2] Departments of Ophthalmology, Medicine, and Cell Biology, Oregon Health & Science University, Portland, OR, United States, [3] Legacy Devers Eye Institute, Portland, OR, United States

Spondyloarthritis (SpA) is a group of immune mediated inflammatory diseases with a strong association to the major histocompatibility (MHC) class I molecule, HLA-B27. Although the association between HLA-B27 and AS has been known for almost 50 years, the mechanisms underlying disease pathogenesis are elusive. Over the years, three hypotheses have been proposed to explain HLA-B27 and disease association: 1) HLA B27 presents arthritogenic peptides and thus creates a pathological immune response; 2) HLA-B27 misfolding causes endoplasmic reticulum (ER) stress which activates the unfolded protein response (UPR); 3) HLA-B27 dimerizes on the cell surface and acts as a target for natural killer (NK) cells. None of these hypotheses explains SpA pathogenesis completely. Evidence supports the hypothesis that HLA-B27-related diseases have a microbial pathogenesis. In animal models of various SpAs, a germ-free environment abrogates disease development and colonizing these animals with gut commensal microbes can restore disease manifestations. The depth of microbial influence on SpA development has been realized due to our ability to characterize microbial communities in the gut using next-generation sequencing approaches. In this review, we will discuss various putative pathobionts in the pathogenesis of HLA-B27-associated diseases. We pursue whether a single pathobiont or a disruption of microbial community and function is associated with HLA-B27-related diseases. Furthermore, rather than a specific pathobiont, metabolic functions of various disease-associated microbes might be key. While the use of germ-free models of SpA have facilitated understanding the role of microbes in disease development, future studies with animal models that mimic diverse microbial communities instead of mono-colonization are indispensable. We discuss the causal mechanisms underlying disease pathogenesis including the role of these pathobionts on mucin degradation, mucosal adherence, and gut epithelial barrier disruption and inflammation. Finally, we review the various uses of microbes as therapeutic modalities including pre/probiotics, diet, microbial metabolites and fecal microbiota transplant. Unravelling these complex host-microbe interactions will lead to the development of new targets/therapies for alleviation of SpA and other HLA-B27 associated diseases.

Keywords: pathobiont, HLA-B27, spondyloarthritis, gut inflammation, dysbiosis

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***Correspondence:**
James T. Rosenbaum
rosenbaj@ohsu.edu

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INTRODUCTION

Spondyloarthritis (SpA) is an umbrella term used for various disorders including ankylosing spondylitis (AS), arthritis associated with inflammatory bowel disease (IBD), acute anterior uveitis, a subset of juvenile idiopathic arthritis (JIA), reactive arthritis (ReA), psoriatic arthritis (PsA), and undifferentiated spondyloarthritis (USpA) (**Figure 1**). These diseases share common clinical features (such as sacroiliitis, enthesitis and dactylitis) and overlapping extra-articular manifestations (i.e., uveitis, psoriasis, and bowel inflammation). Uveitis is the most common extra-articular manifestation of AS. In addition, many AS patients also have gut inflammation, such as Crohn's disease (CD) and ulcerative colitis (UC). On the other hand, axial/peripheral arthritis is the most common extra-intestinal complications in IBD, especially in patients with CD (1, 2). These conditions may occur either simultaneously or sequentially, with almost 50% of AS patients having subclinical gut inflammation and around 15% of IBD patients have peripheral SpA (3, 4). In addition to similar and overlapping disease manifestation, there is a considerable overlap among the genetic risk factors for AS, CD, and PsA, such as *IL23R*, *IL12B*, *STAT3*, *ORMDL3*, and *CARD9* (5), which are associated with IL-23 signaling. The immune and inflammatory response between AS and CD shows considerable overlap dominated by the Th17 helper cell pathways (6, 7). In addition, association with a non-major histocompatibility gene Endoplasmic Reticulum Aminopeptidase 1 (ERAP1) has also been reported in patients with AS (8) and IBD (9) either alone or in combination with polymorphisms in HLA class-I alleles (10). Furthermore, other factors such as environment, host immune regulation, disruption of mucosal barrier, and gut microbial dysbiosis contribute toward pathogenesis of SpA [(11, 12) **Figure 2**]. Host genetic susceptibility is associated with perturbed immune/inflammatory response, which may lead to microbial dysbiosis and pathobiont expansion and loss of barrier function, resulting in inflammation in the gut, joints, eye, and skin (13–16). While our focus for this review is on HLA-B27-associated microbes and their role in various SpAs, we will also discuss pathobionts and host-microbial relationships in IBD that are relevant to this topic.

HLA-B27, SPONDYLOARTHRITIS, AND GUT MICROBIOME

Human leukocyte antigen (HLA)-B27, is a major histocompatibility complex (MHC)-class I molecule associated with various SpAs. Its association with the prototypic SpA- ankylosing spondylitis was discovered almost 50 years ago (17), and is the strongest association

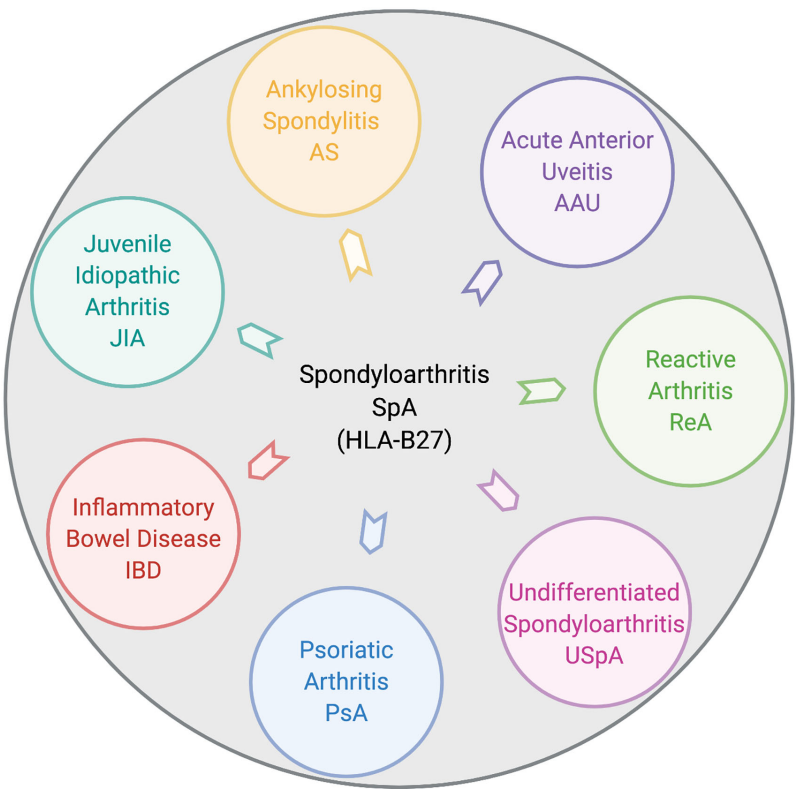

FIGURE 1 | Disease overlap in HLA-B27-associated spondyloarthropathies. Pictorial representation of various disorders broadly included within HLA-B27-associated spondyloarthritis (SpA). These include ankylosing spondylitis (AS), acute anterior uveitis (AAU), reactive arthritis (ReA), juvenile idiopathic arthritis (JIA), inflammatory bowel disease (IBD), psoriatic arthritis (PsA), and undifferentiated spondyloarthritis (USpA). Figure created with Biorender.com.

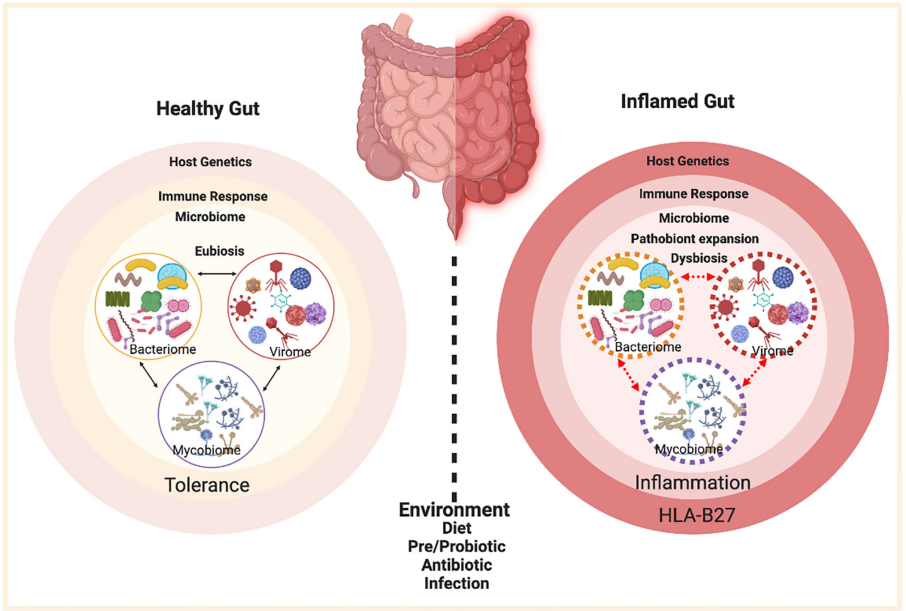

FIGURE 2 | Factors contributing to HLA-B27 associated gut inflammation in SpA. In the healthy gut (left panel) there is microbial eubiosis/homeostasis within the members of bacteriome, mycobiome, and viruses in a stable interdependent microbial community. The gut immune response recognizes commensals and does not mount an inflammatory response. A non-susceptible host genetic background and a lack of environmental stress contribute toward favorable host-microbe interactions and microbial homeostasis. On the contrary, HLA-B27 associated gut (right panel) displays a microbial dysbiosis, which may be associated with pathobiont expansion, dysbiosis in various microbial components (bacteriome, mycobiome, virome) and loss of colonization resistance from the gut commensal microbes. HLA-B27 associated host immune dysregulation can cause loss of barrier protection and therefore bacterial components can activate an aberrant immune response, which in turn triggers an inflammatory response. Figure created with Biorender.com.

known between a genetic factor and a complex, genetic disease. Subsequently, the association of HLA-B27 was found with ReA, axial arthritis in association with IBD, AAU and the axial arthritis subset of PsA (18–20). The MHC complex is located on chromosome 6 (21), and plays a critical role in immunity and recognition of self in almost all cells of the body (22). MHC class I includes HLA-A, HLA-B, and HLA-C, and presents antigen to CD8 T cells. Despite HLA-B27 being a class I molecule, CD8 T-cells have not been associated with disease development; instead CD4+ T cells are thought to drive disease (23). While the actual role of HLA-B27 in triggering inflammation in various disorders remains unresolved, three different theories have been suggested. These include, i) presentation of arthritogenic peptides, which may activate a pathological immune response (24) ii) misfolding of heavy chain of HLA-B27 and its effects on ER-associated degradation (ERAD) and activation of unfolded protein response (UPR) (25–28); and iii) HLA-B27 dimerization during cell surface recycling and recognition by immune receptors on natural killer (NK) cells (29, 30). While these hypotheses may provide some explanation, such as the activation of the inflammatory IL-23/lL-17 axis through UPR or non-canonical activation of CD4+ T cells by HLA-B27 dimers, the exact mechanism by which HLA-B27 leads to development of SpA is unknown (31). In the last decade, HLA-B27-associated perturbation in the gut microbiota has emerged as an underlying mechanism in disease pathogenesis. Indeed, many of the risk genes common to IBD and SpA (e.g., *IL23R*, *NOD2*) are associated with innate immune response pathways (32, 33), consistent with the hypothesis that alteration in the host immune response to gut microbes may play a key role in these disorders.

More than twenty-five years ago, a new insight came from the experimental data revealing the role of gut microbiota in complex inflammatory disorders. In 1994, it was observed that HLA-B27 transgenic (TG) rats raised in a germ-free environment fail to develop either arthritis or colitis (34). Recolonizing the gut with either altered Schaedler's flora (ASF) or just a few commensal microbes was sufficient for disease development (35, 36). Rosenbaum and Davey proposed a hypothesis that 'HLA-B27 predisposes to ankylosing spondylitis by altering the microbiome' (37). Since the last decade many studies in patients and rodent models have shown alteration of gut microbial communities in various HLA-B27 associated disorders (12, 38–40). Based on our extensive studies with the HLA-B27 TG rat model, we demonstrated that HLA-B27-associated microbial dysbiosis is subject to host genetics and environment. This lead to the proposal of an ecological model of microbial dysbiosis (12).

PUTATIVE PATHOBIONTS IN HLA-B27 ASSOCIATED SPONDYLOARTHROPATHIES

Through the advent of high throughput sequencing approaches, many putative pathobionts have been identified in various HLA-B27-associated SpAs and bowel inflammation. A pathobiont is

defined as a microbe that can cause or promote disease only when specific genetic or environmental conditions are altered in the host such as dysregulated host immune response and microbial dysbiosis (41). The term "pathobiont" was first used for *Helicobacter hepaticus*, a bacterium associated with gut inflammation in immunocompromized mice, but not in wild-type animals (42, 43). While most of the sequencing studies have focused on the alterations in bacterial component of the gut microbiota, recent research is also focused to define the fungal and viral component of the gut microbiome (mycobiome and virome respectively) and interkingdom interactions in SpA. In this section, we will discuss important bacterial, fungal, and viral pathobionts in HLA-B27-associated SpAs including overlapping inflammatory diseases such as CD and colitis.

Bacterial Pathobionts

Pathobionts Associated With Reactive Arthritis

Reactive arthritis (ReA) is an immune mediated inflammation of the synovial tissue that usually develops after a urinary or gut infection (44). HLA-B27-associated ReA is a type of SpA triggered by bacteria such as *Campylobacter*, *Chlamydia*, *Salmonella*, *Shigella*, and *Yersinia*, resulting in oligoarthritis of the lower limbs and sometimes with urethritis and conjunctivitis (45). One of the earlier studies isolated *Chlamydia trachomatis* strains from the eye and urethra of a patient with ReA, previously known as Reiter's Syndrome (46). Subsequently, in a study focused on endoplasmic reticulum (ER) stress, which is thought to be downstream to HLA-B27 protein misfolding, *C. trachomatis* was shown to induce IL-23 expression in infected myeloid cells (47). In addition, stimulation through TLR or ER stress can cause the activation of ER stress induced transcription factor CHOP, which in turn increases the expression of IL-23. However, TLR engagement in itself can trigger ER stress through activation of XBP1, essential for production of inflammatory cytokines in macrophages (48). HLA-B27 misfolding was associated with enhanced replication of *Salmonella* by the activation of the unfolded protein response (UPR), through the transcription factor XBP1 (49) Although this study employs HeLa cells that do not express TLR, it is possible that innate immune receptors may be involved in response to bacteria or bacterial products (50). In another study, peripheral blood and serum polymerase chain reaction (PCR) analysis in patients with *Chlamydia* induced ReA showed the presence of DNA from *C. trachomatis* in the peripheral blood cells, but not in the serum (51). This can be explained by the fact that *C. trachomatis* resides in the monocytic cells, prevents their apoptosis and stimulates the production of inflammatory mediators (51). In fact, it is a hallmark of Chlamydia induced ReA, in which bacteria causing synovitis persist in low quantities making it hard to detect using PCR or culture techniques. However, Freise and colleagues later standardized PCR detection for *C. trachomatis* from synovial fluid (52). This may explain why various attempts to cultivate other pathogenic bacteria such as *Yersinia* or *Salmonella* from affected joints have yielded negative results (53, 54). Instead, *Salmonella* and *Yersinia* antigens have been identified in synovial fluid and/or tissue by immunohistochemistry. Antibodies against LPS from these microbes has been shown to be present in the synovial fluid many years after the infection which explains the strong IgA responses in people who have had a bout of ReA (53, 54). In a mouse model of *Salmonella enteritis* induced joint inflammation, increased levels of inflammatory cytokines IL-17 and TNF-α were observed in the mesenteric lymph node and synovium respectively. Neutralizing IL-17 in mice infected with *S. enteritis*, prevented synovitis and curbed the increase in TNF-α, suggesting the role of IL-17 in gut and joint inflammation (55). In addition to these pathogenic microbes, diarrheagenic *Escherichia coli* (DEC) has also shown to increase the incidence of musculoskeletal symptoms in individuals who contracted DEC associated diarrhea during their international travels. Of these patients, a small number of patients also met the criteria for ReA (56). Thus, while ReA may be highly associated with various bacterial enteric pathogens such as *Campylobacter* and *Salmonella* (57), its incidence after DEC infections is low. Recent study in the *Yersinia enterocolitica* murine model of ReA (*TNFRp55-/-* mice) has shown an important role for mesenteric dendritic cells. Intestinal dendritic cells migrated to the regional lymph nodes and contributed toward the immunopathogenesis of ReA (58). It is important to note that in most cases, infections with enteric pathogens do not result in development of ReA. In a comprehensive review by Ajene and colleagues (59), the ReA incidence for *Salmonella*, *Campylobacter*, and *Shigella* ranged from 0.1%–29%, 0%–16%, and 0%–12% respectively. Nevertheless, in studies in which enteric pathogens do lead to the development of ReA, the ability of a bacterium or bacterial antigen to reach the joint or gain access to particular cells such as the macrophages and evade the host defense might play an important role. In view of these different microbes associated with ReA, disease pathogenesis is thought to involve host-microbe interactions as evident with the presence of bacteria or their products in the joint, followed by local immune response.

Altered Schaedler's Flora (ASF)

In 1965, Russell Schaedler developed a model microbial community to colonize germ-free animals to prevent the colonization of opportunistic pathogens (60), which was later modified to be more representative of gut microbiota and renamed as Altered Schaedler's Flora (ASF) (61). 16s rRNA sequencing of ASF was performed to define the phylogeny of the ASF microbes (namely two members of *Clostridium* sp., *Lactobacillus intestinalis*, *Lactobacillus murinus*, *Mucispirillum schaedleri*, *Eubacterium plexicaudatum*, *Pseudoflavonifractor* sp., and *Parabacteroides goldsteinii)* (62, 63). Even though the ASF is a reductionist model microbial community, functional analysis of the ASF metagenome compared with the wild mice metagenome showed the functional similarity between gut microbiome of ASF and wild type mice (64). Early experiments colonizing germ-free HLA-B27 TG rats with ASF played a pivotal role in establishing the role of gut microbiota (especially *Bacteroides*) in the development of gut inflammation (35). Another mouse model for SpA is the SKG model, which has a mutation in the ZAP-70 (T cell receptor signaling gene). Upon injecting with curdlan, a component of bacterial and fungal cell walls, these mice develop SpA with uveitis, arthritis, and CD like

ileitis (65, 66). Germ-free SKG mice recolonized with ASF exhibit increased arthritis incidence, although the severity of arthritis was attenuated in comparison with the specific pathogen free (SPF) mice (66). SPF mice had the highest incidence of Ileitis followed by ASF recolonized mice, while the germ-free mice did not have Ileitis (66). This suggests that dysregulation of mucosal-microbe interface is necessary for the development of ileitis, which fails to occur in germ-free SKG mice. In comparison, ileitis is mild in SKG mice colonized with ASF, highlighting the importance of a diverse microbial community in disease development. ASF studies have paved a way to understand the host-microbe interaction in a measurable way and have emphasized the role of commensal microbes acting as pathobionts in disease development.

Dialister

Dialister is a saccharolytic bacteria, belongs to family *Vellionelaceae* (67), that can convert succinate to propionate (68). Tito and coworkers (69) studied the relationship between the intestinal microbial composition of ileal and colon biopsies from inflamed and non-inflamed tissues, and observed SpA-associated microbial dysbiosis. Of note, they found that *Dialister* was increased in the inflamed tissue and positively correlates with the disease score, whereas the non-inflamed tissue had low frequency of *Dialister*. Another study examining post-infectious SpA reported an increase in the relative abundance of *Dialister*. Subjects who developed enthesitis also had increased abundance of *Campylobacter* and subjects with uveitis and radiographic sacroiliitis had increased abundance of *Erwinia* and unclassified *Ruminococcaceae*, respectively (70). Interestingly, some species of *Dialister* such as *D. pneumosintes* and *D. invisus* are shown to be pathogenic in orthodontic infections (71). Oral pathobionts can colonize the gut during inflammation, as there is increased availability of oxygen and lack of colonization resistance during inflammatory conditions (72). This in turn drives the Th1 response primarily by interferon gamma (IFNγ), and exacerbates gut inflammation (72). Another study focused on microbial dysbiosis associated with HLA alleles in healthy subjects with AS, and rheumatoid arthritis (RA), a chronic autoimmune disease defined by inflammation of the synovium and joint destruction (73). However, the authors did not observe HLA-B27-associated changes in *Dialister* in healthy subjects with either AS or RA associated alleles. While one study focused on the microbiota from biopsies collected from post-infectious SpA patients (69), the latter study focused on the fecal samples from HLA-B27 positive healthy individuals, many of whom will not develop disease. Stated differently, while it is possible for patient cohorts from different geographical locations to have distinct microbes driving disease, differences due to sampling location and disease severity also contributes to the association with distinct pathobionts.

Blautia

Another pathobiont associated with AS is *Blautia*. It is a member of the family *Lachnospiraceae*, which has been associated with gut inflammation in an experimental model of SpA (48, 74). A recent study by Zhang and colleagues (75) on the fecal samples from AS patients in a Chinese cohort has shown *Blautia*, *Megamonas* and *Dorea* associated with AS patients with a concomitant decrease in *Lachnospira*, *Ruminococcus*, and *Clostridium_XlVb*. In a study on patients with ReA, the authors reported an increase in enteropathogens such as *Erwinia* and *Pseudomonas* as well as several other microbes including *Blautia*, *Coprococcus*, *Roseburia*, and *Collinsella* (70). Many of these microbes (e.g., *Blautia*) were thought to be gut commensals, but new studies have shown them to be increased specifically with disease and thus a pathobiont. In a rat model of SpA, *Blautia* has been associated with HLA-B27 and SpA on the Lewis background, but not on the Fischer background (12). Hablot and colleagues (76) compared the microbial dysbiosis in mice with dextran sodium sulfate (DSS) induced colitis from mice with arthritis and colitis (induced with collagen and DSS). They found that mice with arthritis and colitis had increased relative abundance of *Blautia*, *Gemellaceae*, and *Ruminococcus gnavus* as compared with the colitis only group. Both *Blautia* and *Ruminococcus* are closely associated members of the family Lachnospiraceae, which are among the main producers of short chain fatty acids (SCFAs), and many taxa of this family are associated with various inflammatory diseases (77). While the role of *Blautia* in HLA-B27 associated SpAs was discussed in this section, the role of *Ruminococcus gnavus* as a pathobiont is discussed below.

Ruminococcus gnavus

Ruminococcus gnavus is a known pathobiont associated with SpA and associated IBD (51, 78). In a cohort of SpA patients and related as well as unrelated healthy controls, there was an increase in the relative abundance of *R. gnavus*, which correlated with the disease activity and with patients having a history of IBD. This change was not observed in their subjects with RA (78). Increases in *R. gnavus* have been associated with other inflammatory diseases including inflammatory bowel disease (79, 80), CD (81), and pouchitis in UC patients (82). Another instance of increased abundance of *R. gnavus* comes from patients with systemic lupus erythematosus (SLE), an autoimmune disease characterized by a hyperactive immune system which causes inflammation in many tissues, as well as an aberrant antibody response. Most SLE patients will develop either arthritis or synovitis sometime during their disease. A study on SLE patients showed an increased amounts of *R. gnavus* that correlated with disease activity, which was highest in the patients with lupus nephritis (83–85). One plausible mechanism for the contribution of *R. gnavus* in an inflammatory disease, CD, has been shown by Henke and colleagues (86). They found that *R. gnavus* secretes a complex gluco-rhamnan polysaccharide, which can induce the production of inflammatory cytokines like TNFα by activation of TLR4 on the dendritic cells and may explain the mechanism underlying the association between gut inflammation in CD and *R. gnavus*. TNFα is a potent inflammatory mediator in both SpA and IBD. This may explain why an increase in the relative abundance of *R. gnavus* in patients correlates with disease severity in both arthritis and gut inflammation. *R. gnavus* is also shown to provide colonization resistance to the gut microbial community by the

production of bacteriocin ruminococcin A, which is active against pathogenic members of class *Clostridia*, especially *Clostridium perfringens, Clostridium difficile*, and other members phylogenetically related with *R. gnavus* (87), thus providing a competitive edge in gut colonization and inflammation. These studies show the harmful role of R. gnavus through different mechanisms. Association of *R. gnavus* and other pathobionts with both SpA and IBD may partially explain the overlap between the mechanisms underlying these complex inflammatory diseases in many patients.

Akkermansia muciniphila

Many studies on human and animal models of SpA have shown increased abundance of *Akkermansia muciniphila*, a mucin degrading bacteria found in human intestinal content (88). A study on pediatric SpA cohort, the microbiota from patients separated from healthy controls, and was divided subjects into two clusters each dominated by increased levels of either *A. muciniphila* or genus *Bacteroides* (89). Furthermore, to evaluate the pathogenicity of altered microbial composition in children with SpA, the group performed fecal microbial transplant to germ-free K/BxN mice. Transplanted mice displayed over-representation of *Bacteroides* and *Akkermansia*, and the latter positively correlated with disease activity. Addition of *Akkermansia* to ASF also increased the permissiveness to arthritis in these mice, when compared to mice that received ASF alone (90). Metagenomic analysis of fecal samples from patients with enthesitis related arthritis (ERA) showed decrease in the relative abundance of *Faecalibacterium prausnitzii* in both pediatric and adult SpA cohort, while the relative frequency of *Bacteroides fragilis* was increased in pediatric SpA cohort and decreased in adult SpA cohort (91). *A. muciniphila* has also been associated with disease in experimental SpA. In HLA-B27 TG Fischer rats, the relative abundance of *A. muciniphila* was increased along with elevated IgA coating of intestinal microbes (11). In a subsequent study, we compared the effect of host genetic background on microbial dysbiosis and found that the increased level of *A. muciniphila* was found in the Fischer HLA-B27 rats in comparison to Fischer wild-type controls; while in Lewis HLA-B27 TG rats there was an increase in the relative abundance of *Prevotella* when compared to Lewis wild type controls. Addition of *A. muciniphila* in germ-free and SPF *IL10-/-* mice is sufficient to exacerbate gut inflammation. IL10 is an anti-inflammatory cytokine and *IL10-/-* mice develop chronic colitis with marked increase in pathological type-I helper T cell response (92) in SPF but not in germ-free conditions. NLRP6 deficiency in these mice results in the enrichment of *A. muciniphila*, which then acts as a pathobiont in the development of colitis (93), and highlights the ability of NLRP6 in regulating colonization of colitogenic bacteria. Contrary to the role of *A. muciniphila* in inflammation, relative abundance of *A. muciniphila* has been shown to have an inverse correlation with obesity and metabolic diseases (94, 95). In fact, supplementation of *A. muciniphila* reversed high fat diet induced obesity in mice, which was mediated by altered adipocyte metabolism and improved gut barrier function (96). *Akkermansia* is a short chain fatty acid producer (97) and is thought to increase fatty acid oxidation in intestines and adipose tissues (98). Taken together, these distinct and opposite (pathobiont vs commensal) roles of *A. muciniphila* in various disorders highlight the tight regulation of the microbial abundance within the community and the effect of dysbiosis (increase or decrease in relative abundance) in health and disease.

Prevotella

Prevotella is another mucus degrading pathobiont that shows gut inflammation associated increased relative abundance in *NLRP6-/-* mice (99). *Prevotella* can reach to the crypt in the mucosal layer of the gastrointestinal tract, and are associated with SpA. Metagenomic analysis from the gut microbial DNA from a Chinese cohort of AS patients has revealed the abundance of Prevotella *melaninogenica, Prevotella copri*, and *Prevotella* spp. C561 and decreased abundance in *Bacteroides* spp (100). Interestingly, they also observed increased abundance of the *Bifidobacterium* genus, a commensal gut bacteria commonly found in probiotics. On the contrary, there was a decrease in the abundance of family *Prevotellaceae* in AS patients. This may be due to differences in the cohorts (Chinese vs caucasian), sampling location (fecal vs ileal biopsy), or it could also be due to the relationship between other members of the microbial community and/or the effect of host genetics (100). Scher and colleagues observed increased relative abundance of *P. copri* has also been reported in 16s microbiome sequencing of fecal samples from patients with new onset RA (101). They performed metagenomic sequencing of patient derived *Prevotella* strains and also compared the metagenomes between healthy controls and new-onset RA patients. Patients with new onset RA had decreased abundance of vitamin metabolism (i.e., biotin, pyroxidal, and folate) and pentose phosphate pathway which was consistent with Prevotella genomes lacking these functions. To determine whether Prevotella copri was sufficient to drive gut inflammation, they gavaged antibiotic treated mice with P- copri and after 2 weeks they found that P. copri had dominated the gut microbiota in these mice and exacerbated the susceptibility to DSS induced colitis (101). Consistent with these mouse studies, another study found enrichment of *P. copri* in patients during the pre-clinical phase of RA, before disease onset (102), which suggests a role of *P. copri* in intestinal dysbiosis and disease susceptibility. A recent cross-sectional study utilized data from a previous TwinsUK cohort, and used genotyping and microbiota data after excluding patients with RA and their twins. The authors found *Prevotella* spp in the gut microbiota of individuals who had RA associated genotype associated without the disease. again suggesting a role for host-microbe interactions prior to disease onset (103).

Mucispirillum schaedleri

Mucispirillum schaedleri, a Gram negative pathobiont, is a member of ASF, and is known to colonize the gut mucus layer in rodents (104). It was reported that in mice having a combined deficiency of two susceptibility genes for CD, namely nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and NADPH oxidase, disease can be induced by

M. schaedleri (105). *NOD2* pays an important role in microbial regulation in the ileum (106), whereas *NADPH oxidase* is known to regulate the gut intestinal barrier through the production of reactive oxygen species (107). The authors demonstrated that in the absence of both *NOD2* and phagocytic *NADPH oxidase*, there is accumulation of *M. schaedleri* in the gut lumen and mucosa associated with gut inflammation. Since, *Mucispirillum* is a bacterium found in rodents, it is an unlikely contributor to human diseases. However another bacterial taxa, *Proteobacteria*, which is closely related to *Mucispirillum* have been reported to have increased abundance in both patients with SpA (108) and CD (109). In contrast, another study reported that *M. schaedleri* can protect mice against *Salmonella typhimurium* virulence factors (110). This was supported by data from our study with the HLA-B27 TG rats, in which we found a decrease in the relative abundance of *M. schaedleri* as compared to the wild type rats (12), which may suggest a protective effect of the microbe. Since M. *schaedleri* is a mucolytic bacterium like *A. muciniphila*, we can hypothesize that it may be beneficial to the host at low relative abundance within the microbial community. However, increased abundance of *M. schaedleri* may compromise the spatial segregation by bringing luminal microbes close to the intestinal epithelial cells, thereby triggering an inflammatory response.

Adherent Invasive Escherichia coli

Invasive properties of various bacteria such as *E. coli*, *S. typhimurium*, and *Citrobacter rodentium*, may be critical for their ability to colonize the host. Of these, *E coli* has been associated with the induction of gut inflammation in CD (111). A study characterizing adherent invasive *Escherichia coli* (AIEC) found that these *E. coli* may harbor genes associated with bacterial adhesion and invasion and therefore regulate barrier permeability contributing to gut inflammation (112). Another study assessed the prevalence of AIEC associated with the intestinal mucosa of patients with CD, UC, and of healthy controls (113). They found that AIEC was found associated with the inflamed regions in the ileal mucosa in patients with CD, but was not observed in the ileal mucosa of patients with UC. In another study on the IgA coated bacterial fraction from patients with CD-associated SpA detected enrichment of *E. coli* in comparison to patients with CD alone. This IgA coated *E. coli* fraction displayed genotypic and phenotypic similarities to AIEC. Colonization of these AIEC in germ-free mice induced inflammatory Th17 mucosal immune response in comparison to colonization with non-AIEC strains of *E. coli* (114). This study identified immune reactive pathobionts that provide a link between mucosal immunity and systemic inflammation in CD-associated SpA and may guide future therapies.

Fungal Pathobionts

While microbial dysbiosis and pathobiont enrichment have been associated with SpA and associated CD for almost two decades, most of the work has been focused on the gut bacteria and some *Archaea*. However, fungal products such as β-glucan have been known to trigger SpA and ileal inflammation in SKG mice model (BALB/c ZAP-70W163C mutant) of SpA (115). Another study has shown the association of anti-*Saccharomyces cerevisiae* antibodies (ASCA) with intestinal inflammation in patients with Ax SpA and associated CD (116). In the last decade, with new and advanced approaches to characterize the fungal component of the gut microbiome (mycobiome), their role in disease pathogenesis is being studied more extensively. In a recent study in patients with SpA, treatment with IL-17 inhibitors was associated with a shift in bacterial and fungal taxa, specifically the bacteria from the family Clostridiales and the yeast *Candida albicans* (117). In these patients, the changes in the gut microbiome were associated with the perturbations in metabolic pathways and overexpression of IL-17/23 cytokines and the expansion of IL-25/17 producing tuft cells as well as type 2 innate lymphoid cells (ILC2), both of which are implicated in helminth immunity (117). A study investigated the mycobiome in IBD (CD and UC) and found increased intestinal fungal diversity in patients with CD in comparison with healthy controls (118). However, they did not observe any difference between the fungal species between the CD and UC groups. Another study focused on the relationship between *Candida albicans* and gut inflammation by using mice that lack Galectin 3 (Gal3-/-), an intestinal lectin that binds specifically to C. albicans, and showed that in Gal3-/- mice, DSS colitis was worse in comparison to wild type mice, with enhanced colonization by *C. albicans* (119). This revealed the role of *C. albicans* in augmenting DSS mediated colitis as well as the role of Gal-3 in preventing colonization by *C. albicans*. In another example of host fungal interaction, Iliev's group (120) illustrated that the fungal community in the gut interacts with the immune system through innate immune receptor Dectin-1. In the DSS colitis model, mice deficient in Dec-1 had exacerbated colitis if challenged with *Candida tropicalis* whereas WT mice did not show an increase in colitis. In a study focused on both micro- and myco-biota in AS patients, the authors showed an increase in the levels of *Ascomycota*, where altered mycobiota was associated with the degree of radiographic damage (121). El Mouzan and colleagues (122, 123) investigated the gut fungi in treatment naive new onset CD in a pediatric Saudi Arabian cohort and found fungal dysbiosis associated with CD patients without the loss of fungal diversity between CD patients and healthy controls (HCs). They found that patients with CD had an increase in *Psathyrellaceae, Cortinariaceae, Psathyrella*, and *Gymnopilus* with a concomitant decrease in *Monilinia*. In a recent study, the authors found *Malassezia restricta*, a common skin commensal fungus, associated with the intestinal mucosa in CD patients (124). *M. restricta* was specifically associated with individuals carrying the IBD risk gene, CARD9-, a signaling adaptor protein with an antifungal role. The study showed that CARD9 variants present in these patients can induce the host immune cells to produce inflammatory cytokines against *M. restricta*. In a mouse model, *M. restricta* exacerbated colitis in germ-free as well as gnotobiotic mice. Taken together, these studies display the importance of fungi in HLA-B27-associated SpAs and highlight the importance of host-bacteria-fungal interactions in these diseases.

Fungi-Bacteria Functional Interaction

Recent studies have shown the role of inter-kingdom fungal-bacterial interactions contributing to various SpAs. One such study showed investigated the bacterial-fungal interkingdom networks in AS patients (121) and showed perturbed relations between gut bacteria and fungi as evident by decreased fungal to bacterial biodiversity ratios in these patients. Another study looking into the mycobiome of Japanese CD patients showed an increase in the abundance of *C. albicans*, *Entyloma*, and *Trichosporon* in the CD patients in comparison with the HCs. In contrast the HCs had increased abundance of *Saccharomyces* and *Sarocladium* in comparison to the CD patients. Microbial dysbiosis was also observed in CD patients as evident by decreased microbial diversity and increased abundance of *Enterococcus* in CD patients (125). Bacterial-fungi correlations showed positive correlation between *Enterococcus* and *Malassezia*. CD patients showed a positive correlation between *Ruminococcus* and *Sarocladium* and *Ustilago* (125). These associations are especially interesting as these bacteria and fungi have been separately associated with various spondyloarthropathies. In another study, it was shown that patients with CD are associated with increased levels of the fungus *Candida tropicalis* and two bacteria *E. coli* and *Serratia marcescens*. *C. tropicalis* positively correlated with *E. coli* and *S. marcescens* in these patients and was observed to associate closely in biofilms in comparison to other microbes (126). Studies are also investigating specific bacteria-fungi relationships in rodent models. Mice treated with DSS to induce colitis showed an increase in disease severity when supplemented with *Candida albicans*. On the other hand, colitis improved in these mice with the addition of *Saccharomyces boulardii*. Treatment with antibiotics affected the disease severity and the effects of fungi on colitis. While treatment with vancomycin that targets all Gram negative microbes protected mice from colitis, treatment with colistin to target *Enterobacteraceae* specifically retained the colitis phenotype (127). Disease was not affected by addition of either *C. albicans* or *S. boulardii*. Fungal-bacterial correlations were decreased severely in the colistin treated mice, suggesting that effect of fungi on colitis was due to its interaction with bacteria belonging to family *Enterobacteraceae*. Restoring the *Enterobacteraceae* in these mice restored the effect of both *C. albicans* and *S. boulardii* on the colitis (127). These studies highlight that microbial functions are considerably affected by various positive and negative trans-kingdom interactions between the members of the bacteria and fungal community.

Viral Pathobionts

The human gut virome is another emerging component of the gut microbiome, which is thought to impact human health either directly or *via* the modulation of the bacteriome through bacteriophages. However, the virome has been more of a dark matter with studies on a limited number of known viruses. A recent study by Norman and others (128) showed that the virome was altered in IBD with a significant expansion of *Caudovirales* bacteriophages. They compared the bacterial and *Caudovirales* bacteriophage communities and found distinct relationships in CD and UC patients. It was associated with increased richness but decreased viral diversity and these changes were concomitant to the changes in the bacterial communities suggesting a role of viral perturbations in IBD. Studies on the human virome have shown that viruses display bacteria like inter-individual variability and also respond dynamically to various environmental influences like diet (129, 130). In the healthy gut the viral core is made of virulent phages. However, in patients with CD, the virome shifts toward a temperate viral core and the changes in the viral community affect the bacterial community (131). Determining the virome in HLA-B27 mediated diseases may shed light on pathogenesis and may be crucial for the development of phage biomarkers.

So far, we have focused on specific bacterial, fungal, and viral pathobionts associated with HLA-B27-assocated SpA and other immune/inflammatory disorders that overlap clinically with SpA. However, determination of disease associated microbes may also depend upon other environmental factors such as geographical location, diet, genetic factors (other than HLA-B27), as well as technical factors like sampling location, related vs unrelated controls, methods of sequencing and data analysis. Spatial heterogeneity of microbial community profile through the gastrointestinal tract has shown to vary immensely. For example, the fecal microbiota provides a view of the microbial diversity at a given time point, and is used in majority of the microbiome studies, it neglects the mucosa-associated microbes (132). In addition, effects of related and unrelated healthy controls along with related healthy controls that cohabit need to be considered, as they can introduce variability. While efforts are being made to standardize the microbiome studies (133), attention toward the host, environmental and technical difference issues will be highly valuable while inferring results from multiple studies.

PATHOBIONTS VS DYSBIOSIS

The gut microbial community is diverse with enormous inter-individual variability due to host genetics and other environmental factors, which may explain why different studies on SpA with diverse patient cohorts have reported expansion of distinct disease-associated microbes or pathobionts. In healthy individuals, pathobionts are present in relatively low abundance and increase during dysbiosis in disease susceptible individuals, contributing to pathogenesis. This could be an active increase in their relative abundance due to changes in their microenvironment, or they can increase as the colonization pressure from gut commensals is lost due to inflammation. Therefore, it is vital to study these pathobionts in the context of their host genetics and microbial community structure. IL-2 knockout mice have dysregulated T cell functions and develop chronic immune mediated colitis in SPF mice (134), however these mice like the *IL-10-/-* mice discussed earlier fail to develop colitis under germ-free conditions (135). These studies emphasize the interaction between the host genetics and gut microbiome in disease development. This is exemplified in HLA-B27 rats, which also fail to develop colitis in germ-free conditions. Introduction of *Bacteroides vulgatus* in germ-free

HLA-B27 TG rats is sufficient to induce colitis, but its introduction in SPF raised athymic HLA-B27-TG rats fails to induce colitis (136). In contrast, addition of *B. vulgatus* is not sufficient to induce colitis in *IL-10-/-* mice, which suggested that resident enteric bacteria are necessary for immune activation and this development of spontaneous colitis in this model. In fact, presence of *B. vulgatus* protects the *IL-2-/-* mice from developing *E. coli* induced colitis, which underscores that different microbes and their interactions may dictate their ability to trigger disease (36, 137). In $TNF^{\Delta ARE}$ mice model, biosynthesis of TNF is dysregulated, leading to the development of chronic inflammatory arthritis and CD like ileitis. Germ-free $TNF^{\Delta ARE}$ mice did not develop ileitis, even when colonized by a pathobiont *E. coli* LF82. Development of CD like gut inflammation occurs only when these mice were colonized with cecal content from inflamed mice raised in SPF condition (138). Collectively, these studies performed with different genetic susceptibility models pinpoint the joint role of diverse microbiota and genetic susceptibility as a requirement for development of SpA or associated gut inflammation.

Metchnikoff (1908) observed that intestinal microbes are dependent on our dietary intake, and therefore it may be possible to modify the gut microbiota. He defined "dysbiosis" as ecological imbalance in the gut microbial community. While the concept of microbial dysbiosis was forgotten in the following decades with the focus on antibiotics, and lack of ability to culture and classify gut microbes, which are mostly obligate anaerobes and refractory to culture. Recent advances in culture free determination of microbial community members using next generation sequencing abilities, the microbial dysbiosis and its role in human health and disease has been a focus. These studies have classified many distinct features of microbial dysbiosis, such as reduced microbial diversity, pathobiont expansion and loss/alteration of microbial community structure (139). Another important feature of microbial dysbiosis is the perturbation in the metabolic function, which has been associated with many immune and inflammatory disorders including IBD and SpA (139–142).

In our study with the HLA-B27 transgene (12) on three different rat genetic backgrounds namely Lewis, Fischer and Dark agouti (DA), we have demonstrated that the gut microbiome is dependent on the host genetics and environment. Only two of the backgrounds (Lewis and Fischer) were disease susceptible, whereas the DA rats were resistant to HLA-B27-associated SpA. In disease susceptible Lewis and Fischer backgrounds, HLA-B27-associated gut microbial dysbiosis was dependent on the host background, while the immune dysregulation was independent of the host genetics and environmental effects, and showed considerable overlap of inflammatory mediators between HLA-B27 TG Lewis and HLA-B27 TG Fischer rats. Analyzing the predictive metabolome and host-microbe inter-omic analysis suggested that in comparison with their respective wild type controls, HLA-B27-associated with different pathobionts in Lewis (*Prevotella*) and Fischer (*Akkermansia*, members of family *Lachnospiraceae*). However, the microbial functional/metabolic pathways perturbed in both backgrounds were similar. In both these backgrounds, these diverse pathobionts are associated with common host genes for immune/inflammatory pathways (74). This led us to propose an ecological model of dysbiosis where perturbation of the microbial community structure and function contributes to disease pathogenesis, instead of a single microbe driving disease (12). These studies suggest that gut microbial functions are highly dependent on their community structure and the gut microenvironment (143).

Pathobionts Are Context Dependent

Many microbes deemed as commensals can act as pathobionts under certain circumstances. In such cases, the pathogenicity of these pathobionts is dependent on the host genetics as well as the composition of the gut microbiome. In antibiotic treated mice, adding commensal *Bacteroides* spp. can induce the development of colitis in IBD-susceptible background, but not in IBD-non-susceptible background (144). This may explain why adding back commensal microbes such as ASF to germ-free HLA-B27 TG rats is sufficient to drive colitis as well as arthritis (35). Conversely, pathobionts have been shown to exert beneficial effects in certain disorders. As mentioned earlier, supplementation of *A. Muciniphila* in mice and humans has shown to improve various metabolic parameters in obesity (145, 146). These results suggest that a microbe can act as a pathobiont depending on the context of host genetics and their position in the microbial community structure (**Figure 3**). We reported that presence of segmented filamentous bacteria (SFB) in both Lewis and Fischer genetic backgrounds correlates with disease in the presence of HLA-B27 but not in wild type controls (12). SFB adheres to the epithelial cells in terminal ileum in rodents at the time of weaning and induces the development of Th17 cells (147). SFB is absent on the DA rat background and these animals are resistant to gut inflammation and arthritis in the presence of HLA-B27. In contrast to these studies, SFB was shown to protect against rotavirus infection and diarrheal disease (148). In another study, SFB was able to protect from *Citrobacter rodentium* induced colitis (147). This may suggest that the microbial community is complex, and the results from the mono-colonization studies, while important to determine mechanistic pathways, may not be sufficient to recapitulate fully functional microbial communities.

ASSOCIATION VS CAUSATION

The association between the host species and their symbiotic microbes is a result of millions of years of coevolution, which has resulted in a homeostatic balance between the gut microbial community in host health and disease (149, 150). While imbalance in the symbiotic microbial communities inhabiting our body has been linked to various SpAs (151), it is not clear whether microbial dysbiosis is the cause or the effect of these disorders. Experimental models of various SpAs can help elucidate the causal microbes and microbial pathways, which can be confirmed in patients. One such example is

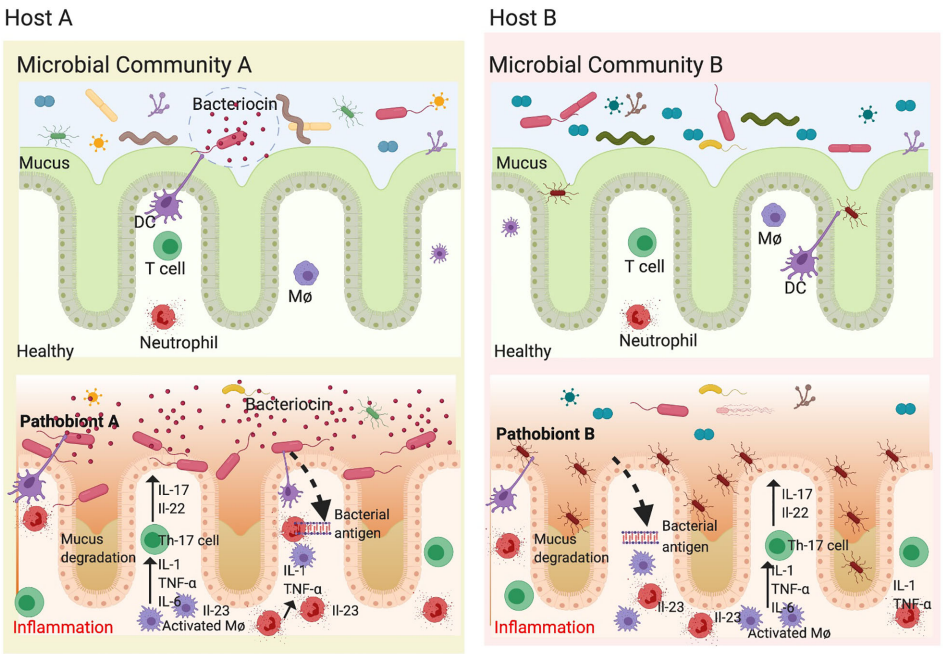

FIGURE 3 | Pathobionts depend on host genetics and gut microbial community. The gut microbiota is highly diverse and varies between healthy individuals depending on host genetics, diet and environment. Each individual microbial community consists mostly of commensals which provide colonization resistance to opportunistic pathobionts. In genetically susceptible individuals (HLA-B27), there is microbial dysbiosis concomitant to loss of epithelial barrier resulting in an inflammatory microenvironment which further increases the loss in commensal microbes. This presents a unique opportunity for pathobionts to thrive and exacerbate inflammation. Since different individuals have different microbial community structure and different pathobionts depending on their genetics and environment, this may explain why we observe different pathobionts associated with various spondyloarthropathies. In microbial community A, only pathobiont A is present and when the conditions change (a trigger). In addition, certain pathobionts can make bactericidal compounds known as bacteriocins to avoids colonization resistance. When the pathobiont A is in bloom, it increases and exacerbates inflammation by degrading mucus and disrupting epithelial barrier. However, in another microbial community on a distinct host genetic background and in the presence of pathobiont B, which is mucous associated, an increased relative abundance can be associated with disease as most microbes are unable to cross the mucous barrier. In both cases, the dendritic cells (DCs) and Macrophages (Mφ) release inflammatory mediators such as IL-23, TNF-α, and IL-6, which activate T helper 17 cells to make IL-17, IL-22 causing inflammation and disrupting epithelial barrier thereby perpetuating the inflammatory cycle. Figure created with Biorender.com.

Porphyromonas gingivalis, a periodontal bacterium, which has been shown to colonize synovial joints and exacerbate collagen induced arthritis (152). This suggests that *P. gingivalis* plays a mechanistic role in arthritis due to its translocation to the joints.

A complication in determining causal microbial mechanisms is that host-microbiome interactions are complex and have a multitude of variables affecting them. This makes it challenging to determine the pathobiont or other microbial species responsible for the disease phenotype (153). While mono-colonization of germ-free animals is a simplistic tool for determining causation, it lacks complex inter-microbial interactions and may not recapitulate the complexities of a stable microbial community. Thus, instead of colonization with single commensal and/or pathobiont, development of complex model microbiomes or synthetic microbial communities (154) and better culture techniques to culture/characterize refractory microbes (155) may help determine disease mechanisms. Development of better animal models and/or methods to colonize selective microbes in a complex microbial community will enable us to directly approach the mechanisms and address causality (156).

DISEASE MECHANISMS

Determination of the causal relationships and the underlying mechanisms with which these microbes interact with each other and their host is vital to develop therapeutic targets. Deciphering the mechanisms of microbial function in health and disease is crucial to ascertain cause and result relationships. Pathobionts are thought to exert their effects on host physiology through mucin degradation, disruption of epithelial barrier function as well as the loss of colonization resistance by commensals, therefore making them foremost targets to investigate disease mechanisms.

Mucin Degradation

The mucus layer provides the first layer of physical barrier by limiting the microbial contact with the host tissue. Mucin degradation by microbial metalloproteinases has been suggested to contribute to IBD pathogenesis (157). Pathobionts such as *Akkermansia* and *Prevotella*, which are implicated in HLA-B27-associated SpAs, are known to have mucin degrading capabilities albeit through different mechanisms. For example,

Prevotella may contribute to inflammation by encoding enzymes (superoxide reductase and a phosphoadenosine phosphosulfate reductase), which enable *Prevotella* to resist host reactive oxygen species and outcompete microbes essential for mucosal homeostasis such as *Bacteroides* spp (68, 101); *Akkermansia* can exacerbate inflammation by degrading the mucus layer over epithelial cells, thereby weakening the epithelial barrier (158). Mucolytic bacteria could contribute to disruption of intestinal barrier and joint inflammation. One such bacteria, *R. gnavus* is known to express ß-glucuronidase, that can convert bile acids into inflammatory secondary bile acids (deoxycholic and lithocholic acids), associated with intestinal inflammation (78, 159).

Mucosal Adherence and Barrier Disruption

Almost 60 percent of patients with HLA-B27-associated SpAs have microscopic gut lesions, with one third having overt gut inflammation (160). First degree relatives of SpA and IBD patients also show signs of subclinical gut inflammation and impaired gut epithelial barrier (161, 162). While the underlying mechanism is not fully understood, animal models of SpAs and IBD have highlighted the importance of gut mucosa for host-microbe interactions. Many pathobionts associated with HLA-B27 such as *Akkermansia*, *Prevotella* and *Mucispirillum* have the ability to adhere and degrade the mucus layer (11, 12, 101). When epithelial barrier is disrupted, gut commensal microbes such as *Bacteroides vulgatus* are also sufficient to cause and perpetuate IBD in immunocompromized mice (163). During dysbiosis, increase in the pathobionts can degrade the mucus layer and may activate local inflammatory response, which could lead to disruption of the epithelial barrier. While we do not know if pathobionts cause barrier disruption, or if barrier disruption triggers pathobiont bloom, both events are related and critical to disease pathogenesis.

Loss of Colonization Resistance

Of the many symbiotic functions performed by gut commensals, formation of stable microbial communities is perhaps the most important function, since it provides colonization resistance to infections and pathobiont expansion. The gut microbial community is dynamic with constant struggle for niche and resources between the gut commensals and the opportunistic pathobionts for energy, resources and niche. In a healthy individual, the gut commensals can either kill the pathobionts by production of bacteriocins, outcompete them for resources or activate the immune response to produce antimicrobial peptides (164). One such mechanism is the fucosylation of epithelial cells that promotes colonization by commensals and resistance to pathogens (165). Under eubiosis, the host immune system has the ability to distinguish gut commensals from pathobionts, although the mechanisms are not clear. In a recent study, the authors demonstrate that pathobionts such as *Citrobacter rodentium* can trigger inflammatory Th17 cells, while gut commensals like SFB trigger tissue-resident homeostatic Th17 cells (166). These tissue resident Th17 cells do not make inflammatory cytokines or participate in inflammatory reactions and have a slow metabolism. On the contrary, Th17 cells in response to *C. rodentium* have an inflammatory effector potential (166). During dysbiosis, mucosa associated pathobiont/s can expand at the expense of gut commensal microbes, degrade mucus and activate host inflammatory response (11).

THERAPEUTIC IMPLICATIONS

Gut microbe/s or microbial metabolites may provide novel treatment opportunities in HLA-B27-associated SpAs. These approaches aim to answer the basic question- what parameters affect the reorganization of a stable microbial community after dysbiosis and/or inflammatory insult? Here we focus on the role of pre and probiotics, short chain fatty acid, diet and fecal microbiota transplant in alleviation or amelioration of the disease.

Pre- and Pro-Biotics

Prebiotics and probiotics can contribute to maintaining healthy gut community and therefore play and important role in the overall health of the gastrointestinal tract (167). Prebiotics are plant-based fiber, which may enhance the activity of beneficial gut bacteria, thereby promoting host health (168). On the other hand, probiotics consists of live bacterial strains, which upon ingestion in adequate amounts may confer health benefit to the host (169). Studies on HLA-B27 TG rats treated with prebiotic compounds inulin and oligofructose, demonstrated reduced colitis severity, associated with increase in the relative abundance of *Lactobacillus* and *Bifidobacterium* species. Prebiotic treatment significantly decreased inflammatory cytokines such as IL-1β and increased the levels of TGF-β, an immunomodulatory cytokine in the cecal tissue of these rats (170). In another study by the same group, the prebiotic combination of inulin and oligofructose was also effective in partially preventing colitis. While the HLA-B27 TG rats showed an altered microbial community, concomitant with an increase in Bifidobacterium, they were unable to observe any changes to the luminal short chain fatty acid concentrations (171). A later study by another group tried to dissect the effects of prebiotic treatment by using either inulin or fructo-oligosaccharide in HLA-B27 TG. All of the HLA-B27 TG rats which were fed fructo-oligosaccharide showed significant reduction in colitis, whereas only half of the HLA-B27 TG rats fed inulin showed improvement in colitis. While both groups were associated with decrease in the *Clostridium cluster XI*, rats fed fructo-oligosaccharides showed increase in *Bifidobacterium*, and inulin fed HLA-B27 TG rats showed an increase in *Bacteroides, Prevotella*, *Porphyromonas* group (172). Another group also measured the impact of a probiotic and prebiotic combination (*Lactobacilli, Bifidobacteria*, and inulin) on the severity of colitis and microbial community in HLA-B27 TG rats. Colitis was attenuated in HLA-B27 rats which received the probiotic, and they showed increase in microbial diversity, specifically an increase in the *Bifidobacterium animalis* (173). These studies show the promise of prebiotic and probiotic supplements as therapy for gut inflammation associated with various SpAs by renewing and restoring host gut microbial communities.

Short Chain Fatty Acids

Another important contribution of gut microbiota is the production of microbial metabolites such as butyrate, propionate, and acetate; collectively known as short chain fatty acids (SCFA). These are the primary products of non-digestible carbohydrates/prebiotics, and their oxidation provides a major source of energy for the colonocytes (174). Microbial dysbiosis is accompanied by perturbations in the microbial metabolic function including changes in production/oxidation of short chain fatty acids (SCFA) and trimethylamine N-oxide (TMAO), which play an important role in modulation of host physiology [reviewed in (175)]. Asquith and colleagues showed that HLA-B27 expression alters the host and microbial metabolic profile with an increase in the levels of histidine, tyrosine, spermidine, N-acetylmuramate and glycerate in HLA-B27 TG rats (140). When supplemented with propionate (a SCFA), HLA-B27 TG demonstrated attenuation in the inflammatory disease. In another study, administration of propionate was shown to attenuate the severity of uveitis in an inducible model of experimental autoimmune uveitis (176). Propionate and other SCFAs such as butyrate can activate G protein-coupled receptors, GPR41 and GPR43 (177) (124). Mice deficient in GRP43 show exacerbation of inflammation in colitis, arthritis and asthma. The authors demonstrated that resolution of inflammatory response was dependent on the activation of GPR43 by SCFA (178). SCFAs such as butyrate have shown to effect the host innate immune function (179). They showed a reduction in inflammatory cytokines produced by macrophages *in vitro* when treated with butyrate, as well as in macrophages isolated from mice whose drinking water was supplemented with butyrate. Taken together, SCFAs play an important role in mucosal homeostasis by not only fueling the colonocytes, but also by suppressing the innate immune cells from mounting an inflammatory response. These diverse roles highlight the importance SCFAs as a therapeutic in various inflammatory (SpA, IBD) as well as metabolic (type I diabetes) diseases.

Diet

Diet plays a major role in microbial community maintenance since it is an important source of small molecules, which are converted to various metabolic products by gut bacteria. High fat westernized diet is thought to be associated with various inflammatory disorders, whereas high fiber diet is associated with amelioration of inflammation (180). In a study by Rodrigues-Cabezas and colleagues, amelioration of colitis was reported in HLA-B27 TG rats given fiber enriched diet (181). This was associated with increased production of SCFA (butyrate and propionate), which in turn acted synergistically to inhibit pro-inflammatory mediators (181). Supplementing the diet with butyrate in mice has been shown to mediate the homeostasis of regulatory T cells (182, 183). In a mouse model, butyrate supplementation increased serotonin derived activation of the aryl hydrocarbon receptor (AhR). AhR is a transcription factor involved in the sensing of environmental signals like the redox potential (184); and recognized as the mediator for various ligands from diet, commensal microbes and host metabolites [Reviewed in (185)]. While AhR has recently been discovered as a B cell transcription factor and induces the development of regulatory B cells (186), its effect on immune modulation has been shown in IBD. Indole derivatives from cruciferous vegetables activate the AhR, and plays an important role in the maintenance of innate lymphoid and intraepithelial lymphocytes (187, 188). AhR receptors are expressed by peripherally derived regulatory T cells in the gut, and their expression has been shown to play a key role in the gut homing and anti-inflammatory functions of gut regulatory T cells (189). They are known to be involved in the maintenance of epithelial barrier and dampen various inflammatory conditions (190, 191). A study by Maslowski et al. (178), showed that decreased intake of complex plant polysaccharide fiber perturbs the microbial community leading to decreased production of SCFAs. Thus, diet has a role in maintenance of gut microbial community, which promotes gut health by increasing host beneficial metabolites as well as by maintaining community resistance by gut commensals to pathogenic microbes.

Fecal Microbiota Transplant (FMT)

The role of microbiota in disease pathogenesis and evidence from FMT for *Clostridium difficile* infection makes FMT a promising treatment for HLA-B27 associated spondyloarthropathies. While FMTs are currently not available for spondyloarthropathies, FMT trials are underway in patients with RA (ClinicalTrials.gov identifier: NCT03944096), who are refractory to methotrexate treatment, as well as in a European cohort for psoriatic arthritis (ClinicalTrials.gov identifier: NCT03058900). Trials are also underway for the FMT treatment of AS patients (ASGUT-ClinicalTrials.gov Identifier: NCT03726645). In a recent report by (192), FMT treatment for *C. difficile* infection resulted in a decrease in disease activity for PsA. Recently, FMT has been proven to be effective for the induction of clinical remission associated with endoscopic improvement in active ulcerative colitis concomitant with persistent increase in microbial diversity (193). A major concern with FMT trials is development of a standardized fecal sample, and the concerns for long term colonization of a foreign microbial community in a different host microenvironment. While patients with *C. difficile* pseudomembranous colitis offer very little colonization resistance to FMT, in other inflammatory diseases the resident microbial community can prevent the colonization of the microbial community from FMT. To standardize the gut microbiota for FMT, researchers have developed methods to develop a synthetic microbial community which mimics the fecal microbiota (154). This synthetic microbial FMT is under trials for *C. difficile* infection (Clinical trial registration number: CinicalTrials.gov NCT01372943). Together, these studies show a remarkable role of FMT in repopulating the gut microbiota and its role in disease amelioration.

CONCLUSIONS AND FUTURE DIRECTIONS

HLA-B27 has been recognized to associate with various SpAs, especially AS for almost 50 years. With the advent of culture free

sequencing techniques, the role of microbiome and microbial dysbiosis in disease pathogenesis is now well accepted. However, we are beginning to appreciate the complexity of gut microbial communities which consist of bacteria, archaea, viruses, and fungi. If we include the inter-kingdom interactions as well the host-microbiota relationships as factors affecting the role of individual microbes, these interactions in a stable microbial community become very complex. However, spatial differences in microbial community structure along with difference due to host, environmental, and technological factors should be considered before we cement the role of certain microbes and their functions in HLA-B27 associated SpAs.

The emerging picture suggests an important role of pathobionts in contributing toward HLA-B27 associated SpAs. Mechanistic studies on these pathobionts in germ-free and gnotobiotic rodent models have provided fundamental insights into the role of microbes in disease pathogenesis. While this review has focused on bacteria and fungi in SpA and associated disorders, studies of viruses especially bacteriophages in the gut mucosal environment could help explain whether viruses play a role in development of SpA in genetically susceptible hosts. However, the gut microbiome is a complex ecosystem of polymicrobial communities and therefore understanding the functional/metabolic implications of these microbial perturbations in context of an established gut microbial community is of immense value in determining host-microbe causal relations in SpA. The mechanistic effect of pathobiont expansion and dysbiosis in the context of host genetics and environment, will provide opportunities to develop novel therapeutic targets for disease alleviation/amelioration in HLA-B27 associated SpAs.

AUTHOR CONTRIBUTIONS

TG and JR jointly created the review outline. TG wrote the review and JR provided editing. All authors contributed to the article and approved the submitted version.

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Epstein-Barr Functional Mimicry: Pathogenicity of Oncogenic Latent Membrane Protein-1 in Systemic Lupus Erythematosus and Autoimmunity

Melissa E. Munroe[1], Jourdan R. Anderson[1], Timothy F. Gross[1], Laura L. Stunz[2], Gail A. Bishop[2,3,4,5] and Judith A. James[1,6]*

[1] Arthritis and Clinical Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, United States, [2] Department of Microbiology & Immunology, The University of Iowa, Iowa City, IA, United States, [3] Department of Internal Medicine, The University of Iowa, Iowa City, IA, United States, [4] Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA, United States, [5] Iowa City VA Medical Center, Iowa City, IA, United States, [6] Department of Medicine and Pathology, Oklahoma University Health Sciences Center, Oklahoma City, OK, United States

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***Correspondence:**
Melissa E. Munroe
melissa-munroe@omrf.org

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Systemic lupus erythematosus (SLE) and other autoimmune diseases are propelled by immune dysregulation and pathogenic, disease-specific autoantibodies. Autoimmunity against the lupus autoantigen Sm is associated with cross-reactivity to Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1). Additionally, EBV latent membrane protein-1 (LMP1), initially noted for its oncogenic activity, is an aberrantly active functional mimic of the B cell co-stimulatory molecule CD40. Mice expressing a transgene (Tg) for the mCD40-LMP1 hybrid molecule (containing the cytoplasmic tail of LMP1) have mild autoantibody production and other features of immune dysregulation by 2–3 months of age, but no overt autoimmune disease. This study evaluates whether exposure to the EBV molecular mimic, EBNA-1, stimulates antigen-specific and concurrently-reactive humoral and cellular immunity, as well as lupus-like features. After immunization with EBNA-1, mCD40-LMP1 Tg mice exhibited enhanced, antigen-specific, cellular and humoral responses compared to immunized WT congenic mice. EBNA-1 specific proliferative and inflammatory cytokine responses, including IL-17 and IFN-γ, were significantly increased ($p<0.0001$) in mCD40-LMP1 Tg mice, as well as antibody responses to amino- and carboxy-domains of EBNA-1. Of particular interest was the ability of mCD40-LMP1 to drive EBNA-1 associated molecular mimicry with the lupus-associated autoantigen, Sm. EBNA-1 immunized mCD40-LMP1 Tg mice exhibited enhanced proliferative and cytokine cellular responses ($p<0.0001$) to the EBNA-1 homologous epitope PPPGRRP and the Sm B/B' cross-reactive sequence PPPGMRPP. When immunized with the SLE autoantigen Sm, mCD40-LMP1 Tg mice again exhibited enhanced cellular and humoral immune responses to both Sm and EBNA-1. Cellular immune dysregulation with EBNA-1 immunization in mCD40-LMP1 Tg mice was accompanied by enhanced splenomegaly, increased serum blood urea nitrogen

(BUN) and creatinine levels, and elevated anti-dsDNA and antinuclear antibody (ANA) levels ($p<0.0001$ compared to mCD40 WT mice). However, no evidence of immune-complex glomerulonephritis pathology was noted, suggesting that a combination of EBV and genetic factors may be required to drive lupus-associated renal disease. These data support that the expression of LMP1 in the context of EBNA-1 may interact to increase immune dysregulation that leads to pathogenic, autoantigen-specific lupus inflammation.

Keywords: autoimmunity, systemic lupus erythematosus, Epstein-Barr virus, molecular mimicry, functional mimicry, EBNA-1, LMP1, mouse

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease driven by dysregulated cellular and humoral immunity (1–4). Increased immune dysregulation is associated with increased clinical disease activity and flare (5–7) and places patients at risk of permanent end-organ damage, SLE-associated morbidity, and early mortality (8). Such immune dysregulation begins years before clinical disease onset and amplifies through a feed-forward aggregation of altered innate and adaptive immune pathways as patients progress to SLE classification. Concurrent with or following these changes in innate and adaptive immune pathways, pathogenic SLE-associated autoantibody specificities accumulate (9). These autoreactive responses commonly target nuclear antigens such as Ro/SSA, La/SSB, Sm, RNP, and dsDNA (3, 4), the latter two specificities associated with lupus nephritis (10). Despite improved disease management and treatment approaches to suppress and circumvent dysregulated immunity, patients with SLE exhibit persistent and waxing/waning dysregulation of innate and adaptive immune pathways.

Numerous studies over the past two decades have elucidated genetic and genomic contributions to SLE risk and heritability. Despite a twin concordance rate of up to 25% (11) and identification of over 100 lupus associated genetic variants (12, 13), genetics alone explain no more than 50% of SLE risk (14, 15). This supports roles for environmental factors as contributors to SLE etiology (16, 17). Infections, such as Epstein-Barr virus (EBV), are associated with both pediatric (18, 19) and adult (17, 20, 21) SLE. EBV, a member of the herpes virus family, is tropic for B-lymphocytes and promotes cellular dysregulation, including lymphoproliferation (22–24), malignancy (25–27), and autoimmunity (28–30). Compared to unaffected individuals, SLE patients have higher EBV viral loads (31, 32), are more likely to exhibit infection in peripheral blood mononuclear cells (PBMCs) (32, 33) and exhibit aberrant expression of EBV latent genes, including EBV nuclear antigen-1 (EBNA-1) and latent membrane protein-1 (LMP1). These differences in SLE patients may be attributed to immune dysregulation that drives latent protein expression as well as an inability to control viral reactivation (17, 19, 24, 33–37). EBV reactivation is increased in SLE patients, evidenced by increased antibodies to EBNA-1 in conjunction with IgG antibodies against EBV early antigen (EA) and viral capsid antigen (VCA) (17, 38). This viral reactivation is associated with transition to classified SLE (17) as well as clinical disease activity and flare (18, 31).

Both pediatric and adult SLE patients exhibit altered humoral immunity to EBNA-1 (9, 19, 39, 40). EBNA-1 is a structural, molecular mimic with known SLE autoantigens. By eliciting antibodies that structurally cross-react with autoantigens, we and others have shown that EBNA-1 contributes to autoimmunity against Ro/SSA and spliceosomal proteins Sm B, Sm D1, and RNP A (9, 19, 40–42); additional studies by the Spatz laboratory have further found cross-reactivity between EBNA-1 and the SLE-associated autoantigen dsDNA (42–44). Although structural molecular mimicry may be due to random chance, EBNA-1 utilizes and binds to the same nuclear spliceosomal machinery as host cells (45), including Sm and RNP proteins (46), to maintain lytic and latent EBV infection (47). Therefore, functional and structural overlap may drive molecular mimicry between EBNA-1 and SLE-associated autoantigens. Over time with continued cross-reactivity, broken immune tolerance creates a positive feedback loop where autoantibodies mediate cellular damage that releases additional autoantigens, leading to continued immune reactivity, epitope spreading (35, 48), and the accumulation of autoantibody specificities (3, 4, 49) that themselves cross react with EBNA-1 (35). Anti-EBNA-1 antibodies alone may not be enough to break tolerance and drive autoimmunity, as over 90% of individuals have been exposed to EBV and most never develop autoimmunity (50, 51).

Cellular immune dysregulation may facilitate the initial break in tolerance in SLE, as SLE-associated autoantibody specificities associated with EBNA-1 molecular mimicry are detected after evidence of cellular immune dysregulation in pre-clinical SLE (3, 4). Interestingly, EBV encodes proteins that disrupt cellular immune regulation, including LMP1, a functional mimic of CD40. As a costimulatory molecule expressed on antigen-presenting cells, such as B-lymphocytes, dendritic cells, and macrophages, CD40 is vital for B-lymphocyte activation and function and bridges innate and adaptive immunity. Interacting with CD154 on T-lymphocytes (52), CD40 itself triggers B-lymphocyte activation, proliferation, cytokine secretion, and antibody production (52), acts as a co-stimulatory molecule for the B cell receptor (BCR) (53, 54), and amplifies innate signals driven by toll like receptors (TLRs) (55), including TLR7 (56, 57), implicated in SLE pathogenesis (58–60). EBV-encoded LMP1 has been studied *in vitro* (61–66) and *in vivo* (62, 67–71) and is a functional mimic of CD40, although it does so in an enhanced and dysregulated manner (**Figure 1**). Unlike CD40, which

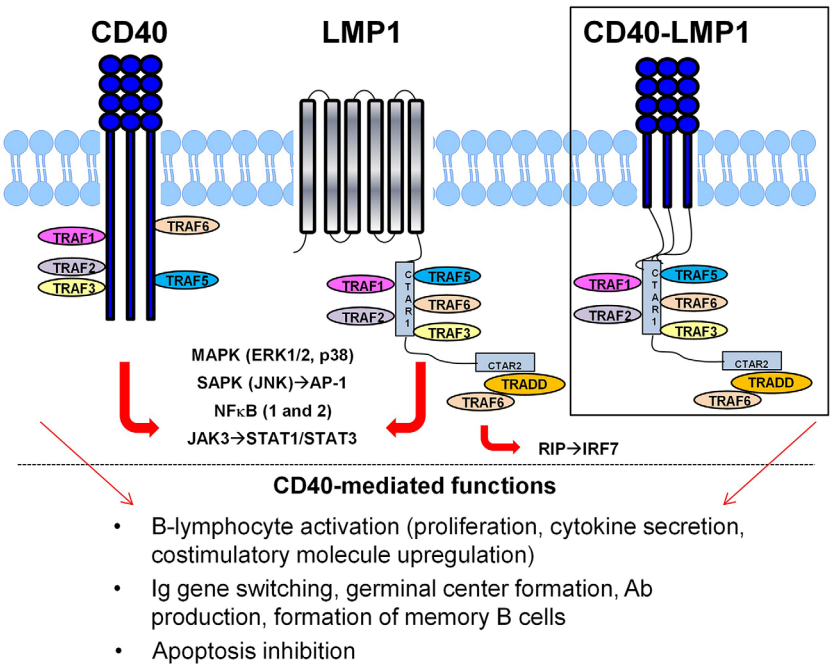

FIGURE 1 | Latent Membrane Protein 1 (LMP1) acts as a viral mimic of the costimulatory molecule CD40. Similar to CD40, LMP1 binds TRAFs through its cytoplasmic domain to mediate proximal signaling/transcriptional regulation and downstream function, including B-lymphocyte activation, antibody production and isotype switching, and apoptosis inhibition. However, LMP1 does this in a dysregulated manner, partially through its ligand-independent, self-aggregating six-transmembrane domains. Replacing the extracellular/transmembrane domain of LMP1 with CD40 demonstrates that the cytoplasmic tail of LMP1 is necessary and sufficient for its enhanced and dysregulated functional mimicry of CD40 (inset).

requires interaction and trimerization with CD154, the six transmembrane domains of LMP1 are able to self-aggregate in a ligand-independent and uncontrolled manner to drive downstream proximal signaling and subsequent distal functional activities that overlap with CD40, including B-lymphocyte activation, germinal center formation, as well as antibody and cytokine production (71). This ability of LMP1 to spontaneously self-aggregate, without the need for CD154 expressed on T-lymphocytes, may allow for its ability to evade the immune system and contribute to the natural selection of EBV to latently persist within B-lymphocytes (72, 73).

Like CD40, the cytoplasmic domain of LMP1 does not have enzymatic activity, but instead utilizes TNF-receptor associated factors (TRAFs) to facilitate its signaling and biologic activities. Replacing the LMP1 extracellular/transmembrane domains with those of CD40 (**Figure 1, inset**) demonstrated that the cytoplasmic tail of LMP1 is necessary and sufficient to mimic CD40 activity and do so in a dysregulated manner (71, 74). LMP1 interacts with TRAFs *via* two carboxy-terminus activating regions (CTAR), CTAR1 and CTAR2 (66, 68). CTAR1, similar to CD40, contains the TRAF binding motif, PXQXT, to bind TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 (63, 68). Yet there are key differences in the way LMP1 utilizes TRAFs compared to CD40. CD40 drives B-lymphocyte activation primarily through TRAFs 2 and 6 (75, 76), as well as TRAF1 (77), with TRAF3 acting as an *inhibitor* (65, 78). In contrast, LMP1 utilizes TRAF3 (63–65) in an *activating* manner alongside TRAF5 (62), as well as TRAFs 1 and 2 (79, 80). Furthermore, CD40-mediated signaling results in ubiquitination and degradation of TRAFs 2 and 3 to downregulate its signal; this does *not* occur in LMP1 signaling (63, 74). In addition, LMP1 indirectly binds TRAF6 *via* TRADD in its CTAR2 domain (81, 82), allowing for additional CD40 signals *via* IRAK1 (83), as well as IRF7 activation *via* RIP (84).

These dysregulated, pro-activation differences in utilization of TRAFs by LMP1 have been shown to translate into an autoimmune disease phenotype *in vivo*. The mCD40-LMP1 transgenic (Tg) mouse model expresses a hybrid molecule with the mouse (m)CD40 extracellular domains and the LMP1 cytoplasmic tail, as described above. The transgene is driven by an MHCII promoter on a C57BL/6 (B6), CD40-deficient background, so that the only CD40 present is mCD40-LMP1. Compared to congenic mCD40 Tg and B6 mice that express full-length, wild-type mCD40 (mCD40 WT mice), mCD40-LMP1 Tg mice exhibit both splenomegaly and lymphadenopathy, with expanded immature/activated B-lymphocyte populations and ectopic germinal center formation. In addition, these mice produce autoantibodies, including anti-dsDNA, and exhibit aberrant cytokine levels, including IL-6 (62, 67, 68, 71). Yet, the mCD40-LMP1 Tg mice are capable of driving T-dependent antibody responses, with normal isotype switching, affinity maturation, and germinal center formation (71).

We have previously demonstrated that in the context of type II collagen, an autoantigen that induces inflammatory arthritis in a murine model of rheumatoid arthritis (85), mCD40-LMP1 Tg mice exhibit accelerated and exacerbated inflammatory arthritis compared to their congenic WT counterparts (70). *Ex vivo*, these mice exhibit enhanced innate and adaptive cellular immunity in antigenic recall responses, particularly TNF-α, IL-6, and IL-17A, as well as enhanced TNF-α and IL-6 secretion in activated B-lymphocytes. This enhanced cellular immunity is accompanied by an increase in total and collagen-specific antibody production (70), with immune pathway specific isotype switching, suggesting that LMP1 is able to drive enhanced and dysregulated cellular and humoral adaptive immunity.

Because mCD40-LMP1 drives an autoimmunity phenotype that leads to overt pathology in the context of the autoantigen collagen (70), we hypothesized that LMP1 may enhance the onset of autoimmunity in conjunction with molecular mimicry between EBNA-1 and the SLE-associated autoantigen, Sm (86). Therefore, the current study investigates antigen-specific cellular and humoral immune responses to EBNA-1 and its cross-reactive lupus autoantigen, Sm, in the context of mCD40-LMP1-mediated adaptive immunity. Based on our previous epitope mapping studies, this includes reactivity in EBNA-1 and Sm immunized mice to the antigenic epitope, PPPGRRP (EBNA-1) and its homologous, comparable antigenic epitope sequence, PPPGMRPP (Sm) (19, 51, 86, 87). Further, we evaluated these mice for enhanced splenomegaly, the presence of ANA and anti-dsDNA autoantibodies, and altered renal function.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased at 5–8 weeks of age from the National Cancer Institute (Bethesda, MD). Mice transgenic for the molecule mCD40-LMP1 (mCD40-LMP1 Tg) or full length mCD40 (mCD40 Tg), driven by the MHC Class II Eα promoter were transferred from the Bishop Lab (The University of Iowa) to the Oklahoma Medical Research Foundation (OMRF). In addition to B-lymphocytes, EBV can also infect myeloid cells (88, 89), so it is reasonable to express LMP1 on these cell types and B-lymphocytes. Tg mice were maintained on the C57BL/6 CD40-deficient background (B6.129P2-CD40tm1Kik/J from The Jackson Laboratory, Sacramento, CA) at OMRF, as previously described (71). Mice were age- and sex-matched and analyzed at 3–4 months of age. All mice were housed in specific pathogen-free barrier facilities with restricted access, all animal care and housing requirements of the National Institutes of Health Committee on Care and Use of Laboratory Animals were followed, and all procedures were approved by the OMRF Animal Care and Use Committee.

Immunizations

Mice were immunized based on protocols described (86, 90) (**Figure 2**). Briefly, all mouse strains either remained naïve (n=6 mice/strain) or were immunized with sterile saline (adjuvant control; n= 6 mice/strain), 100 μg EBNA-1 mosaic (n = 8 mice/strain; EBNA-1 antigen with truncated glycine-alanine repeat; BiosPacific, Inc./Bio-techne, Emeryville, CA), or 100 μg Sm

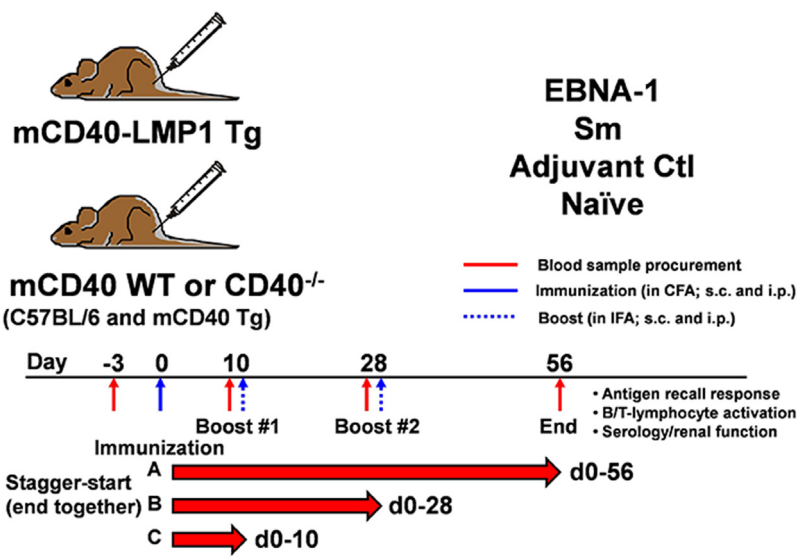

FIGURE 2 | Immunization of mCD40-LMP1 Tg mice with EBNA-1, Sm, or controls to assess antigen-specific immune response and antigen cross-reactivity. As described in *Materials and Methods*, mCD40-LMP1 Tg, mCD40 WT (C57BL/6 and mCD40 Tg), and mCD40$^{-/-}$ mice remained naïve or were immunized with either saline (adjuvant only), EBNA-1, or Sm on Day 0 in CFA, then received boost injections (in IFA) on Days 10 and Day 28. Mice were euthanized on Day 56, spleen and lymph nodes removed, and cell cultures completed. Blood samples were procured for serum prior to initial immunization (Day -3), and on Days 10, 28, and 56 after initial immunization. Separate groups of EBNA-1 immunized mice completed Days 0–10 or Days 0–28 of the protocol. Each experimental group contained 6–8 mice across two separate experiments.

antigen (n= 6 mice/strain; Immunovision; Springdale, AR). Saline and immunogens were emulsified 1:1 in either Complete Freund's Adjuvant (CFA; Sigma-Aldrich/MilliporeSigma, St. Louis, MO) for initial immunization (Day 0), or Incomplete Freund's Adjuvant (IFA; Sigma-Aldrich/MilliporeSigma) for booster immunizations (Day 10, Day 28). Emulsified adjuvant control (saline) and immunogens were injected in equal portions intraperitoneally (50 μg/100μl) and subcutaneously in alternating flanks (50μg/100μl). Blood samples for sera were collected on Days -3, 10, 28, and 56 relative to initial immunization *via* tail vein sampling. A subset of EBNA-1 immunized mice completed 10 or 28 days (n = 6 mice/strain) of the protocol (mice groups were staggered so that all of the mice completed the experimental protocol on the same day).

Lymph Node (LN) Cell Culture

Single cell suspensions (4 x 10^6/ml) of axillary, mesenteric, and inguinal draining LNs from mice were cultured in RPMI 1640 with 5% heat-inactivated fetal calf serum (FCS; VWR International, Radnor, PA), 10 uM 2-mercaptoethanol (Life Technologies/Thermo Fisher Scientific, Waltham, MA), penicillin and streptomycin. Cells were cultured in medium alone or in the presence of 50 ug/ml EBNA-1 mosaic (EBNA-1 with truncated glycine-alanine rich region), Sm antigen, the EBNA-1 homologous antigenic peptide PPPGRRP, the Sm homologous antigenic peptide PPPGMRPP, or 5 ng/ml PMA + 500 ng/ml ionomycin (positive control; purchased from Sigma-Aldrich/MilliporeSigma). Bulk quantities of the peptides PPPGRRP and PPPGMRPP were constructed on polylysine backbones (MAP™, Applied Biosystems, Foster City, CA) by the University of Oklahoma Health Sciences Center Molecular Biology-Proteomics facility. Antigen specific proliferation was determined in 72 h 96-well cultures by pulsing with 1 μCi/well [3H]TdR (GE Healthcare/Amersham Biosciences) at 48 h, and cpm was determined by liquid scintillation 24 h later. Culture supernatants were collected at optimal culture times for cytokine analysis: 48 h for IL-6, TNF-α, and IL-10, and 72 h for IFN-γ and IL-17A.

Cytokine ELISA

Cytokine concentrations in culture supernatants were determined by ELISA, using cytokine-specific coating and biotinylated detection antibodies diluted per manufacturer's protocol (eBioscience/Invitrogen/Thermo Fisher Scientific). Streptavidin-HRP (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) binding to biotinylated detection antibodies was visualized with TMB substrate (KPL/Seracare, Milford, MA) and the reaction was stopped with 0.18 M H_2SO_4. Plates were read at 450 nm *via* Emax Plus Reader (Molecular Devices, San Jose, CA). Data were analyzed with SoftMax Pro software (Molecular Devices); unknowns were compared with a standard curve containing at least five to seven dilution points of the relevant recombinant cytokine (eBioscience/Invitrogen/Thermo Fisher Scientific) on each assay plate. In all cases, the coefficient of determination for the standard curve (r^2) was ≥0.98. ELISA unknowns were diluted to fall within the range of standard values.

Anti-EBNA-1 and Anti-Sm Serology

Standard solid-phase assays were used to measure the antibody reactivity in mouse sera, as described previously (86). One μg of Sm (Immunovision, Springdale, AR) or EBNA-1 mosaic (BiosPacific) was coated per well in each of 96 polystyrene wells/plate. Mouse sera at a dilution of 1:100 (Sm) or 1:1000 (EBNA-1) were incubated in each well for 3 hrs. After incubation, plates were washed and incubated with anti-mouse alkaline phosphatase-conjugated γ-chain-specific goat IgG (Sigma-Aldrich/MilliporeSigma) at 1/10,000 dilution. Para nitrophenyl phosphate disodium (PNPP, Sigma-Aldrich/MilliporeSigma) was used as a substrate for alkaline phosphatase, and plates were read at 405 nm *via* Emax Plus reader (Molecular Devices). ELISA tests were considered positive if the optical density (OD) was at least two standard deviations above the naïve/adjuvant control mean.

Solid-Phase Peptide Synthesis and Anti-Peptide Assays

Sequential, overlapping octapeptides from EBNA-1 and Sm BB' were synthesized at the ends of radiation-derivatized polyethylene pins arranged in a 96-well microtiter plate format, as described previously (19, 51). All unique octapeptides (EBNA-1 aa 1–103 and 288–641; Sm BB' aa 1-233) were synthesized, while duplicate octapeptides (especially in the glycine–alanine-rich region of EBNA-1 [aa 97–321]) were omitted. Positive control pins were synthesized from a known reactive sequence of the Sm B' protein (PPPGMRPP) and used with previously characterized reactive (positive) and non-reactive (negative) sera as standards. Sera from mCD40-LMP1 Tg, mCD40 WT, and CD40-deficient mice were tested for binding with the EBNA-1 or Sm BB' octapeptides by a solid-phase ELISA-based immunoassay, as previously described (19, 51, 86, 91). Briefly, individual solid-phase peptides were incubated with a 1:100 dilution of mouse sera for 2 h at room temperature. Each pin block was washed and incubated with anti-mouse IgG Fc-specific alkaline phosphatase conjugate or with anti-human IgG alkaline phosphatase conjugate for the positive controls (Jackson Immunoresearch Laboratories), overnight at 4°C. Pin blocks were washed, then incubated at 37°C with PNPP substrate until positive control wells had absorbance readings of 1.0 at 405 nm. A well-characterized human positive control serum was used to normalize the results among multiple plates. Reactivity against an octapeptide was considered positive if the absorbance was at least four standard deviations above the naïve/adjuvant control mean.

Autoantibody Detection and Renal Function Tests

Sera were assessed for anti-nuclear antibodies (ANA; Alpha Diagnostic International, San Antonio, TX), anti-dsDNA antibodies (Alpha Diagnostic Int'l), blood urea nitrogen (BUN; Arbor Assays, Ann Arbor, MI), and serum creatinine (Arbor Assays) per manufacturers' protocols. For ANA and anti-dsDNA assays, sera were measured in duplicate at a 1:100 dilution in a 96-well plate format, and the HRP-coupled secondary Ab was goat anti mouse IgG (H and L). Negative and positive control

sera, as well as 5 point calibration curve samples, provided by the manufacturer, were run concurrently with the unknown samples. Sera were diluted 1:10 for BUN assays and 1:30 for creatinine assays, per manufacturers' protocols. Sera were run in duplicate alongside a 5 (creatinine) or 7 (BUN) point standard curve. All assays were read at 450 nm using an Emax Plus Reader (Molecular Devices). Unknowns were compared with a calibration curve containing five dilution points on each assay plate. In all cases, the coefficient of determination for the standard curve (r^2) was ≥0.98.

Statistical Analyses

Analyses were performed with GraphPad version 7.02 Instat software. Student's paired t-test was used to determine significance between paired groups. One-way ANOVA with Dunnett's multiple comparison test was used to determine significance between >2 groups. P-value ≤0.05 was considered significant.

RESULTS

Assessment of LMP1 Functional Mimicry in the Context of EBNA-1

We have previously demonstrated that mCD40-LMP1 Tg mice exhibit mild autoimmunity, marked by lymphadenopathy, splenomegaly, enhanced cytokine secretion, and autoantibody production (71). We therefore asked how mCD40-LMP1 would influence antigen-specific inflammatory responses and lupus-like pathogenic features in the context of EBNA-1. Based on our previous studies assessing EBNA-1 humoral immunity in animal models (86, 90), mCD40-LMP1 Tg mice and congenic controls (B6, mCD40Tg, and B6.CD40-deficient mice) were immunized (in CFA) with EBNA-1 or its cross-reactive autoantigen, Sm, and boosted (in IFA) over a 56-day course (**Figure 2**). Additional mice completed either a 10-, or 28-day EBNA-1 immunization/booster protocol to determine cellular and humoral immune response kinetics. Sera were collected for serology and renal function testing, lymph nodes for assessment of antigen recall responses, and spleens for assessment of splenomegaly and activation capacity of T- and B-lymphocytes.

mCD40-LMP1 Tg Mice Mount Accelerated and Enhanced Cellular Immune Response to EBNA-1 Immunization

To compare the cellular immune response to EBNA-1 and its antigenic epitope PPPGRRP, homologous to a comparable sequence in the lupus autoantigen Sm, draining lymph node cells from EBNA-1-immunized mCD40-LMP1 Tg, congenic WT, or CD40-deficient mice were cultured in the presence or absence of antigen (**Figure 3** and **Supplementary Figure 1**). All three strains of mice were able to mount a proliferative antigen recall response against EBNA-1 56 days after initial EBNA-1 immunization (**Figure 3A**). However, mCD40-LMP1 mice showed a significantly greater response to EBNA-1 ($p<0.01$) and PPPGRRP ($p<0.001$), even after the proliferative response in mCD40-LMP1 mice shifted over time away from an EBNA-1 antigenic response toward PPPGRRP (from 10 to 28 to 56 days post-immunization). Similarly, all strains of mice produced cytokines after PMA/ionomycin stimulation as a positive control, but antigen-specific cytokine secretion was significantly enhanced in mCD40-LMP1 mice, with some unique differences between EBNA-1 and PPPGRRP stimulation (**Figures 3B–F**). Both EBNA-1 and PPPGRRP stimulated high levels of IL-17 (**Figure 3B**) and IFN-γ (**Figure 3C**) in EBNA-1-immunized mCD40-LMP1 mice, where IL-17 and IFN-γ responses increased over time, reaching the maximum levels seen in day 56 WT mice by day 10. The IL-10 response (**Figure 3D**) was also elevated, though relatively delayed compared to IL-17 and IFN-γ. Interestingly, IL-6 was secreted much more robustly in response to EBNA-1 than to PPPGRRP in mCD40-LMP1 mice, with minimal response to either antigen in CD40 WT mice (**Figure 3E**). Conversely, TNF-α secretion increased after both EBNA-1 and PPGRRP antigenic stimulation as early as 10 days post-immunization in mCD40-LMP1 mice and by day 56 in mCD40 WT mice, but not in CD40-deficient mice (**Figure 3F**).

Concurrently Reactive Cellular Immune Response Between EBNA-1 and Sm in EBNA-1 Immunized mCD40-LMP1 Mice

The humoral response to EBNA-1 cross reacts to lupus autoantigens, including, Sm (35, 44, 51). Given the strong antigen-specific cellular immune response in mCD40-LMP1 mice ((70) and **Figure 3/Supplementary Figure 1**), we asked if cellular concurrent reactivity occurred between EBNA-1 and Sm in the context of LMP1 (**Figure 4** and **Supplementary Figure 2**). We therefore measured antigen recall responses to Sm and its critical humoral epitope homologous to EBNA-1, PPPGMRPP, in the same mice where EBNA-1 antigenic recall responses were measured in **Figure 3**. Similar to the EBNA-1 response, mCD40-LMP1 mice had an enhanced cellular immune response to Sm and PPPGMRPP compared to mCD40 WT and CD40-deficient mice, with respect to both proliferation (**Figure 4A**) and cytokine secretion (**Figures 4B–F**). In addition to proliferation, Sm and PPPGMRPP antigen stimulation elicited a robust IL-17A response in mCD40-LMP1 mice immunized with EBNA-1 (**Figure 4B**). Sm and PPGMRPP also stimulated IFN-γ (**Figure 4C**) and IL-10 (**Figure 4D**) responses in EBNA-1 immunized mCD40-LMP1 mice, but to a lesser degree than the primary antigen, EBNA-1 (**Figures 3C, D**). Of note, IL-6 (**Figure 4E**) showed a response to Sm, but not PPPGMRPP, and TNF-α (**Figure 4F**) only exhibited PPPGMRPP cellular responses in mCD40-LMP1 mice. The response to Sm in mCD40 WT and CD40-deficient mice immunized with EBNA-1 suggests that these mice do mount a response to EBNA-1, and that concurrent/cross-reactivity of this response may have a CD40-independent component.

Primary and Concurrently Reactive Response After Sm Immunization in mCD40-LMP1 vs. mCD40 WT and mCD40-Deficient Mice

Because EBNA-1 immunization of mCD40-LMP1 mice produced a strong EBNA-1 cellular immune response that

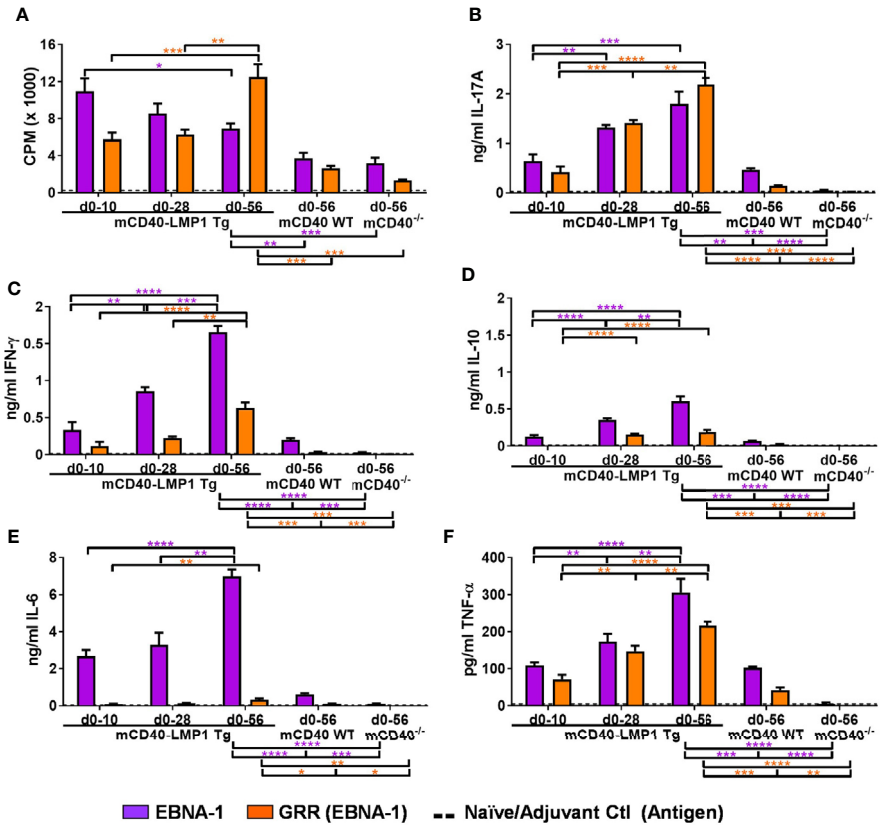

FIGURE 3 | Enhanced antigen recall response to EBNA-1 and homologous region PPPGRRP in mCD40-LMP1 Tg mice. Lymph node cells (4x 10^6/ml) were cultured in the presence of 50 μg/ml EBNA-1 (mosaic) vs. 50 μg/ml PPPGRRP (GRR; EBNA-1 antigenic epitope). Cell cultures were assessed for proliferation **(A)** and cell culture supernatant IL-17A **(B)**, IFN-γ **(C)**, IL-10 **(D)**, IL-6 **(E)**, and TNF-α **(F)**. Antigen recall response from mCD40-LMP1 Tg mice was compared to that of CD40 WT mice and $CD40^{-/-}$ mice (**Figure 2**). Data presented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p < 0.001$, ****$p < 0.0001$ one way ANOVA with Dunnett's multiple comparison test. Significance between experimental groups of mice designated above bar graphs (mCD40-LMP1 mice over time) and below bar graphs (mCD40-LMP1 mice vs. CD40 WT vs. $CD40^{-/-}$ mice); purple = EBNA-1; orange = GRR. A minimal cellular response was exhibited by naïve/adjuvant control mice (dotted line near bottom of y-axis). A minimal cellular response was exhibited by d0-10 and d0-28 mCD40 WT and CD40-deficient mice; only d0-56 data are shown. Antigenic stimulation vs. medium only and PMA/ionomycin is presented in **Supplementary Figure 1**.

concurrently reacted with Sm and its homologous epitope PPPGMRPP (**Figures 3**, **4**), we tested whether Sm immunization of mCD40-LMP1 mice would produce heightened primary (Sm) and concurrently-reactive (EBNA-1) cellular immune responses (**Figures 5**, **6** and **Supplementary Figures 3**, **4**). Indeed, compared to control mice, mCD40-LMP1 mice exhibited enhanced proliferative (**Figure 5A**) and cytokine (**Figures 5B–F**) responses to Sm as the primary antigen, as well against as its antigenic peptide PPPGMRPP, except for a lack of IL-10 after PPPGMRPP stimulation (**Figure 5D**). Unlike EBNA-1 immunization, Sm immunization did lead to a detectable Sm-specific cellular response in mCD40 WT, and to a lesser extent, mCD40-deficient mice.

Compared to the robust concurrently-reactive Sm response after EBNA-1 immunization (**Figures 3**, **4**), Sm immunization produced a more muted concurrently reactive EBNA-1 response (**Figures 5**, **6**). Nonetheless, mCD40-LMP1 Tg mice did mount a concurrently reactive proliferative and cytokine response to EBNA-1, and to a lesser extent, PPPGRRP, particularly through IL-17 (**Figure 6B**), IFN-γ (**Figure 6C**), and TNF-α (**Figure 6E**). No IL-10 was produced in any Sm-immunized mouse strain in response to EBNA-1 or PPPGRRP (**Supplementary Figure 4D**). The limited concurrently-reactive EBNA-1 cytokine response in Sm-immunized CD40 WT mice was primarily reflected by readily detectable TNF-α, while mCD40-deficient mice showed no concurrently reactive cytokine response (**Figure 6E**).

The enhanced, antigen-specific cellular response exhibited in mCD40-LMP1 mice was reflected in an increased presence of activated CD4 T cells before and after EBNA-1 immunization. Further, these mCD40-LMP1 derived T cells were more readily activated by CD3 ± CD28 (**Supplementary Figure 5**). Although follicular and marginal zone B cells are not different between mCD40-LMP1 and mCD40 WT or CD40-deficient mice, mCD40-LMP1 mice showed enrichment of a CD23lo, CD21/CD35lo immature/activated B cell population, as well as increased proliferative and cytokine responses driven by BCR ± CD154 (CD40L) stimulation (**Supplementary Figure 6**).

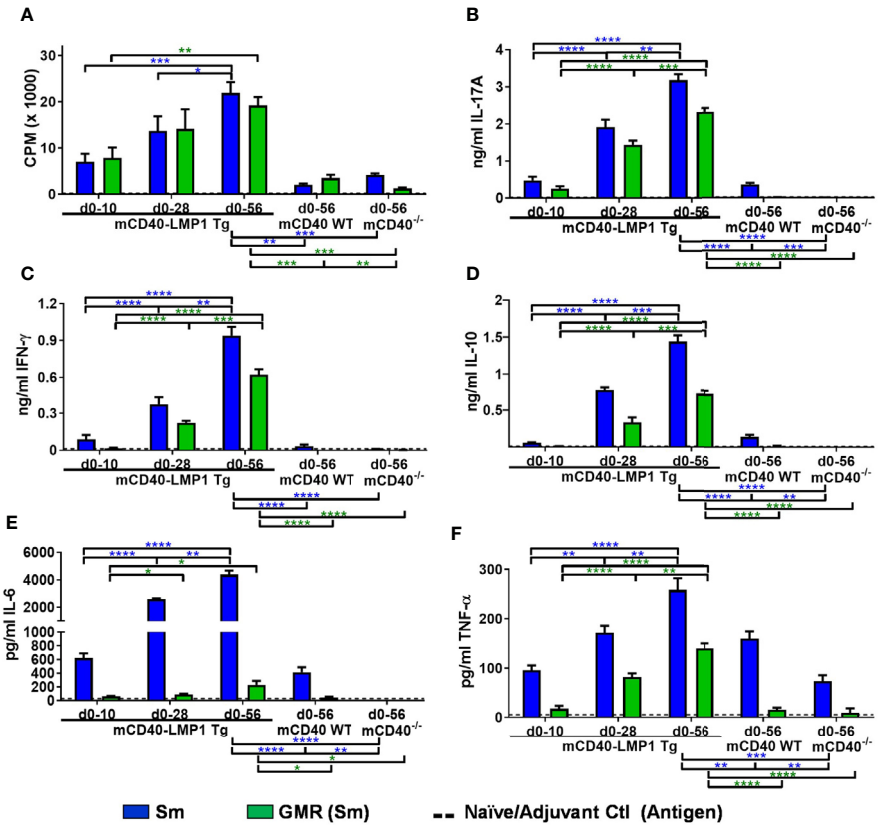

FIGURE 4 | EBNA-1 immunization leads to lupus autoantigen Sm and homologous region PPPGMRPP cellular T cell responses in CD40-LMP1-Tg mice. Lymph node cells (4e6/ml; carried over from **Figure 3**) were cultured in the presence of 50 μg/ml Sm vs. 50 μg/ml PPPGMRPP (GMR; Sm antigenic epitope). Cell cultures were assessed for proliferation **(A)** and cell culture supernatant IL-17A **(B)**, IFN-γ **(C)**, IL-10 **(D)**, IL-6 **(E)**, and TNF-α **(F)**. Antigen recall response from mCD40-LMP1 Tg mice was compared to that of CD40 WT mice and CD40$^{-/-}$ mice (**Figure 2**). Data presented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p < 0.001$, ****$p < 0.0001$ one way ANOVA with Dunnett's multiple comparison test. Significance between experimental groups of mice designated above bar graphs (mCD40-LMP1 mice over time) and below bar graphs (mCD40-LMP1 mice vs. CD40 WT vs. CD40$^{-/-}$ mice); blue = Sm; green = GMR. A minimal cellular response was exhibited by naïve/adjuvant control mice (dotted line near bottom of y-axis). Antigenic stimulation vs. medium only and PMA/ionomycin is presented in **Supplementary Figure 2**.

Enhanced Primary and Concurrently Reactive Humoral Immune Responses Between EBNA-1 and Sm in mCD40-LMP1 Tg Mice

Both primary and concurrent, cross-reactive antibody specificities to EBNA-1 and Sm have been observed in SLE (19, 35, 39) and EBNA-1 drives a strong humoral immune response in animal models (42, 44, 86). Therefore, we evaluated whether the anti-EBNA-1 and anti-Sm antibody responses would be enhanced in mCD40-LMP1 mice compared to mCD40 WT or CD40-deficient mice, after EBNA-1 or Sm immunization (**Figure 7**). Unlike cellular immune responses to either EBNA-1 or Sm, all strains of mice exhibited a readily detectable humoral immune response, although mCD40-deficient mice mounted significantly weaker responses, as expected. Both mCD40-LMP1 Tg and mCD40 WT mice showed a similarly strong, global anti-EBNA-1 antibody response, even as early as 10 days after initial EBNA-1 immunization (**Figure 7A**). However, mCD40-LMP1 mice mounted an earlier and more robust concurrently-reactive Sm antibody response after EBNA-1 immunization (**Figure 7B**). After Sm immunization, mCD40-LMP1 mice once again mounted a concurrently-reactive EBNA-1 response (**Figure 7C**), as well as an enhanced primary response (Sm antigen, **Figure 7D**) compared to control mice, suggesting that the dysregulated cellular immune response driven by the cytoplasmic tail of LMP1 also extends to humoral immunity.

To further characterize the global antibody response to EBNA-1 after EBNA-1 immunization, we mapped the epitope specificity of these responses. Serum reactivity to overlapping octapeptide EBNA-1 epitopes across the EBNA-1 antigen was measured for mCD40-LMP1 Tg, mCD40WT, and mCD40-deficient mice at 10, 28, and 56 days after initial EBNA-1 immunization, compared to adjuvant controls (**Figure 8** and **Supplementary Figure 7**). The patterns of serum interactions across EBNA-1 antigen domains (**Figure 8A**) showed particular regions of reactivity within the N-terminus (one region displayed in **Figure 8B**) and C-terminus (one region displayed in **Figure 8C**) for mCD40-LMP1 Tg mice, mCD40 WT mice, or both. Both mCD40-LMP1 Tg and mCD40 WT mice showed increased responses across the N-terminus (**Figure 8D**) and C-terminus (**Figure 8E**) over time (with additional time/EBNA-1 booster

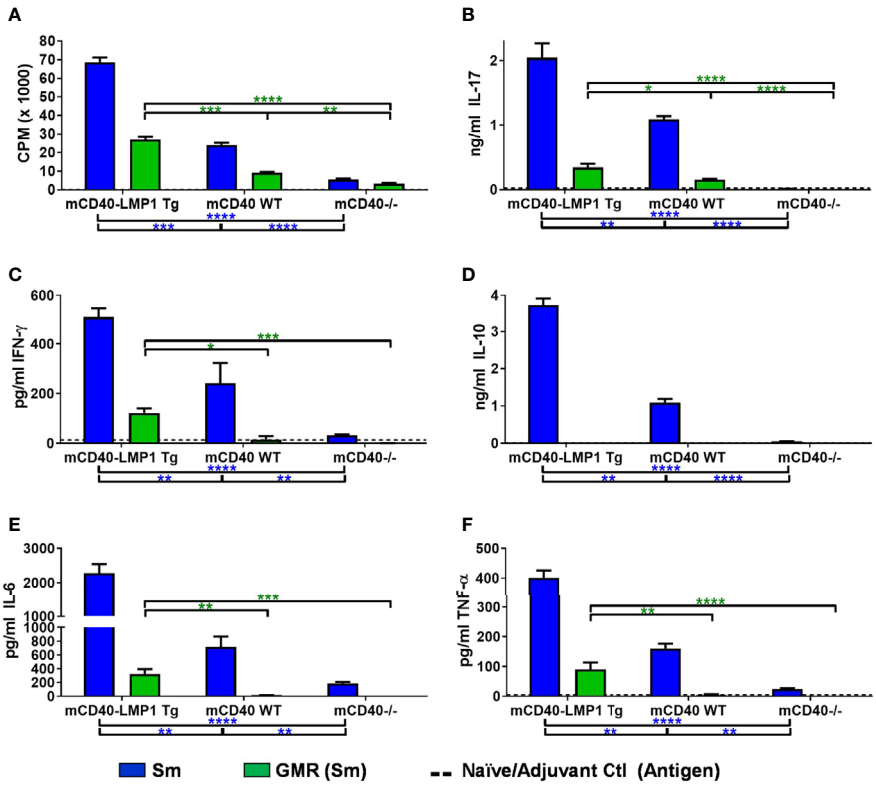

FIGURE 5 | Enhanced antigen recall response to lupus autoantigen Sm and unique reactivity to homologous region PPPGMRPP in mCD40-LMP1 Tg mice. Lymph node cells (4e6/ml) were cultured in the presence of culture medium alone vs. 50 μg/ml Sm, 50 μg/ml PPPGMRPP (GMR; Sm antigenic epitope), and 5 ng/ml PMA/500ng/ml ionomycin. Cell cultures were assessed for proliferation **(A)** and cell culture supernatant IL-17A **(B)**, IFN-γ **(C)**, IL-10 **(D)**, IL-6 **(E)**, and TNF-α **(F)**. Antigen recall response from mCD40-LMP1 Tg mice was compared to that of CD40 WT mice and CD40$^{-/-}$ mice (**Figure 2**). Data presented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p < 0.001$, ****$p < 0.0001$ one way ANOVA with Dunnett's multiple comparison test. Significance between experimental groups of mice designated above bar graphs (mCD40-LMP1 mice over time) and below bar graphs (mCD40-LMP1 mice vs. CD40 WT vs. CD40$^{-/-}$ mice); blue = Sm; green = GMR. A minimal cellular response was exhibited by naïve/adjuvant control mice (dotted line near bottom of y-axis). Antigenic stimulation vs. medium only and PMA/ionomycin is presented in **Supplementary Figure 3**.

immunizations); mCD40-LMP1 mice displayed an enhanced immune response across all time points. Conversely, CD40-deficient mice had a decreasing response over time after initial EBNA-1 immunization, suggesting that an initial CD40-independent antibody response converted to a primarily CD40-dependent response over time.

Similar serum reactivity to overlapping octapeptide epitopes within the Sm BB' antigen was measured for mCD40-LMP1 Tg, mCD40WT, and mCD40-deficient mice 56 days after initial Sm immunization, compared to adjuvant controls (**Figure 9** and **Supplementary Figure 8**). Regions of reactivity to Sm BB' domains (**Figure 9A**) showed enhanced reactivity in Sm-immunized mice across the Sm1 region in the N-terminus (**Figure 9B**), with additional reactivity across the C-terminus, including in the PPPGMRPP antigenic region (**Figure 9C**). Similar to EBNA-1 immunization, immunizing with Sm led to a significantly increased humoral response across both the N-terminus (**Figure 9D**) and the C-terminus (**Figure 9E**) in mCD40-LMP1 Tg mice compared to mCD40 WT and CD40-deficient mice; mCD40 WT mice also mounted a significantly greater anti-Sm response across both N- and C-terminal regions compared to CD40-deficient mice. That CD40-deficient mice mounted a small, but measurable response suggests a CD40-independent component to the anti-Sm humoral immune response.

Autoimmune Phenotype in mCD40-LMP1 Tg vs. mCD40 WT Mice in Response to EBNA-1 Immunization

Unimmunized mCD40-LMP1 Tg mice show a mild autoimmune phenotype (71) that can be pushed to an inflammatory arthritis phenotype in the context of specific antigen, type II collagen (70), while the congenic mCD40 WT and mCD40-deficient strains are not prone to lupus-like disease. Given the enhanced cellular responses, humoral immunity, and dual-reactivity to lupus autoantigen Sm in mCD40-LMP1 Tg mice immunized with EBNA-1, we assessed the presence of other lupus-like features in this mouse strain (**Figure 10**). As expected, adjuvant/naïve mCD40-LMP1 Tg mice had enlarged spleens compared to mCD40 WT mice (**Figure 10A**). EBNA-1 immunization resulted in increased spleen weight in both strains of mice, but

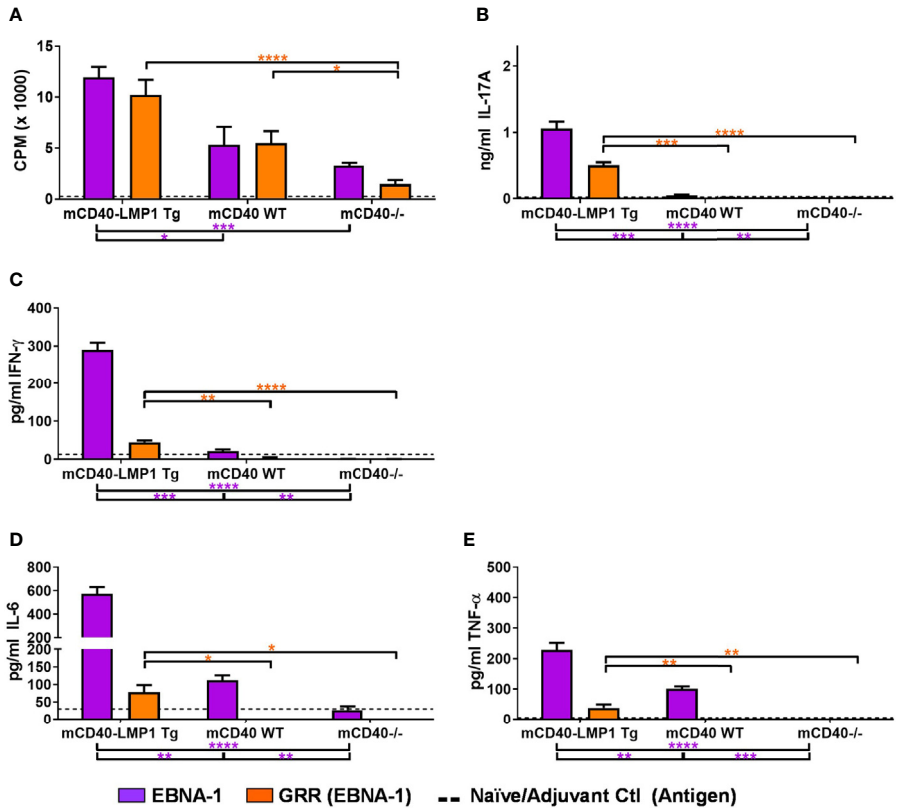

FIGURE 6 | Selective Sm cross-reactivity to EBNA-1 and homologous region PPPGRRP in mCD40-LMP1 Tg mice. Lymph node cells (4e6/ml; carried over from **Figure 5**) were cultured in the presence of 50 μg/ml EBNA-1 (mosaic) vs.50 μg/ml PPPGRRP (GRR; EBNA-1 antigenic epitope). Cell cultures were assessed for proliferation **(A)** and cell culture supernatant IL-17A **(B)**, IFN-γ **(C)**, IL-6 **(D)**, and TNF-α **(E)**. Antigen recall response from mCD40-LMP1 Tg mice was compared to that of CD40 WT mice and CD40$^{-/-}$ mice (**Figure 2**). Data presented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p < 0.001$, ****$p < 0.0001$ one way ANOVA with Dunnett's multiple comparison test. Significance between experimental groups of mice designated above bar graphs (mCD40-LMP1 mice over time) and below bar graphs (mCD40-LMP1 mice vs. CD40 WT vs. CD40$^{-/-}$ mice); purple = EBNA-1; orange = GRR. A minimal cellular response was exhibited by naïve/adjuvant control mice (dotted line near bottom of y-axis). Antigenic stimulation vs. medium only and PMA/ionomycin is presented in **Supplementary Figure 4**.

to a greater extent in the mCD40-LMP1 Tg mice. With respect to autoantibodies, both mCD40-LMP1 Tg and mCD40 WT mice exhibited increased ANA levels after EBNA-1 immunization, and this was enhanced in the mCD40-LMP1 Tg mice (**Figure 10B**). In particular, anti-dsDNA autoantibodies were markedly elevated in mCD40-LMP1 Tg mice 56 days after initial EBNA-1 immunization (**Figure 10C**). Given that anti-dsDNA is associated with lupus nephritis in SLE patients and select lupus nephritis-like mouse models (10), we also assessed serum BUN (**Figure 10D**) and creatinine (**Figure 10E**) levels. Both mCD40-LMP1 Tg and mCD40 WT mice showed increases in BUN (**Figure 10D**) and creatinine (**Figure 10E**) over time, particularly 56 days after initial EBNA-1 immunization in mCD40-LMP1 mice. However, no overt renal pathology nor areas of inflammatory cell recruitment were observed upon histological examination in either strain (data not shown). These data suggest that while some aspects of lupus-associated autoimmunity seen in mCD40-LMP1 Tg mice, enhanced with EBNA-1 immunization, that this may not be enough to drive classic immune complex glomerulonephritis in the time period assessed.

DISCUSSION

More complete understanding of immune dysregulation in SLE will facilitate proactive interventions with the potential to delay and minimize transition to disease classification, clinical disease flare, and permanent organ damage (5, 92). Despite the ability of a multitude of studies to elucidate genetic risk and highlight immune parameters that may be influenced by genetic variance (12, 13), genetic variation alone incompletely explains lupus pathogenesis. Turning our attention to environmental factors such as EBV with its latent immune mimics has the potential to help us further identify underlying mechanisms of immune dysregulation and opportunities for intervention. The current study expounds on the ability of the EBV-encoded functional immune mimic, LMP1, and molecular mimic, EBNA-1, to dysregulate both cellular and humoral immunity, resulting in reactivity to the SLE-associated autoantigen Sm.

Preclinical SLE is marked by the development of cross-reactive antibodies recognizing both EBNA-1 and autoantigens. Similarly, when immunized with EBNA-1 or Sm, B6 mice expressing WT mCD40 mount both a primary humoral

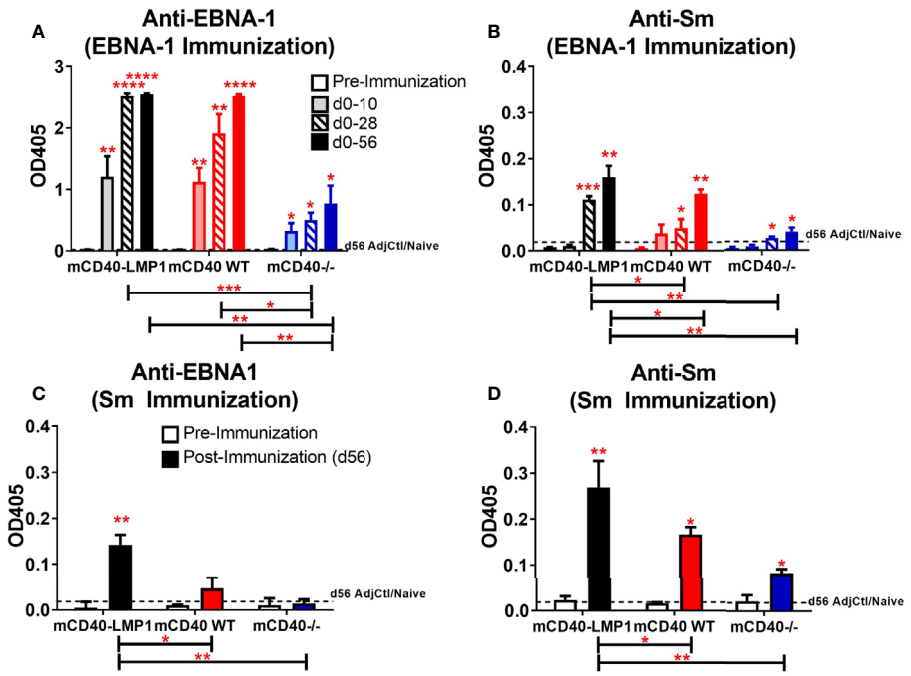

FIGURE 7 | Accelerated humoral reactivity to EBNA-1 and enhanced reactivity/cross-reactivity between EBNA-1 and Sm in mCD40-LMP1 Tg mice. Sera collected at days 10, 28, and 56 **(A, B)** or day 56 only **(C, D)** post-immunization with either EBNA-1 **(A, B)** or Sm **(C, D)** were assessed for EBNA-1 (**A** and **C**, 1:1,000 serum dilution) and Sm (**B** and **D**, 1:100 serum dilution). Antibody response from mCD40-LMP1 Tg mice was compared to that of CD40 WT mice and CD40$^{-/-}$ mice (**Figure 2**). Data presented as mean ± SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ one way ANOVA with Dunnett's multiple comparison test.

response against the immunizing antigen and a cross-reactive response to Sm or EBNA-1, respectively. In mCD40-LMP1 Tg mice, where the cytoplasmic tail of EBV-encoded CD40 mimic LMP1 drives dysregulated signaling, this response is enhanced, particularly to Sm (primary) and EBNA-1→Sm and Sm→EBNA-1 concurrent reactivity. Although mCD40 and mCD40-LMP1 mice exhibited a similar response to total EBNA-1, particularly in the C-terminus near the homologous epitope PPPGRRP (aa398-404), mCD40-LMP1 Tg mice had increased reactivity across the N- and C-terminal domains. This was also the case in mCD40-LMP1 Tg mice immunized directly with Sm, with increased reactivity across both N- and C-terminal domains of Sm BB', particularly in the C-terminus near its homologous epitope PPPGMRPP (aa 191-231). Increased epitope reactivity may allow for enhanced epitope spreading and molecular mimicry/cross-reactivity to lupus associated autoantigens such as Sm. Of note, the modest antibody response to EBNA-1 and Sm in CD40-deficient mice suggests that part of the humoral immune response to these antigens was CD40-independent. The areas of EBNA-1 reactivity were far smaller, but overlapping with mCD40 WT or mCD40-LMP1. A T-independent component of humoral immunity to T-dependent antigens has been demonstrated and likely relies on another TNF-R superfamily member, BLyS/BAFF (93–95).

This study used an immunization protocol designed to induce EBNA-1 humoral immunity in animal models (86), similar to what is observed in SLE patients (19, 39). In addition to an enhanced humoral response, using this immunization strategy in mCD40-LMP1 Tg mice resulted in an enhanced cellular response to EBNA-1/PPPGRRP and Sm/PPPGMRPP, particularly with respect to proliferation, IFN-γ (Th1), and especially IL-17 (Th17) responses, as well as IL-6 and TNF-α. This is not unlike what was observed in the context of type II collagen immunization in the collagen-induced inflammatory arthritis model (70). Of note, the IL-10 response lagged behind other mediators assessed, had less reactivity to the EBNA-1 homologous epitope PPPGRRP, and had no reactivity to the Sm homologous epitope PPPGMRPP. It is possible that the regulatory IL-10 response occurs later than the pro-inflammatory mediator response or that the reactive antigenic region(s) driving an IL-10 response lie(s) outside of the homologous reactive domain for EBNA-1 and Sm. A similar lack of reactivity to the EBNA-1 and Sm homologous domains was also observed with IL-6 secretion. Given that naïve mCD40-LMP1 Tg mice already have increased systemic levels of IL-6 (71), the peptide antigen signal may not be sufficient to drive additional IL-6 production, yet allows for downstream IL-17A secretion. Alternatively, like IL-10, the antigenic region that drives IL-6 production may be outside of the EBNA-1 and Sm homologous domains.

The ability of LMP1 to drive a cellular, concurrently reactive response between EBNA-1 and Sm *in vivo* suggests a possible route for EBV to contribute to cellular molecular mimicry and immune pathway dysregulation. In the present study, EBNA-1 to

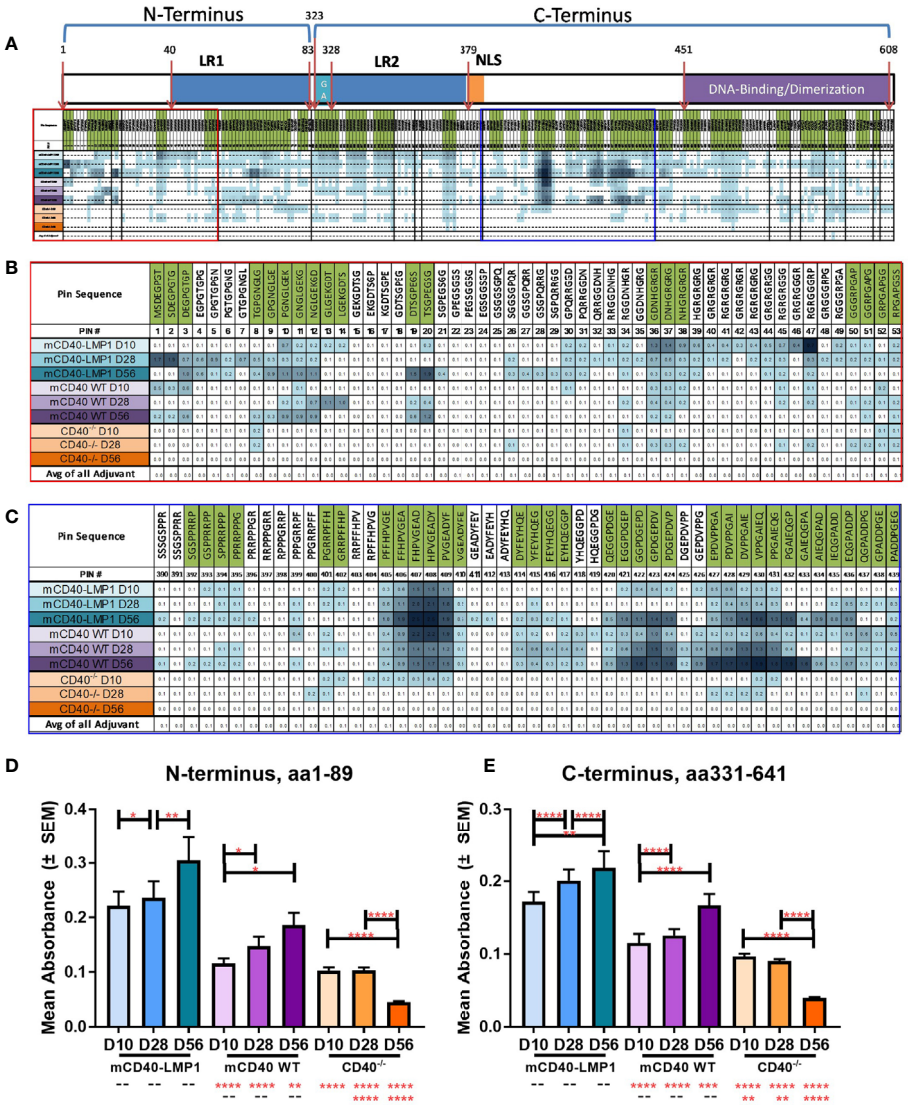

FIGURE 8 | Enhanced EBNA-1 domain-specific humoral response in mCD40-LMP1 Tg mice. EBNA-1 epitope-specific humoral immunity was compared in sera from mCD40-LMP1 Tg mice vs. CD40 WT and CD40-/- mice at 10, 28, and 56 days post-immunization with EBNA-1 (vs. adjuvant/naïve control). EBNA-1 domain map and average reactivity to each EBNA-1 epitope is presented in **(A)**, with green indicating positive epitopes (≥4 SD above adjuvant control). N-terminus area of reactivity from **(A)** is presented in **(B)**. C-terminus area of reactivity from **(A)** is presented in **(C)**. Color intensity of each sample block increases with anti-EBNA-1 epitope reactivity (green shaded epitopes are considered positive if ≥4 SD above adjuvant/naïve control). Corresponding epitope mapping histograms are presented in **Supplementary Figure 7**. Mean ± SEM response to N-terminus (aa1-89, **D**) and C-terminus (aa331-641, **E**) are presented. $*p \leq 0.05$, $**p \leq 0.01$, $***p < 0.001$, $****p < 0.0001$ one way ANOVA with Dunnett's multiple comparison test.

Sm dual-reactivity was more robust than Sm to EBNA-1 dual-reactivity, suggesting that EBNA-1 drives concurrently reactive cellular immunity to Sm, and not the reverse. It is not unusual for concurrent, cross-reactive T-lymphocytes to drive immune and autoimmune processes (96). A cellular immune response to EBNA-1 that cross reacts with autoantigen (myelin) has been demonstrated in multiple sclerosis (97–100), despite the common lack of T-lymphocyte control of EBV infection in multiple sclerosis (101, 102) and SLE (103, 104). A cross-reactive cellular immune response to EBNA-1 has yet to be demonstrated in human SLE, but may be best detected during preclinical SLE when cellular immune dysregulation first gives rise to humoral autoimmunity (2–4, 17). Additionally, cross-reactive cellular immunity with EBNA-1 may be apparent during periods of EBV reactivation, when PBMCs are likely to express EBNA-1 and LMP1 (17), especially since LMP1 positive PBMCs coincide directly with immune dysregulation that leads to clinical disease flare (34, 105). Together, our current and previous findings suggest that LMP1 contributes to immune dysregulation that may set the stage for SLE pathogenesis. Sustained and dysregulated cellular immunity driven by LMP1 may allow for a break in tolerance that allows for the production

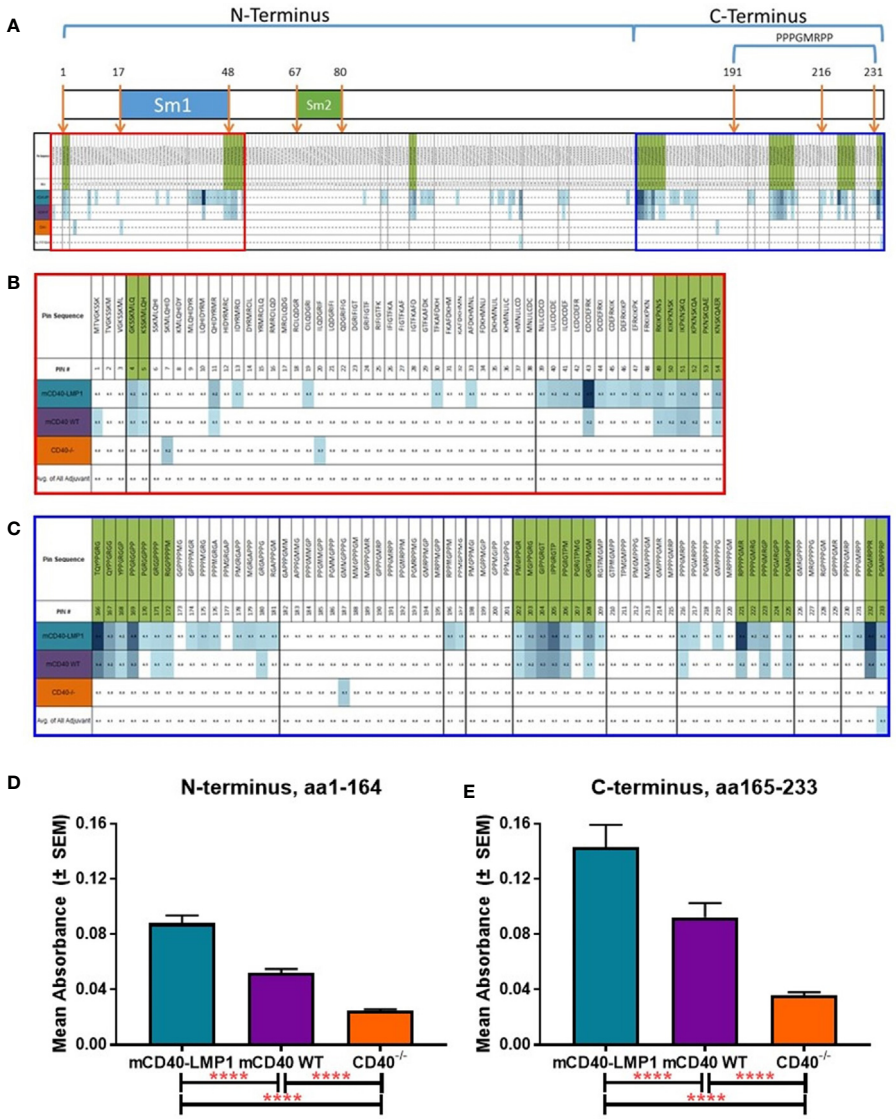

FIGURE 9 | Enhanced Sm BB' domain-specific humoral response in mCD40-LMP1 Tg mice. Sm BB' epitope-specific humoral immunity was compared in sera from mCD40-LMP1 Tg mice vs. CD40 WT and CD40-/- mice at 56 days post-immunization with Sm antigen (vs. adjuvant/naïve control). Sm BB' domain map and average reactivity to each Sm BB' epitope is presented in **(A)**, with green indicating positive epitopes (≥4 SD above adjuvant control). N-terminus area of reactivity from **(A)** is presented in **(B)**. C-terminus area of reactivity from **(A)** is presented in **(C)**. Color intensity of each sample block increases with anti-Sm BB' epitope reactivity (green shaded epitopes are considered positive if ≥4 SD above adjuvant/naïve control). Corresponding epitope mapping histograms are presented in **Supplementary Figure 8**. Mean ± SEM response to N-terminus (aa1-164, **D**) and C-terminus (aa165-233, **E**) are presented. *****p < 0.0001* one way ANOVA with Dunnett's multiple comparison test.

of SLE-associated autoantibodies, including ANA and anti-dsDNA (62, 67, 69, 71). The concurrent expression of EBNA-1 and its role as a molecular mimic may then contribute to accumulation of additional SLE-associated autoantibody specificities, including Sm (9, 19, 35, 39).

Although mCD40-LMP1 Tg mice immunized with EBNA-1 developed ANA and anti-dsDNA autoantibodies, as well as some renal dysfunction with increased BUN and creatinine, no overt renal pathology was noted on histological examination. It is possible that the mCD40-LMP1 Tg mice were just starting to develop nephritis 56 days after initial EBNA-1 immunization and may have developed overt renal pathology if given more time. Alternatively, the B6 strain may be resistant to immune complex glomerulonephritis, thus requiring additional genetic influence even in the context of mCD40-LMP1. Phenotypically, naïve mCD40-LMP1 Tg mice appear similar to B6.Sle2 mice, which exhibit polyclonal antibodies and activated T-cell immunity, but require genes from B6.Sle1 mice to develop

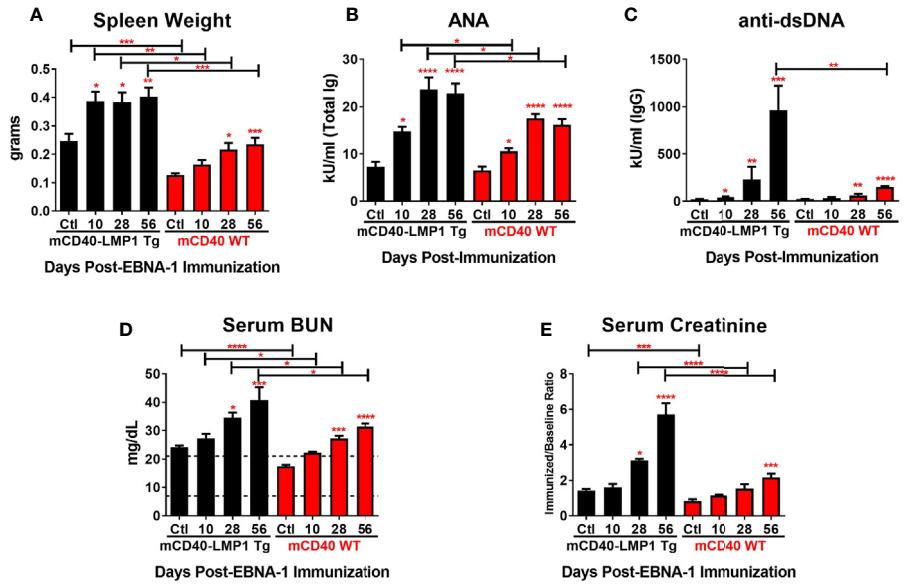

FIGURE 10 | Increased spleen size, autoantibodies, and renal dysfunction in mCD40-LMP1 vs. mCD40 WT over time after EBNA-1 immunization. Mean ± SEM spleen size **(A)**, serum ANA **(B)**, serum anti-dsDNA **(C)**, serum BUN **(D)**, and serum creatinine **(E)** levels at 10, 28, and 56 days post-EBNA1 immunization (vs. adjuvant/naïve control) in mCD40-LMP1 Tg vs. mCD40 WT mice. *$p \leq 0.05$, **$p \leq 0.01$, ***$p < 0.001$, ****$p < 0.0001$ one way ANOVA with Dunnett's multiple comparison test.

overt nephritis (106–108). Crossing mCD40-LMP1 Tg mice with B6.Sle1 mice, but not B6.Sle3 mice, accelerates autoimmunity, including increased cellular immunity, development of anti-dsDNA autoantibodies, and overt renal pathology evidenced by glomerular inflammatory infiltrates (69). Further, LMP1 is expressed in the kidneys of human SLE patients, particularly patients who are positive for anti-Sm autoantibodies (37, 109). This suggests the possibility that enhanced and dysregulated cellular immunity associated with LMP1 functional mimicry (62, 67, 69, 71) may foster anti-Sm and anti-dsDNA autoantibody specificities associated with EBNA-1 molecular mimicry (35, 42–44) to propel some aspects of immune complex-driven lupus nephritis.

We propose that LMP1, in potential conjunction with genetic risk (17, 34, 69, 105), may contribute to immune dysregulation that fosters broken tolerance, enhancing EBNA-1 molecular mimicry and fueling autoantibody production, downstream cellular and tissue damage, and SLE pathogenesis. Although findings in the current study were driven by an mCD40-LMP1 hybrid molecule in the absence of CD40, similar cellular and humoral immune dysregulation has been noted in both *in vitro* (65) and *in vivo* (69) mouse studies in the presence of endogenous CD40, as well as in human patients with confirmed LMP1 expression (34, 110–114). Together, these findings suggest a model whereby EBV-encoded latent immune mimics initiate a network of feed-forward loops that contribute to SLE pathogenesis with LMP1 driven immune dysregulation, and EBNA-1 stimulated autoantibody production (**Figure 11**).

A positive autoregulatory loop that maintains LMP1 expression (**Figure 11**-1) could perpetuate this cycle. Both SLE-associated genetic polymorphisms (12, 13) and EBV infection can upregulate TLR7 expression *via* IRF7 (115). TLR7 stimulates LMP1 expression (112), and LMP1, in turn, stimulates IRF7 *via* RIP, promoting further LMP1 expression. The dysregulation of immune mediators by LMP1 further promotes LMP1 expression (34, 110–112, 116) (**Figure 11**-2). Of particular interest, the regulatory mediator IL-10, which is upregulated during periods of non-flare in SLE patients (6, 7), promotes LMP1 expression (110), which then has the potential to drive inflammatory immune dysregulation leading to a subsequent period of increased clinical disease activity and flare (34, 105).

LMP1 drives additional forms of immune dysregulation that contribute to SLE disease pathogenesis (3), clinical disease activity and flare (5–7), including type I IFN and Th1-, Th2-, and Th17-type immunity. Type I IFN is produced in response to LMP1-mediated IRF7 stimulation (84), in conjunction with other TLRs, including TLR3 and TLR9 (117–120) (**Figure 11**-3). Together, type I IFNs (innate response) and the adaptive immune responses enhanced by LMP1 [current study and (70, 113, 114, 116)] can contribute to T cell-mediated antibody/autoantibody production, facilitating cross-reactive responses between molecular mimic EBNA-1 and lupus autoantigens (35) (**Figure 11**-4). In addition to our findings in the current study, we and others have demonstrated cross-reactivity between EBNA-1 and lupus autoantigens, both in animal models and human SLE patients, including Ro/SSA (19, 39, 40, 86), Sm (19, 39, 44, 121), RNP (39, 51, 86), and dsDNA (39, 40, 42–44). Autoantibodies and ongoing inflammation (122) cause cellular

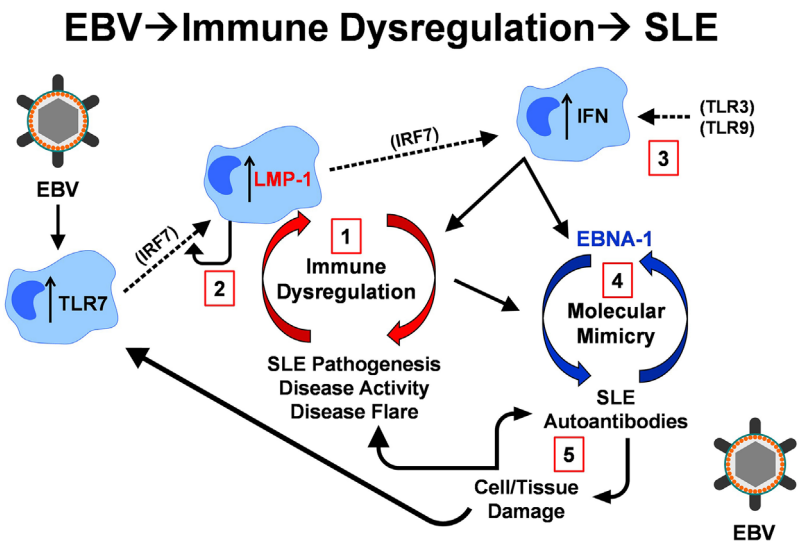

FIGURE 11 | Feed forward model of LMP1 expression, immune dysregulation, and SLE autoimmunity. EBV infection can drive increased TLR7 expression *via* IRF7, which also stimulates increased LMP1 expression. LMP1, in turn, stimulates IRF7 *via* RIP to continue this positive autoregulatory loop **1**. The very immune dysregulation (innate and adaptive) that LMP1 propels also upregulates LMP1 expression **2**. In addition, LMP1 drives interferon (IFN) production *via* IRF7, in conjunction with other TLRs, including TLR3 and TLR9 **3**. Type I IFNs (innate response), in addition to the adaptive immune response that LMP1 promotes, can contribute to T cell-mediated antibody/autoantibody production, allowing for the cross-reactive, molecular mimic response between EBNA-1 and lupus autoantigens Ro, Sm, and RNP **4**. Autoantibodies, in conjunction with ongoing inflammation, lead to cell and tissue damage, releasing additional lupus-associated autoantigens that interact with TLR7 and continue to drive cellular and humoral autoimmunity **5**.

and tissue damage that releases more lupus associated autoantigens, which can interact with TLR7 to further propagate cellular and humoral autoimmunity (112, 117, 123–126) (**Figure 11**-5).

In addition to cellular expression of LMP1, the LMP1 transmembrane domain enables extracellular expression on vesicles and exosomes (127), where it can be internalized (128), including by dendritic cells (129). This allows for LMP1-induced cellular proliferation and activation (128, 130, 131), as well as antibody production and class-switching in non-infected B cells (132). This would allow for LMP1 expression in cells other than B-lymphocytes [and epithelial cells, which are also tropic for EBV (133)] and drive additional pathogenicity.

Our findings suggest that LMP1 can both promote cellular immune dysregulation and potentiate EBNA-1 humoral immunity and dual-reactivity with the lupus autoantigen Sm. Such dysregulation may be necessary, yet insufficient, to explain SLE pathogenesis. Over 90% of the general population is EBV seropositive (134), yet only a subset of individuals develop SLE or other autoimmune diseases. Indeed, EBNA-1 molecular mimicry and LMP1-mediated immune dysregulation have been noted in patients with mononucleosis (51, 135–137), but this does not lead to autoimmune disease in most patients. It is possible that immune dysregulation fostered by EBV latent mimics provides a break in immune tolerance that creates an opportunity for SLE-associated genetic risk variants to drive SLE pathogenesis (12, 30, 138–140). In a mouse model of lupus-like disease that associates phenotype with genetic risk [B6.Sle1.Sle2.Sle3 mice (107)], we have previously demonstrated that mCD40-LMP1 Tg mice accelerate lupus-like autoimmunity in B6.Sle1, but not B6.Sle3 mice [mCD40-LMP1 Tg mice are phenotypically similar to B6.Sle2 mice (71, 107)], including histologic evidence of glomerulonephritis) (69). Further, SLE patients experiencing heightened clinical disease activity and flare have been shown to exhibit an altered type I IFN gene signature that is associated with LMP1 expression in PBMCs (34). Immune dysregulation that contributes to SLE pathogenesis, clinical disease activity, and organ damage may be further augmented by lifestyle and other environmental factors, including smoking (141–144), UV exposure (145–147), and changes in gut microbiome (148–150). Future studies that further elucidate the relationship in gene-environment interactions, including EBV-encoded latent mimics, have the potential to better define windows of therapeutic opportunity for targeted treatments.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Oklahoma Medical Research Foundation Institutional Animal Care & Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

MM managed mouse colonies, designed and carried out experiments, completed data analysis, and principally wrote manuscript. JA and TG provided technical support. LS and GB provided transgenic mice and Hi5 (WT and mCD154) insect cells, as well as experimental and editorial guidance. JJ provided additional support, as well as experimental and editorial guidance. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.606936/full#supplementary-material

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REVIEW
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Epstein Barr Virus and Autoimmune Responses in Systemic Lupus Erythematosus

Neelakshi R. Jog[1*] *and Judith A. James*[1,2]

[1] Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, Oklahoma City, OK, United States,
[2] Departments of Medicine, Pathology, Microbiology & Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, United States

Systemic lupus erythematosus (SLE) is a complex systemic autoimmune disease. Infections or infectious reactivation are potential triggers for initiation of autoimmunity and for SLE flares. Epstein-Barr virus (EBV) is gamma herpes virus that has been associated with several autoimmune diseases such as SLE, multiple sclerosis, Sjogren's syndrome, and systemic sclerosis. In this review, we will discuss the recent advances regarding how EBV may contribute to immune dysregulation, and how these mechanisms may relate to SLE disease progression.

Keywords: Epstein-Barr virus, systemic lupus erythematosus, inflammation, autoimmune disease, viral homologs of host genes, molecular mimicry

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***Correspondence:**
Neelakshi R. Jog
Neelakshi-Jog@omrf.org

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a multifaceted systemic autoimmune disease (1) stemming from immune dysregulation. A characteristic feature is the presence of autoantibodies directed towards nuclear antigens (ANA), which can be detected up to a decade before disease onset. Although not completely characterized, studies suggest that cellular dysfunction, dysregulated inflammatory responses and autoantibody -mediated damage leads to progression of autoimmune disease and organ damage (2).

The underlying factors responsible for disease transition and pathogenesis likely involve an interplay between genetic and environmental factors. SLE has a twin concordance rate of 24% to 40% (3, 4) and over 100 genetic associations have been identified and confirmed (5).

Infections or pathogens have been proposed to lead to autoimmunity. Epstein Barr virus (EBV) is one such pathogen that has been repeatedly associated with SLE since the first report in 1969. EBV adopts several strategies to exploit host immune response for its persistence. Consequences to the host are increased acute inflammation and autoantibody generation, which are usually transient and self-limited, as seen in patients with infectious mononucleosis (6). However, a growing body of research suggests that these effects in certain individuals, possibly based on genetic risk factors, can cascade into a chronic inflammatory state. Due to its strong association with tumorigenesis, EBV has been studied extensively for its ability to overcome immune surveillance and approached to combat tumorigenic effects.

In this review we provide a compilation of the current understanding of how EBV may contribute to immune dysregulation, including strategies used by EBV to combat immune surveillance, and how these processes may relate to SLE pathogenesis (**Figure 1**).

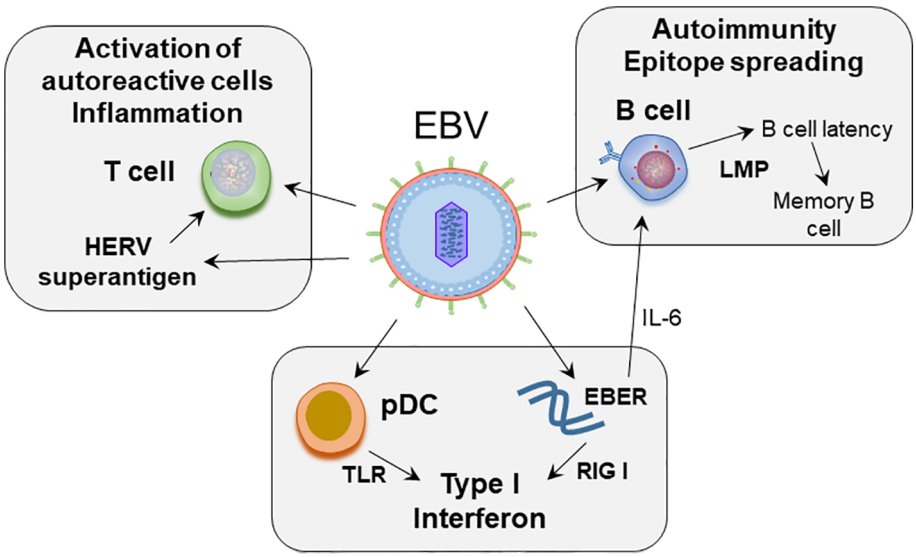

FIGURE 1 | Proposed role of EBV in SLE pathogenesis. EBV infects naïve B cells. The infected B cells enter the memory B cell compartment through germinal center like reaction, mediated by the expression of latent membrane proteins. EBV maintains latency in the resting memory B cells. EBERs, non-coding RNA expressed by EBV, can mimic dsRNA, and activate RIG-I leading to production of type I interferons. EBERs also induce growth factor IL-6 and regulate B cell survival. EBV can act on plasmacytoid dendritic cells (pDC). Initial binding of virus is mediated by class II MHC on pDCs, following which through engagement of TLR7 and 9, EBV RNA and DNA can induce type interferon secretion by pDCs. EBV induces superantigen on HERV-K18, which can induce unregulated T cell activation.

EBV LIFE CYCLE

Acute primary EBV infection, which is also a common cause of infectious mononucleosis, is characterized by fatigue, atypical lymphocytosis, splenomegaly, and lymphadenopathy. Although the host immune response eventually controls viremia, the virus maintains latency in memory B cells with occasional reactivation to infect naïve B cells. EBV genomes in latently infected B cells are thought to exist as episomes (7), although it is possible that the genomes exist as integrated DNA. EBV expresses nine latent proteins; six EBV nuclear antigens (EBNA, EBNA-1, 2, 3A, 3B, 3C, and leader protein), and three latent membrane proteins (LMP 1, 2A, and 2B). In addition to latent proteins, expression of small non-polyadenylated RNAs, EBER1 and 2, is also observed (8).

Unique forms of latency that differ in the latent protein expression have been identified (**Table 1**). Latency III, where all latency gene products are expressed, is the predominant latency observed in lymphoblastoid cell lines, acute infectious mononucleosis, and certain immunocompromised individuals.

TABLE 1 | Latency forms of EBV.

Latency	Genes expressed
Latency 0	EBER1/2
Latency I	EBNA-1, EBER1/2, miRNA
Latency II	EBNA-1, EBER1/2, miRNA, LMP1/2
Latency III	EBNA, EBNA-1, 2, 3A, 3B, 3C, EBNA-LP, LMP 1, 2A, 2B, EBER1/2, miRNA

This form of latency can mediate naïve B cell activation. EBNA1 and LMP1/2A are expressed in the latency II program, which is observed in nasopharyngeal carcinoma and Hodgkin's lymphoma. LMP1 and LMP2 can induce B-cell activation and growth (proliferation). Latency I, which is observed in EBV-positive Burkitt's lymphoma tumors, expresses only EBNA-1. In this form, latent EBV genomes can multiply in dividing memory cells. The Gly-Ala repeats in EBNA-1 inhibit antigen processing, and therefore, CD8 T cells are unable to detect virally infected cells in this form. Latency 0 is observed in quiescent B cells, where no EBV proteins are expressed, however cells switch to Latency I during cell division with expression of EBNA-1, which is required for replication of the episome. Latently infected B cells occasionally reactivate EBV. This allows the virus to re-infect new B cells and epithelial cells, and acts as a source of viral transmission. Although the molecular pathways involved in viral reactivation are studied extensively, the triggers for reactivation *in vivo* are unclear.

The occasional reactivation of the virus can be detected serologically. A primary infection with EBV leads to an IgG response to viral capsid antigen (VCA). The VCA IgG antibodies are maintained throughout the life span of the individual. Following VCA IgG response, IgG responses toward early antigen (EA) are detected. These antibodies are detectable for 6 months to up to two years. During EBV reactivation, EA IgG levels are detectable and there is an increase in VCA IgG levels (9). Therefore, an increase in VCA IgG and presence of EA IgG indicates current or recent reactivation of the virus.

EBV LATENCY AND REACTIVATION IN SLE

Many studies to date have demonstrated an association between SLE and EBV infection. A higher EBV seroconversion rate was observed in both pediatric and adult SLE patients compared to healthy controls (10–12). SLE patients show increased levels of IgG antibodies toward VCA and EA, both indirect markers of increased EBV reactivation. However, the IgG responses towards other herpes viruses such as cytomegalovirus (CMV) and herpes simplex virus (HSV) are similar in SLE patients and controls. These reports suggest that SLE patients may have increased reactivation of the virus. EBV viral load is elevated in SLE patients (13, 14), which may also suggest increased reactivation. A possible reason for increased reactivation is inefficient regulation of the latent phase or enhanced transition from latent to lytic phase. Interestingly, a higher percentage of patients have detectable levels of EBV gene BZLF1 (15), which is an immediate-early gene that is responsible for the switch to lytic cycle. Two other latent genes LMP1 and LMP2A were also detected in SLE patients. The type of latency maintained in SLE patients is not completely understood. LMP1/2A are expressed in latency II, and all latent genes are expressed in latency III (**Table 1**). The presence of two latent genes, BZLF-1 and LMP-1, which cannot be detected in seropositive healthy individuals, suggests that EBV latency may be dysregulated in some SLE patients. Based on the expression pattern of latent genes reported so far, SLE patients may have an intermediate form between latency II and III.

EBV REACTIVATION IN SLE AND UNDERLYING MECHANISMS

Based on serologic evidence and higher viral loads observed in SLE patients, the consensus is that SLE patients have increased EBV reactivation. Dysregulation of anti-viral T cell responses is a proposed mechanism for increased viral loads. SLE patients have higher interferon γ (IFNγ) secreting CD4+ T cells, but lower frequencies of EBV specific CD8+ T cell responses. EBV viral loads in peripheral blood cells positively correlated with EBV specific and IFNγ secreting CD8+ T cells (14). EBV specific CD8+ T cells in SLE patients are functionally impaired (16, 17), although CMV specific responses were unaltered (17). The upregulation of PD1 on EBV specific T cells in SLE patients may be responsible for the suppressed responses to EBV antigen, as blockade of PD1 restored IFNγ production in response to EBV antigens. Based upon these data and the observed diminished responses of SLE T cells to superantigen stimulation, the authors suggest that SLE T cells demonstrate an exhausted phenotype. However, CMV specific T cell responses were unaltered by PD1 blockade. These data suggest that the general immune surveillance mechanisms are intact in SLE patients, but there is an inherent defect in regulating EBV infection (17). Both CD4 and CD8 lytic and latent antigen specific functional T cells were lower in SLE patients. A negative correlation between SLE disease activity index (SLEDAI) and EBV specific functional T cell responses was reported (18), with decreased EBV lytic gene responsive T cells in patients with elevated disease activity. Furthermore, an inverse relationship was observed between EBV specific T cells and levels of anti-EBV antibodies (18). SLE T cells may also contribute to defective regulation of certain B cell functions (19). Absolute numbers of Th17 and Treg cells were reduced in SLE patients with EBV and/or CMV viremia compared to those without viremia or healthy controls. However, there was a direct correlation between viremia and SLEDAI, suggesting that reduction in Th17 and Treg cells may be a consequence of SLE immune dysregulation independent of viremia (20). EBV can transactivate superantigen on human endogenous retroviral (HERV)-K18, which can lead to unrestricted activation of T cells (21).

EBV induced gene 3 protein (EBI3) was identified in EBV transformed B cells (22). It serves as a beta chain for cytokines IL-27, IL-35, and IL-39, and can induce regulatory or suppressive T cells in a murine model (23). The serum IL-35 level and the percentage of CD4+EBI3+ T cells were negatively correlated with the SLE disease activity index, and both of these parameters were increased shortly after treatment of active SLE patients with methylprednisolone (24). However, levels of EBV reactivation were not determined in this study. Although EBI3 was initially reported in EBV transformed B cells and induced by LMP1, the name of the gene is misleading. EBV infection of T cells is not established unequivocally. It was later shown that EBI3 can be induced in naïve T cells by polyclonal stimulation with plate bound anti-CD3 and anti-CD28 (25). This also explains upregulation of EBI3 in experimental murine models, which lack EBV infection. Therefore, the increase in IL-35 observed in SLE patients may be independent of EBV induced gene expression. Studies evaluating the upregulation of EBI3 in SLE patients in the context of EBV infection and subsequent contribution to SLE pathogenesis are lacking.

Differences in cytokine production in response to EBV antigens have been reported. SLE patients exhibited a decreased IL-12, IFNγ, IL-17, and IL-6 response to EBNA-1, and decreased induction of IL-6, TNFβ, IL-1β, and GM-CSF upon EBV-EA-D stimulation. Serologic SLEDAI scores, based solely on anti-dsDNA, complement, thrombocyte, and leukocyte levels, correlated negatively with numerous cytokine responses against EBNA-1 and EA-D (26). These data further support impaired regulation of immune response against latent and lytic EBV antigens in SLE patients.

The numbers of infected B cells positively correlated with SLE disease activity index (15). The EBV viral load in SLE patients with active disease was found to be higher than in inactive cases (27), however, another study did not find this (17). Although this report did not find a consistent increase in EBV viral load immediately prior or at the time of a flare, the viral loads were higher in a majority of patients during elevated disease activity (17), suggesting that EBV may have a role in the pathogenesis and activity of SLE. The overall low number of EBV-infected B cells during latency and the lower numbers of B cells due to

lymphopenia in SLE patients provides a technical challenge in detecting EBV DNA. Assays with higher sensitivities to detect both latent and lytic EBV genes, perhaps partnered with single cell technologies, will be helpful to understand the relation between timing of EBV reactivation and SLE flare. Detailed longitudinal analyses of a larger cohort of SLE patients will improve our understanding of viral reactivation and SLE disease activity.

Newer data have evaluated the association of EBV reactivation with SLE disease onset. Our retrospective analyses of unaffected family members of SLE patients showed that SLE relatives that subsequently transition to classified SLE (>4 ACR criteria), have increased VCA-IgG and EA-IgG at a time-point prior to the transition, when compared to relatives that do not transition to SLE (28). The responses towards CMV and HSV-1 were not different between the two groups of relatives. These data suggest that EBV reactivation observed in SLE patients is not due to immune dysregulation caused by the chronic autoimmune and inflammatory environment in patients, nor is it solely a consequence of immunosuppressive medications. However, as the study involved blood relatives of SLE patients, a genetic component may be involved in increased EBV reactivation. On similar lines, seropositivity for anti-EBV early antigen (EA), a marker of EBV reactivation, was dramatically increased in patients with SLE compared with unrelated controls (92.3% vs 40.4%; OR 17.15(95% CI 10.10, 30.66), p<0.0001) or unaffected first-degree relatives of lupus patients (49.4%; OR 12.04(7.42, 20.74), p<0.0001). The seroprevalence of VCA IgG in patients and first-degree relatives was similar suggesting same level of prior EBV exposure in these two groups (29).

Significant interactions between EBV serology and single nucleotide polymorphisms (SNPs) in genes that are associated with SLE and also involved in EBV infection were observed. The association between VCA IgG level and transitioning to SLE was modified by *CD40* rs4810485 (interaction p = 0.0009). Similarly, the association between VCA IgA and transitioning to SLE was modified by *IL10* rs3024493 (interaction p = 0.008) (28). In line with a genetic component contributing to increased EBV reactivation, a higher frequency of subjects with germ-line mutations in CTLA-4 had detectable EBV viral load when compared with healthy controls. None of the subjects had symptoms of EBV infection the time of analyses. However, none of the 15 subjects included in this study had a SLE diagnosis (30). Parks et al. showed a significant interaction between VCA IgA and CTLA-4 gene polymorphism (-1661AA), and increased VCA IgA seropositivity in African American SLE patients (31). CTLA-4 -1661 mutation was associated with risk of SLE in young African American patients (32).

Harley et al. recently showed that in EBV-immortalized B cells, almost half of SLE European ancestry risk alleles can be occupied by EBNA-2 protein, which is expressed in latency II and III. The authors showed that host transcriptional factors bind to SLE risk loci only in the presence of EBV, and that EBNA-2 is involved in allele dependent transcription complex formation at risk loci. These data provide another potential origin of gene/environment interaction in SLE (33).

Thus, genetic predisposition leading to immune dysregulation may contribute to EBV reactivation eventually resulting in classified SLE.

EBV EFFECTS ON THE IMMUNE SYSTEM IN SLE

In order to evade the host immune system and to establish a persistent latent infection, EBV encodes several viral homologues of human proteins. These homologues either accentuate the effect of human proteins on immune cells, inhibit, or allow the virus to hijack the immune response to its benefit.

EBV IL-10

EBV IL-10 (vIL-10) is a late viral gene expressed during the lytic phase of virus replication encoded by the viral BCRF1 gene, which is highly homologous to the human IL-10 (hIL-10) gene (34, 35). Due to the high homology, vIL-10 shares some of the suppressive and stimulatory functions of hIL-10. vIL-10 can inhibit inflammatory cytokine (IFNγ, TNFα) production and can promote proliferation and differentiation of B cells, as well as immunoglobulin production. Functional differences between hIL-10 and vIL-10 have also been reported. vIL-10 cannot co-stimulate mouse thymocyte proliferation and mast cell proliferation and cannot up-regulate MHC class II on B cells (36–38).

We recently showed that in contrast to hIL-10, vIL-10 can induce a pro-inflammatory phenotype in monocytes. vIL-10 induced a unique gene expression profile in monocytes, and monocytes exposed to vIL-10 showed defective clearance of apoptotic cells. vIL-10 signals through the same receptor subunit as hIL-10, can act as a competitive inhibitor of hIL-10, and inhibit suppressors of immune response induced by hIL-10. vIL-10 levels were significantly higher in SLE patients plasma compared to matched controls (39). As vIL-10 is a lytic gene, these data also support increased reactivation of EBV in SLE patients.

Increased vIL-10 in SLE patients may increase pro-inflammatory responses by monocytic cells, while inhibiting hIL-10 functions. These pro-inflammatory mediators, along with a reduced clearance of apoptotic infected cells, may lead to increased antigen presentation and activation of cytotoxic T cell responses towards EBV. Indeed Stewart et al. showed that vIL-10 enhances the generation of allo-specific CTL, EBV-specific CTL, and HLA-unrestricted activated killer cells (40). Although this allows the virus to enter latency and persist, in a genetically prone individual with defective tolerance checkpoints, the defective clearance and increased antigen presentation may lead to autoimmune responses (**Figure 2**). Further longitudinal studies evaluating vIL-10 levels in preclinical samples, as well as in SLE patients, pre and post flare, and associations of these levels with monocyte activation status are needed to confirm the role of vIL-10 in induction of an autoimmune response.

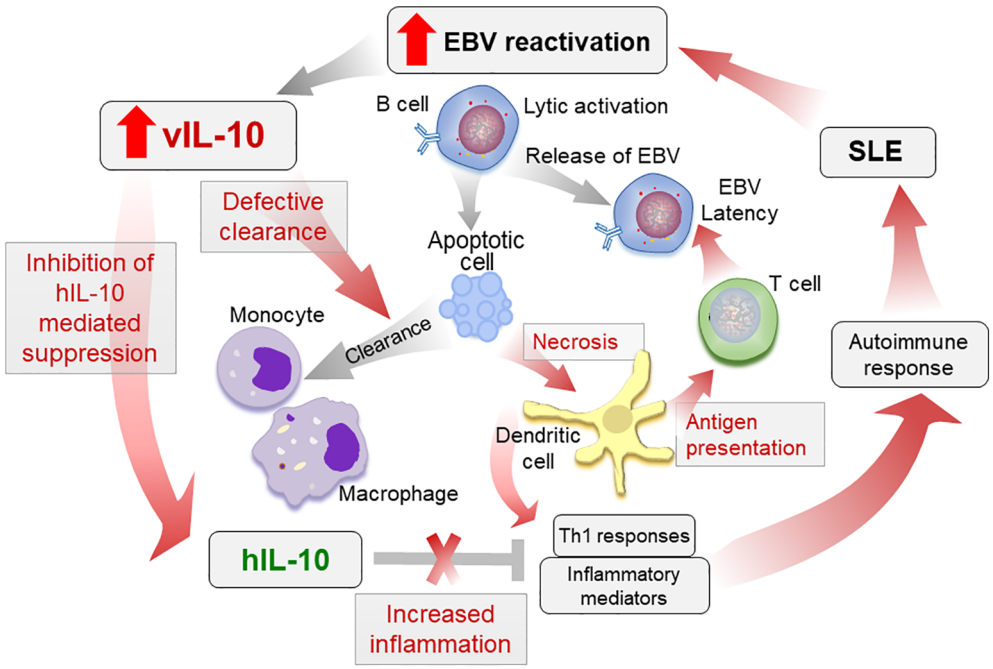

FIGURE 2 | Proposed role of vIL-10 in SLE pathogenesis. Increased reactivation of EBV in SLE patients increases vIL-10. vIL-10 competes with hIL-10 for IL10R1 and inhibits the suppressive effects of hIL-10 on myeloid cells. vIL-10 also reduces the ability of monocytes/macrophages to clear apoptotic cells. This leads to increased secondary necrosis, increased presentation of antigens by dendritic cells (DCs), and allows virus to establish latency. Reduced clearance of apoptotic cells leading to secondary necrosis along with increased antigen presentation and inflammatory responses exacerbate autoimmune response in SLE. The processes possibly regulated by vIL-10 are shown in red arrows.

How vIL-10 induces a unique gene expression in monocytes and can inhibit hIL-10-mediated immune suppression is not clear. vIL-10 has lower affinity to IL-10R1 compared to hIL-10. However, vIL-10 is more potent than hIL-10 in inducing B cell proliferation, and therefore the lower affinity may not explain the differences in monocyte activation by vIL-10. The vIL-10: IL-10R1 interaction is very transient, while with hIL-10 is more sustained (41). A transient interaction may interfere with ligand-dependent receptor internalization and proteasomal degradation. vIL-10 may be sequestering receptors and compete with hIL-10. Although not reported in the literature yet, it is possible that the vIL-10 monomer forms a heterodimer with hIL-10 and inhibits signaling by hIL-10.

Latent Membrane Proteins

How EBV maintains latency in memory B cells is also not completely understood. It is hypothesized that EBV enters the memory B cell compartment through differentiation of the latently infected B cell blasts into resting memory B cells, also known as the germinal center (GC) model. The observations that the viral infection is strictly latent in resting memory B cells in the periphery, but active infection of naïve B cells and virus shedding can be detected in tonsillar lymphoid tissue, support this hypothesis [Reviewed in (42)].

EBV expresses three latent membrane proteins (LMP, 1, 2A, 2B) that can mimic signals necessary to rescue normal B cell differentiation in absence of T cell signals. Despite the lack of significant protein homology, LMP1 is a functional homologue of CD40, and acts as a constitutively active receptor (43). LMP1 induces B cells to express B cell-activating factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL), which mediate B cell survival and T cell-independent antibody production, and therefore can induce class switch recombination (CSR) in absence of a GC reaction (44, 45). Thus, EBV may block B cells from entering GC, and induce extra-follicular B cell activation through the expression of LMP1. The expression of a chimeric molecule with the mouse CD40 extracellular domain and the LMP1 intracellular signaling regions in lupus-prone mouse strain accentuated the autoimmune phenotype. This suggests that LMP1 acts synergistically with host predisposing genetic factors and contributes to exacerbation of an autoimmune response (46).

LMP2A mimics the B cell receptor (BCR), and contains an immunoreceptor tyrosine based activation motif (ITAMs) which associates with downstream signaling kinases. LMP2A mimics the BCR signal and can rescue B cells lacking surface immunonoglobulin from death (47). Conditional expression of LMP2A in murine GC B cells enhanced BCR signals, facilitated plasma cell differentiation, and resulted in selection of low affinity antibody producing cells. The conditional GC expression also led to SLE-like autoimmune phenotype including anti-double stranded DNA (dsDNA) antibody production, and immune complex deposition in the kidneys (48). Expression of LMP2A transgene in anti-Sm heavy chain

transgenic mice resulted in increased anti-Sm antibodies (49). In these mice transgenic for anti-Sm and LMP2A, anti-Sm B cells bypassed the pre-plasma cell tolerance checkpoint and differentiated into antibody secreting cells, suggesting that LMP2A can modify GC B-cell selection and may contribute to persistent EBV infection.

EBV RNA (EBERs and MIRNA)

Additional genes that are expressed during EBV latency are two noncoding RNAs, EBER1 and EBER2, and 44 microRNAs (miRNAs), derived from two loci, the BART and BHRF clusters. BART transcript encodes 22 miRNA precursors (miR-BART1-22) with 40 mature miRNAs, whereas the BHRF1 transcript expresses three miRNA precursors (miR-BHRF1-1, -2, and -3) producing four mature miRNAs (50). EBV miRNA from infected cells were secreted in exosomes, which can be internalized by monocyte derived dendritic cells (51) and modulate their gene expression. In individuals with increased EBV viral load, EBV miRNA were detected in both B and non-B cells in peripheral blood. Although the levels of EBV miRNA have not been compared between SLE patients and unaffected donors, EBV miRNA may be contributing to differences in gene expression profiles observed in non-B cells in SLE patients.

EBER1 and EBER2 are present in all four latency stages (52, 53). Several reports have suggested a role for EBERs in the tumorigenic process *in vivo*, which are also supported by murine studies where transgenic mice expressing EBER1 developed lymphoid hyperplasia and an increase in lymphoma incidence (54). EBERs form a stem–loop structure by intramolecular base-pairing, which can give rise to dsRNA-like molecules (55, 56). EBERs can bind to dsRNA activated protein kinase PKR, inhibit its phosphorylation and can confer resistance to IFN-induced apoptosis in Burkitt's lymphoma cells (57). EBERs can contribute to B cell transformation and growth by inducing the growth factor IL-6 (58). EBERs can regulate target regulation of several miRNAs. Expression of EBER can enhance the inhibitory effect of miR143-mediated downregulation of the inflammatory gene IL1α (59), however, the significance of these effects in the development or progression of autoimmune diseases is unclear. Expression of EBER in EBV-negative B lymphoma cell line resulted in upregulation of kinases involved in B cell pro-survival signaling, which were previously considered to be regulated solely by LMP1, suggesting a redundancy in function between EBERs and LMP1 during latency (60). EBERs are recognized by retinoic acid-inducible gene I (RIG-I) through the helicase domain and can activate signaling to induce type I interferon and interferon-stimulated genes (61).

SLE patients show increased levels of type I interferon in serum, and SLE disease activity correlates with IFNα levels and the strength of the interferon signature (62, 63). EBV increases IFNα secretion by plasmacytoid dendritic cells (pDCs) through toll-like receptors (TLR). The recognition of EBV is mediated by class II MHC molecules (64). The increased LMP1 gene expression in SLE patients correlated with SLE disease activity index (SLEDAI) and interferon induced gene expression (65). The levels of EBERs were not evaluated in this study. The contribution of EBV or EBER mediated interferon activation and the significance of this induction in progression of SLE needs further evaluation.

EBV AND AUTOIMMUNE HUMORAL RESPONSE IN SLE

In SLE patients, EBV EA IgG positivity correlated with lupus antibodies (29). EBV IgG also correlated with anti-Ro and anti-La antibodies in SLE patients (66).

Molecular mimicry between SLE autoantigens and EBV antigens may lead to autoimmune response. Antibodies towards different regions of EBNA-1 protein cross-react with SLE autoantigens SmB, SmD, as well as Ro (67). Monoclonal antibodies generated from mice immunized with EBNA-1 cross-react with dsDNA (68, 69). Cross-reactivity between the anti-EBNA-1 response and anti- complement component C1q response has also been shown. Anti-C1q antibody towards A08 epitope of C1q isolated from SLE patients can bind a peptide derived from EBNA-1, EBNA348, and SLE patients that showed reactivity to EBNA348 peptide had higher levels of anti-C1q. This cross-reactivity was shown to be dependent on amino acid identity (70). Peptides derived from EBV EA and LMP1 increased ANA positivity in mice. Both these peptides increased anti-SmB and anti-SmE. While EA derived peptide, EP4, additionally increased anti-SmD and anti-Ro, LMP1 derived peptide increased anti-rRNP. Levels of EP4 antibodies were higher in SLE patients and correlated with SLEDAI. Interestingly, both these peptides had about ~60% amino-acid sequence similarities with self-peptides, but the percentage of similarities with amino-acid characteristics was 75 and 70% respectively for each peptide (71).

Immunization of experimental animals with peptides from regions of EBNA-1 lead to lupus-like autoimmune disease (72–74). In these studies, immunization with a single peptide lead to the generation of cross-reactive antibodies, but the autoimmune response also spread to several different epitopes, and was not restricted to the cross-reactive epitope. Furthermore, injection of mice with plasmids expressing either full-length EBNA-1 or EBNA-1 lacking 15 amino acids in in the Gly-Ala repeats, resulted in anti Sm, and anti-dsDNA antibodies (75). Epitope spreading has been suggested as a possible mechanism for accrual of antibody specificities, and has been shown to occur with immune response towards spliceosomal and other proteins (72, 74, 76).

Taken together, these reports suggest that molecular mimicry with EBV epitopes may allow loss of tolerance to self-antigens. Through the process of epitope spreading, these responses may target additional self-epitopes, eventually leading to pathogenic responses and to clinical SLE (**Figure 3**).

ANIMAL MODELS OF EBV INFECTION

Although a significant effort has been made to understand EBV biology, understanding how EBV contributes to autoimmune pathogenesis, and the causal relationship between EBV infection,

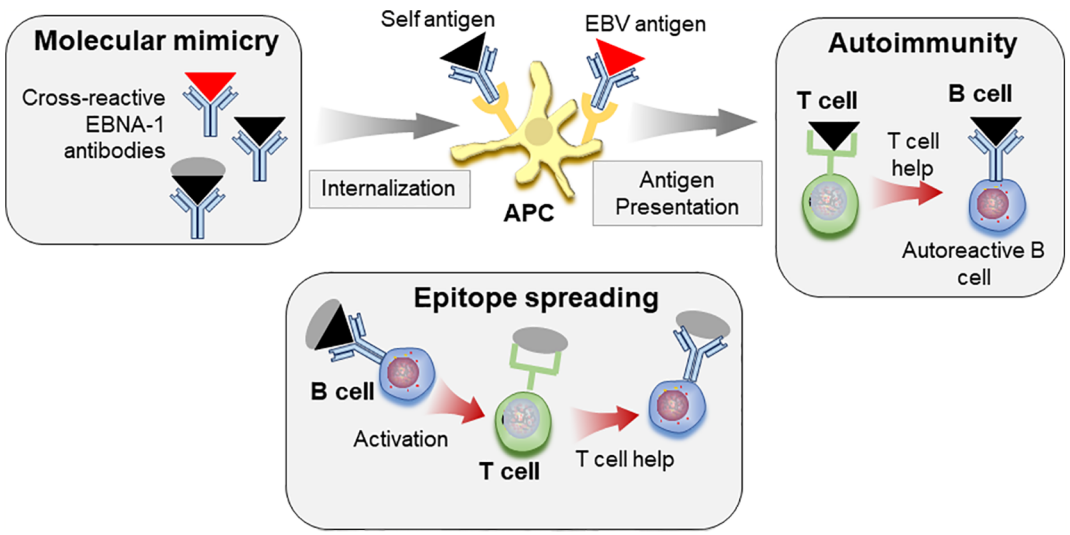

FIGURE 3 | Molecular mimicry and epitope spreading in autoimmunity. Antibodies to viral antigens such as EBNA-1 (red triangle) cross-react with autoantigens (black triangle) due to structural similarities. Immune complexes consisting of autoantigen/antibody complexes are internalized by antigen presenting cells (APC), antigen is processed and peptides presented to T cells, which can allow for loss of tolerance. Defective T cell tolerance possibly contributed by genetic susceptibility may be responsible for this loss of tolerance. These autoreactive T cells in turn provide help to auto-reactive B cells leading to autoreactive antibody response. The self-protein bound to B cell receptor is internalized, processed, and presented to T cells. The autoimmune response can be further diversified by epitope spreading. B cells specific for viral antigen (red triangle) can recognize similar structures on self-antigen (black triangle). However, these cells can internalize and present peptides from whole protein that carries the cross-reactive epitope (black triangle+gray circle) to T cells, which then provide help for antibody response towards additional epitopes on the protein by B cells.

reactivation and autoimmunity are limited due to lack of an appropriate animal model. Peptide immunizations have been instrumental in establishing molecular mimicry between EBV antigens and autoantigens. Transgenic mouse model approaches allowed a better understanding of the ability of EBV latent proteins to modulate B cell function. However, the expression of EBV encoded oncogenes in absence of the entire EBV genome has limitations. These knowledge gaps warrant a suitable animal model that recapitulates the features of EBV infection.

Non-human primates are infected naturally with EBV-related herpesviruses, or lymphocryptoviruses (LCV), and are therefore considered as models for EBV infection [reviewed in (77)]. A primary EBV infection can be established in healthy New Zealand white rabbits, and EBV can also infect Owl monkey and marmosets (78–80). These animal models may prove to be useful for understanding role of EBV in malignancies. However, none of these are characterized as animal models for human autoimmune diseases.

A major advance in establishing a mouse model for EBV came from utilization of humanized models on an immune-deficient murine background. The reconstitution of severe combined immune-deficient (SCID) mice with human peripheral blood leukocytes results in mice with inducible human immune function (81) and development of EBV+ lymphomas by transfer of peripheral blood leukocytes from EBV positive donors (82). However, several limitations such as transient nature of the graft, low engraftment levels, and frequent graft-versus-host disease caused by human T cells attacking mouse tissues, limit the use of this model.

Reconstitution of recombination activating gene 2 (Rag2) deficient IL2 receptor gamma (IL2Rγ) deficient mice also supported EBV infection (83). The deficiency of IL2Rγ allows for T cell re-constitution, and T cells are selected on murine tissue. However, as the T cell are selected on murine and not human tissue, the response in these mice is still suboptimal. This limitation can be overcome by implanting Non-obese diabetic (NOD)/SCID mice with human fetal liver and thymic tissue to provide human T cells appropriate thymic environment, with subsequent autologous CD34+ cell implantation following sub-lethal irradiation (BLT mice) (84). BLT mice showed marked increase in memory T cells, and the T cells could respond to autologous antigen presenting cells upon EBV infection, suggesting that human T cells in BLT mice can mount human-MHC-restricted response and can be used to reproduce human T and B cell interactions. Although an attractive approach, humanized models of EBV infection have not been utilized for SLE research yet. Reconstitution of immunodeficient mice with hematopoietic stem cells from EBV positive and EBV negative SLE patients and matched controls may provide useful insights into pathways regulating increased reactivation in SLE and/or role of EBV in disease progression.

EBV infection of NOD/SCID IL2Rγ-/- (NSG) mice reconstituted with human cord blood hematopoietic cells resulted in erosive arthritis in 65% of mice (85). However, neither anti-citrullinated peptide antibodies nor rheumatoid factor were detected in the blood of affected mice. The serological response to EBV infection observed in humans was also not detected, suggesting that the arthritis observed in

these mice was by mechanisms different from those in patients. However, the genetic factors associated with rheumatoid arthritis were not considered in this study.

The study does point out a possible limitation of using humanized mouse models to replicate EBV infection. During both primary infection and subsequent reactivation, lytic replication of EBV occurs in oropharyngeal epithelial cells, where infectious virus particles are produced and shed. Although EBV is hypothesized to infect and to maintain latency only in B lymphocytes (86), EBV can replicate in epithelial cells and viral gene expression patterns differ when the virus emerges from epithelial cells versus B cells, which suggests passage back and forth (87). Due to differences in routes of infection and lack of the epithelial infection, humanized mice do not recapitulate the complete life cycle of EBV infection, and therefore do not reflect the immune response to EBV infection. These models also lack final lytic replication in oropharyngeal epithelial cells, which the virus uses to amplify infectious virus production during shedding into saliva. This limitation may be overcome by human epithelial tissue grafts in humanized mice followed by infection through the natural route. However, whether the transient infection in epithelial cells that produces virus with increased tropism to B cells is necessary to establish latent EBV infection in B cells and whether this transient infection occurs during EBV reactivation are not known.

A murine virus similar to EBV is an alternate approach. The most probable is murine gamma herpes virus 68 (MHV68). Although not identical to EBV, MHV68 shares several features. MHV68 is found in class switched B cells that have undergone GC reaction and reflect memory B cells. MHV68 is a natural pathogen of free-living murid rodents. Virus neutralizing antibodies are detectable in the natural hosts (88). The infection of mice with MHV72, a gamma herpesvirus strain related to MHV68, leads to detectable anti-viral antibodies, and these correlate with viral reactivation (89).

MHV68 infection is associated with an expansion of lymphocyte populations that drives an infectious mononucleosis-like response marked by enlarged lymph nodes and splenomegaly (90, 91). Productive infection in the lungs following intranasal infection of mice with MHV68 lasts for ~10 days. During this time the virus spreads to spleen through infected B cells and establishes latency in GC B cells (92). Long term latency is detected in IgD- subset of splenic B cells (93). MHV68 has been shown to maintain latency in peritoneal macrophages, which has not been reported for EBV. However, similar to EBV, the splenic latency is solely dependent on B cells (94).

MHV68 increased anti-Sm antibodies in wild type and lupus prone mice during acute phase of infection, however, chronic infection protected mice from lupus-like disease (95). The frequency of infected cells and viral load was not determined, and single high dose of virus was used, which was administered intra-peritoneally. Lower doses of virus do not impact establishment of latency but can delay the acute-phase replication peak. Small numbers of pre-formed virus particles were detected in splenocytes of mice infected with lower doses of the virus (96). Although this small increase in the numbers of virus particles did not constitute significant reactivation in the non-autoimmune wild type C57/Bl6 strain used in that study, it may contribute to immune response in a mouse strain genetically prone to immune dysregulation. Therefore, administration of lower doses of MHV68 to lupus-prone mice by oral and/or intranasal routes, may recapitulate EBV infection in SLE patients. MHV68 does not encode a homologue for human IL-10. However, a recombinant MHV72 expressing EBV IL-10 showed exacerbated acute-phase pathogenicity (97). The effect of this recombinant virus on lupus like disease in murine models has not been evaluated. Detailed analyses of humoral response to MHV68, frequency of viral reactivation, and frequency of infected memory B cells in lupus prone mice are necessary to understand the role of MHV68 in murine lupus-like disease.

CONCLUDING REMARKS

EBV can modulate immune responses in a myriad of pathways, including generation of cross-reactive antibodies, IFNα secretion, antigen independent B cell activation, gene expression modification, and anti-inflammatory response suppression. SLE patients show evidence of increased reactivation of EBV, possibly resulting from dysregulated immune responses together with genetic risk factors. Furthermore, the viral homologues such as vIL-10 modulate immune response in a manner that can exacerbate autoimmune response in genetically susceptible subjects. A longitudinal study that closely follows levels of viral latent and lytic gene expression and cellular changes, in the context of genetic risk alleles will provide an improved understanding of EBV reactivation in SLE and how this reactivation may contribute to autoimmune response.

Mouse models, either humanized or MHV infection of lupus prone mice, may be an alternate approach to decipher the role of EBV. CD34+ hematopoietic stem cells generated *in vitro* from induced pluripotent stem cells (iPSC), which are EBV negative, to reconstitute BLT mice described by Melkus et al. can overcome the effects of prior exposure to EBV in patient cells. The use of iPSC also allows for introducing (or reverting) specific mutations to further clarify the gene/environment interactions, and determining immune dysregulation immediately following EBV infection.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Monica Guma, University of California, San Diego, United States

***Correspondence:**
Kristine A. Kuhn
kristine.kuhn@cuanschutz.edu

†Present address:
Emilie H. Regner, Division of Gastroenterology and Hepatology, Department of Medicine, Oregon Health Sciences University, Portland, OR, United States
Alison E. Freeman, Cascade Gastroenterology, Bend, OR, United States

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Multi 'Omics Analysis of Intestinal Tissue in Ankylosing Spondylitis Identifies Alterations in the Tryptophan Metabolism Pathway

Adam J. Berlinberg[1], Emilie H. Regner[2†], Andrew Stahly[1], Ana Brar[3], Julie A. Reisz[4], Mark E. Gerich[2], Blair P. Fennimore[2], Frank I. Scott[2], Alison E. Freeman[2†] and Kristine A. Kuhn[1]*

[1] Division of Rheumatology, Department of Medicine, University of Colorado, Aurora, CO, United States, [2] Division of Gastroenterology and Hepatology, Department of Medicine, University of Colorado, Aurora, CO, United States, [3] Department of Medicine, University of Colorado, Aurora, CO, United States, [4] Department of Biochemistry and Molecular Genetics, University of Colorado, Aurora, CO, United States

Intestinal microbial dysbiosis, intestinal inflammation, and Th17 immunity are all linked to the pathophysiology of spondyloarthritis (SpA); however, the mechanisms linking them remain unknown. One potential hypothesis suggests that the dysbiotic gut microbiome as a whole produces metabolites that influence human immune cells. To identify potential disease-relevant, microbiome-produced metabolites, we performed metabolomics screening and shotgun metagenomics on paired colon biopsies and fecal samples, respectively, from subjects with axial SpA (axSpA, N=21), Crohn's disease (CD, N=27), and Crohn's-axSpA overlap (CD-axSpA, N=12), as well as controls (HC, N=24). Using LC-MS based metabolomics of 4 non-inflamed pinch biopsies of the distal colon from subjects, we identified significant alterations in tryptophan pathway metabolites, including an expansion of indole-3-acetate (IAA) in axSpA and CD-axSpA compared to HC and CD and indole-3-acetaldehyde (I3Ald) in axSpA and CD-axSpA but not CD compared to HC, suggesting possible specificity to the development of axSpA. We then performed shotgun metagenomics of fecal samples to characterize gut microbial dysbiosis across these disease states. In spite of no significant differences in alpha-diversity among the 4 groups, our results confirmed differences in gene abundances of numerous enzymes involved in tryptophan metabolism. Specifically, gene abundance of indolepyruvate decarboxylase, which generates IAA and I3Ald, was significantly elevated in individuals with axSpA while gene abundances in HC demonstrated a propensity towards tryptophan synthesis. Such genetic changes were not observed in CD, again suggesting disease specificity for axSpA. Given the emerging role of tryptophan and its metabolites in immune function, altogether these data indicate that tryptophan metabolism into I3Ald and then IAA is one mechanism by which the gut microbiome potentially influences the development of axSpA.

Keywords: ankylosing spondylitis, spondyloarthritis, metabolomics, tryptophan, metagenomics, indole, microbiome

INTRODUCTION

Ankylosing spondylitis is a form of axial spondyloarthritis (axSpA) resulting in inflammation of the axial spine, peripheral joints, and entheses (1). The clinical overlap between axSpA and bowel inflammation has long suggested an interaction between the gut and joint in its pathogenesis (2), and the condition of reactive arthritis caused by intestinal pathogens indicates that intestinal bacteria may trigger some forms of disease. Intestinal microbiome studies in humans with axSpA have shown significant ecological alterations (dysbiosis) in bacterial taxa such as *Ruminococcus gnavus, Dialister*, and *Akkermansia muciniphila* as compared to healthy controls (3–7). Few studies have demonstrated consensus taxa, though, which may be due to geographic and other study-specific influences, yet raises the question of how disparate bacterial species can contribute to pathogenesis of disease.

In the HLA-B27/β2m transgenic rat model that develops spontaneous SpA and bowel inflammation, transcriptional changes in IL-17, IL-23, and TNF, key cytokines in the pathophysiology of SpA (8), in the intestinal tissues are associated with metabolic changes as well as microbial changes that are found in human SpA (9). This finding suggests that dysbiosis can influence pathogenic immunity of SpA. Additional work utilizing rats with different genetic backgrounds indicates that community functions rather than specific taxa may be key to disease development. Using two susceptible rat strains for the development of intestinal inflammation in the setting of B27, dysbiosis was vastly different between the two strains, lacking a common taxonomic profile to associate with disease and specific cytokine production. However, when metabolic pathways were imputed from the dysbiotic ecosystem, common features of vitamin synthesis and short-chain fatty acid synthesis emerged (10). Indeed, murine studies have addressed the concept of intestinal bacterial metabolites such as riboflavin and short chain fatty acids like butyrate influencing immune cells, particularly Th17 cells (8, 11–14). In the B27 rat model, both intestinal bacteria and short and medium-chain fatty acid metabolites are likely important for establishing Th17-mediated inflammation (15, 16). Thus, community function leading to metabolic alterations that affect mucosal immunity may be more relevant than specific taxa in influencing pathophysiology.

Despite the described dysbiosis in axSpA, a number of knowledge gaps remain: First, what are the microbial community functions in human axSpA? As described above, there are metabolic changes in rats with B27-related SpA. Furthermore, alterations in a number of bacterially-generated metabolites have been found to be altered in a similar SpA disease in humans, psoriatic arthritis (PsA) (3, 17, 18). Yet, such analyses have not been performed in human axSpA. Second, microbiome studies in axSpA to date have been inclusive of subjects with intestinal inflammation, which is present in nearly 50% of axSpA patients on a histologic level (19). How this serves as a confounding factor remains unclear. In this study, we hypothesized that the intestinal microbiome associates with an altered metabolomic profile in axSpA distinct from controls and confounding by intestinal inflammation. Within this work, we utilize an unbiased metabolomics approach followed by metagenomics methods to investigate alterations in metabolic byproducts of the bacterial communities and how these can relate to the gut microbiome using a pure axSpA cohort compared to those with intestinal inflammation.

METHODS

Subject Recruitment

Utilizing a case-control format, patient and control study subjects were recruited at the University of Colorado Hospital between November 2017 and November 2018. Subjects were identified from the endoscopy schedule if undergoing a routine colonoscopy as part of their clinical care or recruited to undergo an elective flexible sigmoidoscopy. Recruited healthy controls (HC) (n=24) were undergoing colonoscopy for routine cancer screening or a change in bowel habits. AxSpA cases (n=21) underwent elective flexible sigmoidoscopy for the purpose of this study or were undergoing colonoscopy due to changes in bowel habits (n=2), and were only included as cases when IBD was excluded macroscopically and histologically. Subjects with CD (n=27) were undergoing colonoscopies for disease activity assessment and colon cancer/dysplasia screening. Only patients without endoscopic or histologic evidence of dysplasia were recruited into the study. Additionally, patients with both CD and AxSpA (CD-axSpA) (n=12) were recruited and similarly underwent either elective flexible sigmoidoscopy for the purpose of this study (n=8) or standard of care colonoscopy (n=4). Subjects with CD were eligible if they had biopsy-proven CD with terminal ileum involvement during the history of their disease. Subjects recruited as axSpA cases fulfilled the 2009 Assessment of SpondyloArthritis International Society (ASAS) criteria for axSpA (20), including evidence of axial disease by either MRI or radiographs. Individuals with CD-axSpA met study criteria for both CD and axSpA. Exclusion criteria for all groups included: presence of bowel disease, pregnancy, use of antibiotics in the two weeks prior to study entry, cancer or cancer history, inability to stop aspirin or non-steroidal anti-inflammatory drugs seven days before and after endoscopy, use of anticoagulation, HIV, and *Clostridium difficile* infection within the past 3 months.

At the time of endoscopy, subjects completed questionnaires regarding demographic information and disease activity through the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and Harvey Bradshaw Index. These data are presented in **Supplemental Table 1**. Prior to colonoscopy, a rectal swab was also performed on each subject. Swabs (BD FecalSwab) were inserted 3 cm past the anal verge and rotated against the lateral colon wall a minimum of three times. Swabs were then placed immediately on ice and frozen at −80°C until further use. Thirty pinch biopsies from uninflamed rectosigmoid colon were taken during endoscopy, placed into RPMI 1640 (Gibco) on ice, and stored cryogenically in recovery freezing media (Gibco) until further analysis.

This study was conducted according to the principles within the Declaration of Helsinki. All study procedures were approved by the Colorado Multiple Institutional Review Board. All subjects provided written informed consent.

Metabolomics

Following initial colonic pinch biopsies, four previously frozen dry samples were randomly chosen and analyzed *via* ultra-high pressure liquid chromatography-mass spectrometry-based high throughput metabolomics at the University of Colorado School of Medicine Metabolomics Core. Frozen tissue samples were weighed to 15 mg tissue per mL extraction buffer then extracted at 20 mg/mL using ice-cold 5:3:2 methanol:acetonitrile:water (v/v/v) in the presence of glass beads at 4°C. Samples were homogenized using a bead beater for 5 min then vortexed 30 min at 4°C, spun down for 10 min at 18,000 rcf and 4°C, and the supernatants analyzed on a Thermo Vanquish UHPLC coupled to a Thermo Q Exactive mass spectrometer. Metabolites were separated on a 5 min C18 gradient with positive and negative (separate runs) using electrospray ionization. Detailed data acquisition parameters and chromatographic conditions are described in a recent methods paper (21, 22). Quality control was assessed using technical replicates injected every 10 runs. Resulting.raw files were converted to.mzXML format using RawConverter then metabolites assigned and peak areas integrated using Maven (Princeton University) in conjunction with the KEGG database and an in-house standard library of >600 compounds. The targeted data analysis focused on metabolites involved in central carbon and nitrogen metabolism and yielded measurements of 184 metabolites. No *post hoc* normalization was performed; data is available upon request. Samples were normalized relative to each other based upon the same initial starting weight of tissue.

Microbial DNA Extraction, Library Prep, and Metagenomics Sequencing

DNA was isolated from previously described rectal swabs using the Qiagen AllPrep Power Fecal DNA/RNA kit. Standard protocol was followed per kit instructions. Quality control was performed using a Thermo Scientific NanoDrop 2000 spectrophotometer ensuring 260/280 nm light ratios >1.7 for all samples. Libraries were then constructed using the NEB Next Ultra II FS DNA Library Prep Kit in a paired end fashion with 2x150 base pair paired end reads. One hundred fifty nanograms of DNA was utilized for each sample in creating libraries. Libraries underwent quality control *via* tape station prior to multiplexing at a concentration of 4 nM, and sequencing was performed on an Illumina NovaSeq6000 platform (San Diego, CA, USA) at the University of Colorado Genomics core with >6 Gb data output per sample.

Data Processing and Taxonomic Classification

Manual inspection of sequenced reads was performed utilizing FastQC v0.11.9 for all samples. Paired end reads were then concatenated and quality control conducted with Kneaddata 0.7.5 (http://huttenhower.sph.harvard.edu/kneaddata), utilizing Trimmomatic v0.39 (23) and Bowtie2 v2.3.5 (24) to remove unwanted human genome reads and low quality sequences. The processed reads were then entered into the HUMAnN 2.0 pipeline (25), utilizing MetaPhlAn v2.0 (26), which does not account for paired-end relationships, with gene profiling abundance performed using the UniRef90 full universal database. Output data in reads per kilobase was then converted to copies per million prior to downstream application. Taxonomic profiling, alpha diversity, and beta diversity were performed in MicrobiomeAnalyst (27, 28). EdgeR was used at standard settings to perform statistical analysis of previously obtained taxonomic profiling.

Metagenome Analysis

Functional output from HUMAnN 2.0 regarding gene families from all samples was merged in a pairwise manner using the command: humann2_join_tables. Output from HUMAnN 2.0 in reads per kilobase was then converted to copies per million prior to downstream application utilizing the command: humann2_renorm_table. Following this, gene families were then regrouped from Uniref90 to KEGG (Kyoto Encyclopedia of Genes and Genomes) orthology (KO) using the command: humann2_regroup_table. Unbiased metagenomics assessment was conducted using the MicrobiomeAnalyst software on the converted HUMAnN 2.0 output data. The EdgeR feature was utilized for taxonomic and functional assessment, which normalizes read counts followed by low abundance removal and False Discovery Rate (FDR) correction (29). From the overall KO metagenomics set, a comprehensive search was performed for any genes related to the keywords indole or tryptophan. Output from HUMAnN 2.0 pathway analysis was also characterized using the EdgeR feature of MicrobiomeAnalyst in a similar manner for the assessment of pathways related to tryptophan metabolism.

Data Analysis

Subject demographics and medications were compared between all four groups using ANOVA or Fischer's exact test. All tests of significance with p-value <0.05 was considered statistically significant. Metabolomics assessment was performed using MetaboAnalyst software (30). PERMANOVA was performed in R. Taxonomic and functional profiling was performed using MicrobiomeAnalyst software. For taxonomy alpha diversity, students t-test was utilized using the methods of Observed, Chao, Shannon, and Simpson. Beta diversity was assessed utilizing Bray-Curtis dissimilarity and visualized with a Principal Coordinate Analysis (PCoA) plot. PERMANOVA was performed using MicrobiomeAnalyst software of the beta diversity clustering. Statistical analysis of previously obtained Assessment of differential abundance of taxa was performed using EdgeR for sparse data correction, and utilizing a p-value cutoff of 0.05 and FDR of 0.1 given low abundance OTUs, where indicated in the results section. Taxonomic data was then log corrected with correction factor of log(x+1) to account for zero values as has previously been described (31). Metagenomics analysis was performed using MicrobiomeAnalyst, and EdgeR was utilized for sparse data correction with an adjusted p-value

cutoff of 0.05 and FDR of 0.05. Statistical analyses and graphics were conducted with GraphPad 8.2 (GraphPad Software).

RESULTS

Subjects

In total 84 subjects were included in this study: 24 HC, 27 CD, 21 axSpA, and 12 CD-axSpA. Patient demographics are described in **Supplemental Table 1**. Overall groups were not significantly different with regards to age, sex, ethnicity, smoking, and family history of autoimmunity. As expected, a significantly higher prevalence of HLA-B27 was detected in axSpA groups. Given that subjects with overlapping CD-axSpA would be treated for one or the other condition at the time of the second disease diagnosis, obtaining newly diagnosed, untreated subjects was not feasible. Therefore, TNF-inhibitor (TNFi) usage was matched across the three disease groups. All subjects underwent fecal sampling and either colonoscopy or sigmoidoscopy with biopsies as described in the Methods. After analysis for macroscopic and histologic intestinal inflammation in the subjects, gross inflammation in the terminal ileum and histologic evidence of CD was noted in one subject recruited to the axSpA group who, therefore, was recategorized into the CD-axSpA group.

Bacteria-Produced Indoles are Increased in Axial Spondyloarthritis Colon Tissue

We first sought to assess the relevant bacterial metabolites that are taken up by the host in axSpA compared to HC and CD. Therefore, colon biopsies underwent broad assessment of central energy and redox metabolites, yielding 184 named metabolites by LC-MS (**Supplemental Figure 1** and **Supplemental Table 2**). Principal components analysis (PCoA) demonstrated significant separation between axSpA and HC groups but overlap within CD and CD-axSpA ($p<0.001$ by PERMANOVA; **Figure 1A**). Using a VIP plot to identify the top factors driving the separation in the PCoA, several metabolites within the omega-3 fatty acid and amino acid pathways, including tryptophan derivatives, were identified (**Supplemental Figure 2**). Additionally, using Venn diagrams to demonstrate the similarities and differences from pairwise comparisons of the most significantly changed metabolites, we discovered that indole-containing compounds from tryptophan metabolism associated with the presence of axSpA when compared to HC; when compared to CD, the presence of axSpA seemed to associate with omega 3 fatty acids (**Supplemental Figure 3**). Using a volcano plot to demonstrate which metabolites were most significantly different between axSpA and HC, three indole-containing byproducts of tryptophan metabolism were identified as significantly increased in axSpA relative to HC while omega 3 compounds were significantly decreased (**Figure 1B**).

Of the two major pathways affected in our analysis, two of the three byproducts of tryptophan metabolism, indole-3-acetate (IAA) and indole-3-acetaldehyde (I3Ald), are hypothesized to be microbially generated (32–34), making them intriguing products of the gut microbiome. Relative colon tissue levels of these two metabolites demonstrate significant increases in IAA in both axSpA and CD-axSpA when compared to HC and CD (**Figure 1C**). I3Ald was also significantly elevated in axSpA compared to HC and CD; this metabolite was significant also in CD-axSpA versus HC and elevated with a trend towards significance in comparison to CD (**Figure 1D**). For comparison, the parent compounds tryptophan and indole are included, and only demonstrate a significant difference with a decrease in concentration for indole in axSpA compared to HC and CD (**Figures 1E, F**). In sum, these data allude to altered tryptophan metabolism by the microbiome in the setting of axSpA that is not driven by the presence of bowel inflammation.

Taxonomic Classification in Axial Spondyloarthritis Exhibits Minimal Evidence of Gut Dysbiosis

We next sought to identify bacterial associations with the highly defined subject groups in our study using shotgun metagenomics, focusing on the differences between HC and axSpA given their striking metabolic findings. An average of 7,874,610 ± 1,765,741 paired-end reads were obtained from the axSpA subjects, 23,458,021 ± 9,039,626 paired end reads from the CD subjects, 23,982,607 ± 7,473,020 from the CD-axSpA, and 8,487,020 ± 874,485 from the HC subjects. After data processing and quality control with Kneaddata 0.7.5, a total of 1,448,835 ± 2,043,378 paired-end reads were obtained from the axSpA subjects, 1,822,197 ± 2,069,588 paired end reads from the CD subjects, 2,291,875 ± 2,184,367 from the CD-axSpA, and 1,121,841 ± 1,276,699 from the HC subjects (**Supplemental Table 3**). Taxonomic profiling was performed using the MicrobiomeAnalyst software. While there were no statistical differences at class and family taxonomic levels across groups (**Figures 2A, B**, respectively), in pairwise comparisons, two species were found to be significantly higher in axSpA: *Bifidobacterium adolescentis* ($p<7.22x10^{-4}$, FDR 0.055) and *Porphyromonas bennonis* ($p<0.001$, FDR 0.055), and two species were found to be higher in HC: *Streptococcus anginosus* ($p<1.76x10^{-4}$, FDR 0.028) and *Bacteroides dorei* ($p<0.001$, FDR 0.055) (**Figure 2C**). Alpha diversity analyses of richness and evenness as assessed by Observed, Chao1, Shannon, and Simpson indices were not different between the four subject groups (**Supplemental Figures 4A–D**). Beta diversity was assessed by PCoA on the basis of axSpA vs HC using Bray-Curtis dissimilarity and PERMANOVA, and found to have no separation (**Supplemental Figure 4E**). Given that previous studies included subjects with CD-axSpA in their microbiome analyses of axSpA (6, 7), we compared axSpA to CD-axSpA (**Figure 2D**) and found only one species to be different between the two groups, which was higher in the CD-axSpA group, *Prevotella bivia* (mean axSpA 0.059/median 0, mean CD-axSpA 0.145/median 0, $p<3.0x10^{-4}$, FDR 0.051). Further comparisons were performed for all other groups to understand underlying taxonomic differences between the groups based upon the presence of bowel inflammation using an FDR cut-off of 0.1 (**Supplemental Figure 5** and **Supplemental Table 4**). There were no species level differences

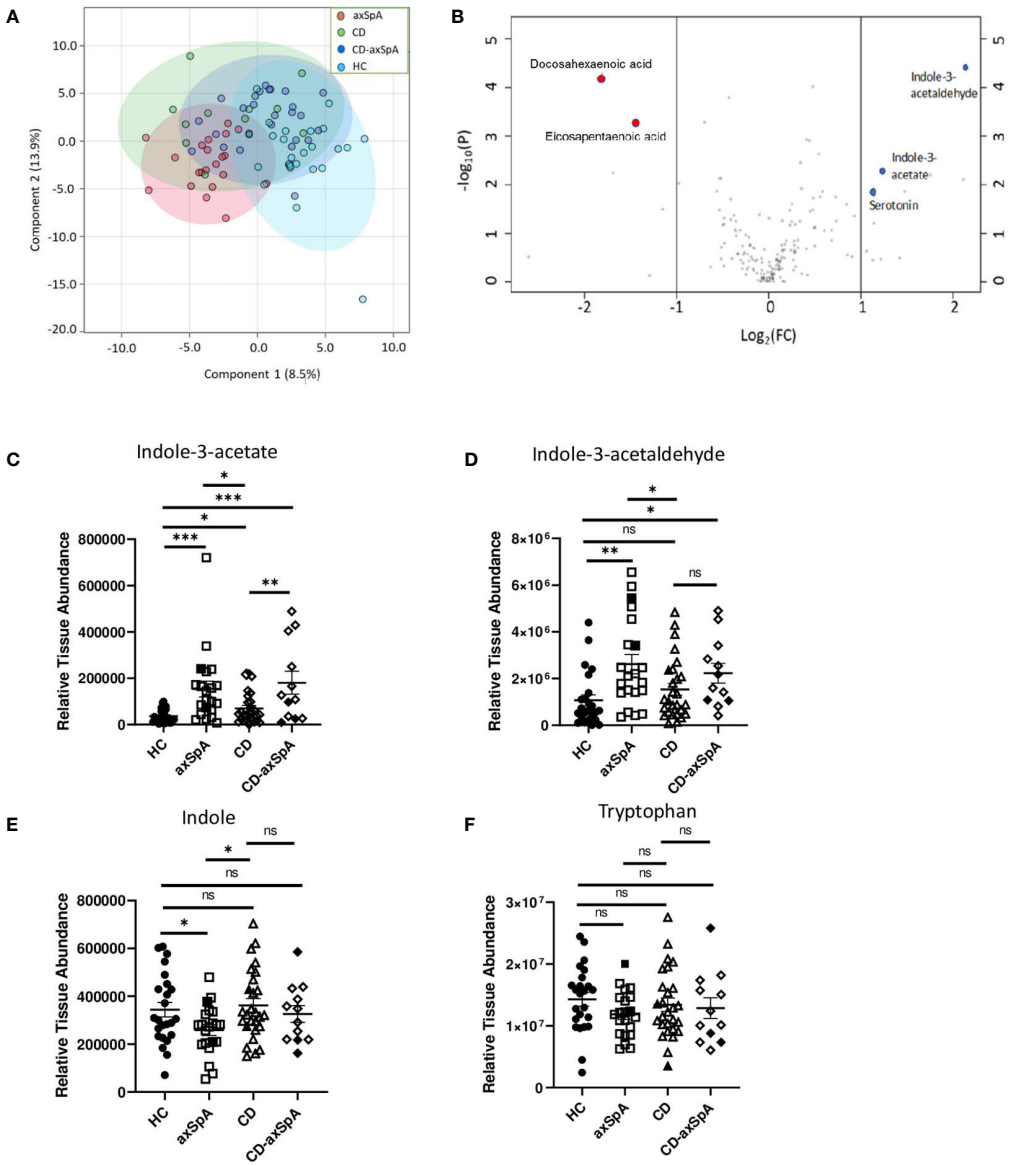

FIGURE 1 | Bacteria-produced indoles are significantly increased in axSpA. Broad screening of metabolites was performed by LC-MS in colon biopsies from HC, axSpA, CD, and CD-axSpA. **(A)** PCoA of the metabolite data in all four groups is shown. By PERMANOVA, p<0.001. **(B)** Volcano plot comparing the fold change differences in metabolites (x-axis) between HC and axSpA versus p-value (y-axis). Positive fold change values indicate higher in axSpA while negative values are those higher in HC. Blue dots represent identified tryptophan derivatives of significance, and red dots represent omega-3 metabolites. Relative tissue concentrations for **(C)** IAA, **(D)** I3Ald, **(E)** indole, and **(F)** tryptophan in each subject is indicated by symbols with bars as the mean ± SEM, measures relative to one another after using the same starting tissue weight. Open symbols represent those subjects taking TNFi. *p < 0.05, **p < 0.005, ***p < 0.0005, and ns (not significant) were determined by ANOVA with Kruskal-Wallis post-hoc test.

between CD-axSpA and HC (data not shown). Because prior microbiome studies compared axSpA and CD-axSpA combined versus HC as stated above, we performed this analysis with our data set and found significantly increased abundances of *Porphyromonas bennonis* (p<0.002, FDR 0.072) as well as decreased abundances of *Prevotella buccalis* (p<0.002, FDR 0.072), *Streptococcus anginosus* ($p<5.12x10^{-6}$, FDR $9.59x10^{-4}$), *Bacteroides dorei* ($p<9.11x10^{-5}$, FDR 0.008), *Bacteroides thetaiotamicron* (p< 0.001, FDR 0.066), and *Sutterella wadsworthensis* (p<0.002, FDR 0.072) in the combined axSpA groups (**Supplemental Figure 5A**). *Fingoldia magna* ($p<3.30x10^{-4}$, FDR 0.021) and *Akkermansia muciniphilia* ($p<3.48x10^{-4}$, FDR 0.021) were significantly expanded in axSpA compared to CD while *Bacteroides dorei* ($p<1.88x10^{-4}$, FDR 0.021) was more abundant in CD versus axSpA (**Supplemental Figure 5B**). *Akkermansia muciniphilia*

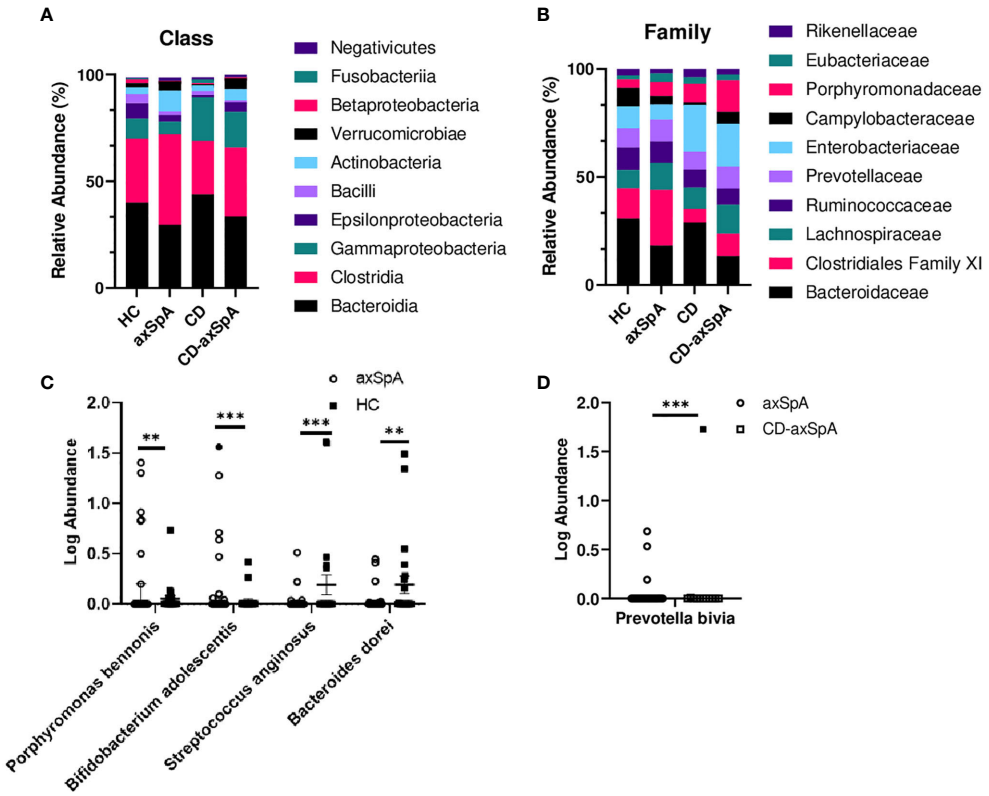

FIGURE 2 | Minimal taxonomic differences are observed in axSpA versus HC. Shotgun metagenomics were performed on rectal swabs from study subjects. The relative abundance of bacterial taxa at the **(A)** class and **(B)** family levels are shown as stacked bars of the mean taxa abundance within the subject group. No significant difference ($p>0.05$) was detected as determined by ANOVA with Kruskal-Wallis post-hoc test. **(C)** Log transformed relative abundances of individual species in the axSpA vs. HC groups and **(D)** axSpA vs. CD-axSpA groups are shown as symbols for each individual, with open symbols representing those subjects taking TNFi. Bars represent the median relative abundance ± interquartile range. $^{**}p < 0.005$ and $^{***}p < 0.0005$ as determined by Kruskal-Wallis post-hoc test.

($p<1.15x10^{-4}$, FDR 0.019) as well as *Porphyromonas somerae* ($p<8.97x10^{-4}$, FDR 0.077) were more abundant in CD-axSpA compared to CD (**Supplemental Figure 5C**). When CD was compared to HC, *Campylobacter hominis* ($p<8.88x10^{-5}$, FDR 0.014) and *Streptococcus anginosus* ($p<7.49x10^{-4}$, FDR 0.058) were significantly more abundant in subjects with HC (**Supplemental Figure 5D**).

Lastly, previously published disease-relevant species of *Ruminococcus gnavus*, *Dialister invisus*, *Akkermansia muciniphila*, and *Eschericia coli* (4–7) were compared against all four groups (**Supplemental Figures 6A–D**, respectively). Of these comparisons, only with *Dialister invisus* were we able to replicate the observations of Tito et al. (7), which was significantly increased in abundance in subjects with axSpA and CD-axSpA, but not CD, compared to HC (**Supplemental Figure 6B**), supporting the specificity of this taxa for axSpA.

Metagenomic Profiling Identifies Altered Tryptophan Metabolism in Axial Spondyloarthritis

Finally, in order to link the bacterial changes in axSpA to our observations of increased IAA and I3Ald from metabolomics screening, we analyzed gene abundance and pathway data from the bacterial sequencing. Taxonomic and functional data from HUMAnN 2.0 was merged among all samples appropriately for pairwise comparisons, then run in MicrobiomeAnalyst using EdgeR software. MicrobiomeAnalyst software takes read per kilobase output from HUMAnN 2.0, converts to copies per million, and streamlines into EdgeR input format. EdgeR was initially developed for RNA-seq analysis, but is effective for the analysis of metagenomics data due to challenges with sparse data analysis utilizing traditional statistics methods (29, 35). The software normalizes total read counts, filters low abundance features, then calculates the $\log_2$ fold change between groups using the Benjamini-Hochberg false discovery rate (FDR) test with a threshold of 0.05. Diversity analysis of the complete data set was performed across KEGG metabolic pathways looking at a global representation of all associated genes in their appropriate metabolic pathways. No statistical difference was noted in the global pathway analysis representing KEGG metabolic pathways between axSpA and HC (**Supplemental Figure 7A**). PCoA was also visualized on the basis of the metagenomics data displaying no difference between groups (**Supplemental Figure 7B**), as well as a dendrogram of all samples present showing no notable

separation (**Supplemental Figure 7C**). Thus, in a global survey of the metagenomics data, we did not identify functional genetic pathways that differed between axSpA and HC.

HUMAnN 2.0 pathway analysis was then performed to identify significantly altered gene function pathways between HC and axSpA, providing additional insight into the overall functional metagenomic status. These data were analyzed as above with the gene level data using the EdgeR feature of MicrobiomeAnalyst, and 61 pathways meeting the criteria of $p<0.05$ and FDR<0.05 are displayed in **Supplemental Table 5** after removal of individual species level pathways; the superpathway of L-tryptophan biosynthesis was identified as significantly increased in HC compared to axSpA. No omega 3 fatty acid pathway was identified. Because our metabolic data heavily implicated changes in tryptophan metabolism by bacteria, a search was then performed for all pathways involved in tryptophan metabolism, without omission of individual species level pathways. Three pathways related to tryptophan biosynthesis were identified, all of which were higher in HC (**Table 1**).

After running the complete metagenomics dataset at a pathway level, we then identified gene level data that significantly differed in relative abundance between axSpA and HC (using $p<0.05$ and FDR<0.05). Without a clear pathway linking these genes from our pathway analysis, we focused on the tryptophan pathway given our metabolic data strongly associating its indole metabolites with axSpA. A search was performed for individual genes associated with the keywords indole or tryptophan. A total of 35 individual genes were found to be statistically significant based upon the cutoff of $p<0.05$ and FDR<0.05 and are displayed in **Table 2**. Values in which the $\log_2$ fold change are positive are considered higher in HC while negative values are higher in axSpA (displayed in gray). Within the tryptophan metabolic pathways, 18/27 genes relevant to tryptophan synthesis were significantly more abundant in HC while 2/2 genes for tryptophan metabolism were significantly more abundant in axSpA (**Table 2**), suggesting that in axSpA, the bacterial community shifts from tryptophan synthesis to metabolism.

Similar analyses were performed in a pairwise manner among remaining groups and demonstrated in **Supplemental Tables 6–10**. When the axSpA and CD-axSpA groups were combined, 13/27 genes relevant to tryptophan synthesis were more abundant in the HC group (**Supplemental Table 6**), suggesting that the microbiome in the presence of bowel inflammation increases tryptophan synthesis. Indeed, tryptophan synthesis genes were overwhelmingly increased in abundance in CD-axSpA compared to axSpA (**Supplemental Table 7**) and in CD compared to HC (**Supplemental Table 8**). However, the gene encoding indolepyruvate decarboxylase, the enzyme that metabolizes tryptophan products to IAA, remained more abundant in the combined axSpA and CD-axSpA comparison to HC (**Supplemental Table 6**) as well as in CD-axSpA compared to HC (**Supplemental Table 9**) and CD-axSpA compared to CD (**Supplemental Table 10**), suggesting that this pathway is specific to axSpA regardless of bowel inflammation. A composite model describing gene abundances in the tryptophan pathway relative to disease status is illustrated in **Figure 3**.

TABLE 1 | Composite tryptophan pathway analysis of metagenomic data.

Pathway	$\log_2$FC (HC:axSpA)	P-value	FDR
PWY-6629: superpathway of L-tryptophan biosynthesis	2.1065	0.002069	0.012391
TRPSYN-PWY: L-tryptophan biosynthesis *Escherichia coli*	1.6584	0.002245	0.01312
PWY-6629: superpathway of L-tryptophan biosynthesis *E. coli*	1.5871	0.004639	0.021526

DISCUSSION

The lack of consensus taxa across multiple studies associating intestinal dysbiosis with axSpA raises the question of relevance to disease pathophysiology. Rather, based on studies in HLA-B27 transgenic rats and emerging data in PsA (3, 17, 18), we hypothesized that the microbial population as a whole may act through bacterially produced metabolites. In this study, we utilized unbiased approaches to characterize and connect gut metabolomics and bacterial metagenomics in axSpA. Screening across central metabolism and redox metabolites by LC-MS in which we were able to detect 184 metabolites, we identified significantly increased indole-containing metabolites of the tryptophan pathway associated with the presence of axSpA with or without confounding bowel inflammation. We also noted several other metabolic pathways that were affected, such as a significant reduction in omega-3 fatty acids that associated with axSpA. Through profiling of the bacterial metagenome, we identified numerous pathways that were altered in subjects with axSpA compared to HC. However, only the tryptophan pathway was consistent in both the metabolomic and metagenomic data sets. Given this overlapping finding, we confirmed tryptophan pathway alterations in our subjects. Altogether, our findings demonstrate that metabolism of tryptophan by the microbiome into indole derivatives to be of significance in axSpA.

Complimentary to our findings, others have shown tryptophan to be decreased in the plasma in axSpA (36), although other studies utilizing independent cohorts and different methods of metabolomic detection (NMR, LC-MS, GC-MS, etc.) have not replicated this finding (37–39). In the feces, one study found decreased cholesterols and steroids in subjects with AS compared to controls (40) suggesting these pathways, including our findings of omega-3 fatty acid alterations, should be further pursued in future studies. However, in juvenile enthesitis-related arthritis, two independently studied cohorts demonstrated reduced tryptophan metabolism in the feces in spite of the lack of taxonomic differences in the microbiomes of these cohorts compared to controls (41). A limitation to these fecal studies, though, is that fecal metabolites may not reflect what the host absorbs, which is the reason why we performed a metabolic analysis on intestinal

TABLE 2 | Relative tryptophan pathway gene abundances in HC vs. axSpA bacterial metagenomics.

Gene	log_2FC (HC:axSpA)	P-value	FDR	Trp Function
K00179: indolepyruvate ferredoxin oxidoreductase, alpha subunit *Bacteroides eggerthii*	−2.7458	1.31E-05	0.001179	Unclear
K00179: indolepyruvate ferredoxin oxidoreductase, alpha subunit *Alistipes finegoldii*	1.5259	0.001195	0.012517	Unclear
K00180: indolepyruvate ferredoxin oxidoreductase, beta subunit *A. finegoldii*	1.6765	0.000462	0.007469	Unclear
K00180: indolepyruvate ferredoxin oxidoreductase, beta subunit *Bacteroides ovatus*	−1.36	0.008443	0.042738	Unclear
K00180: indolepyruvate ferredoxin oxidoreductase, beta subunit *B. fragilis*	1.3126	0.004987	0.02983	Unclear
K01609: indole-3-glycerol phosphate synthase *Roseburia intestinalis*	−2.478	2.33E-05	0.001495	Synthesis
K01609: indole-3-glycerol phosphate synthase unclassified	−2.0789	0.00066	0.008987	Synthesis
K13498: indole-3-glycerol phosphate synthase/phosphoribosylanthranilate isomerase *Escherichia coli*	1.6051	0.001603	0.014665	Synthesis
K13498: indole-3-glycerol phosphate synthase/phosphoribosylanthranilate isomerase	1.5257	0.002535	0.019341	Synthesis
K01609: indole-3-glycerol phosphate synthase *Akkermansia muciniphila*	2.1624	0.002691	0.020043	Synthesis
K01609: indole-3-glycerol phosphate synthase *B. fragilis*	1.4215	0.003307	0.022733	Synthesis
K04103: indolepyruvate decarboxylase	−1.2603	0.004983	0.029816	Metabolism
K04103: indolepyruvate decarboxylase *Corynebacterium aurimucosum*	−1.1923	0.008084	0.041395	Metabolism
K01667: tryptophanase *B. thetaiotaomicron*	1.9251	3.58E-05	0.001874	Synthesis
K01667: tryptophanase *A. finegoldii*	1.4811	0.001467	0.013997	Synthesis
K07185: tryptophan-rich sensory protein *A. finegoldii*	1.8725	0.000181	0.004631	Signaling
K01867: tryptophanyl-tRNA synthetase *E. coli*	2.2716	0.000231	0.005242	Synthesis
K01867: tryptophanyl-tRNA synthetase *Ruminococcus bromii*	−2.1286	0.000352	0.006525	Synthesis
K01867: tryptophanyl-tRNA synthetase *R. intestinalis*	−1.5828	0.002507	0.019205	Synthesis
K01867: tryptophanyl-tRNA synthetase *B. thetaiotaomicron*	1.5689	0.003252	0.022486	Synthesis
K01867: tryptophanyl-tRNA synthetase *Alistipes shahii*	−1.2245	0.007021	0.037604	Synthesis
K01867: tryptophanyl-tRNA synthetase *A. muciniphila*	1.6871	0.009087	0.045048	Synthesis
K01695: tryptophan synthase alpha chain *E. coli*	1.6638	0.00159	0.014609	Synthesis
K01695: tryptophan synthase alpha chain *R. bromii*	−1.6939	0.002344	0.018449	Synthesis
K01695: tryptophan synthase alpha chain *A. muciniphila*	1.7194	0.006817	0.036817	Synthesis
K01696: tryptophan synthase beta chain *R. intestinalis*	−1.982	0.000272	0.005681	Synthesis
K01696: tryptophan synthase beta chain *A. muciniphila*	2.1602	0.000671	0.009074	Synthesis
K01696: tryptophan synthase beta chain *Ruminococcus lactaris*	1.5981	0.000789	0.009917	Synthesis
K06001: tryptophan synthase beta chain *A. muciniphila*	1.8548	0.002536	0.019342	Synthesis
K01696: tryptophan synthase beta chain *B. fragilis*	1.3137	0.004084	0.026096	Synthesis
K06001: tryptophan synthase beta chain *R. intestinalis*	−1.4264	0.004439	0.02757	Synthesis
K01696: tryptophan synthase beta chain *R. bromii*	−1.496	0.008027	0.041207	Synthesis
K01696: tryptophan synthase beta chain *Streptococcus anginosus*	1.1735	0.009845	0.047575	Synthesis
K02846: N-methyl-L-tryptophan oxidase	1.7694	0.001406	0.013714	Synthesis
K02846: N-methyl-L-tryptophan oxidase *E. coli*	1.7694	0.001406	0.013714	Synthesis

tissue. To our knowledge, such an analysis has not been performed previously.

Although the pathophysiologic role of tryptophan metabolism and indole within the intestine in axSpA will need to be demonstrated, an accumulating literature supports its role in local epithelial barrier and immune cell function [reviewed in (32–34)]. Tryptophan is solely derived from dietary intake and absorbed by the host for use in protein synthesis and other metabolic pathways, particularly kynurenine and serotonin derivatives. While bacteria can metabolize tryptophan to kynurenine, they also metabolize dietary tryptophan into indole and using the enzyme tryptophanase as well as other indole derivatives; the host does not generate indole as tryptophanase is exclusive to bacteria (33, 34). Indole-containing derivatives, which can be produced by a variety of microbes, plants, and even recently observed by a human cancer cell line (42), are absorbed across the intestinal epithelium of the host and signal through either the aryl hydrocarbon receptor (AhR) or the pregnane X receptor (PXR) to modulate host responses including barrier and immune functions. Indole and its derivatives have varied effects on the host from promotion of inflammatory responses to regulation and resolution of inflammation depending upon the specific metabolite, receptor, cell, and experimental model (32). For example, in lupus-prone mice, dysbiosis is linked to altered tryptophan catabolism, and feeding a high tryptophan diet correlates with worse disease and greater autoantibody generation (43). Yet, in experimental autoimmune encephalitis, tryptophan metabolism, and specific indole-containing derivatives were linked to reduced CNS inflammation (44). Specifically within the intestine, indole-containing derivatives such as indole-3-propionic acid (IPA) signals directly through epithelial cells to maintain and repair the barrier (45) while IAA and I3Ald, through AhR (14, 46, 47) signaling on innate lymphoid cells, results in increased IL-22 expression in the gut mucosa (46). Thus, there are wide-ranging effects of tryptophan metabolism and its indole-containing derivatives. Although not the main objective of the study, taxonomic profiling in this study failed to reveal differences in alpha or beta diversity by multiple measures (**Supplemental Figures 4A–E**) or in higher order OTUs between the axSpA and HC groups (**Figures 2A, B**). Such a result is likely due to the use of TNFi in our subjects, as TNFi has previously been shown to "normalize" the microbiome in axSpA (47–49). To further assess the role of specific bacterial differences across axSpA, a comparison was made across the most previously

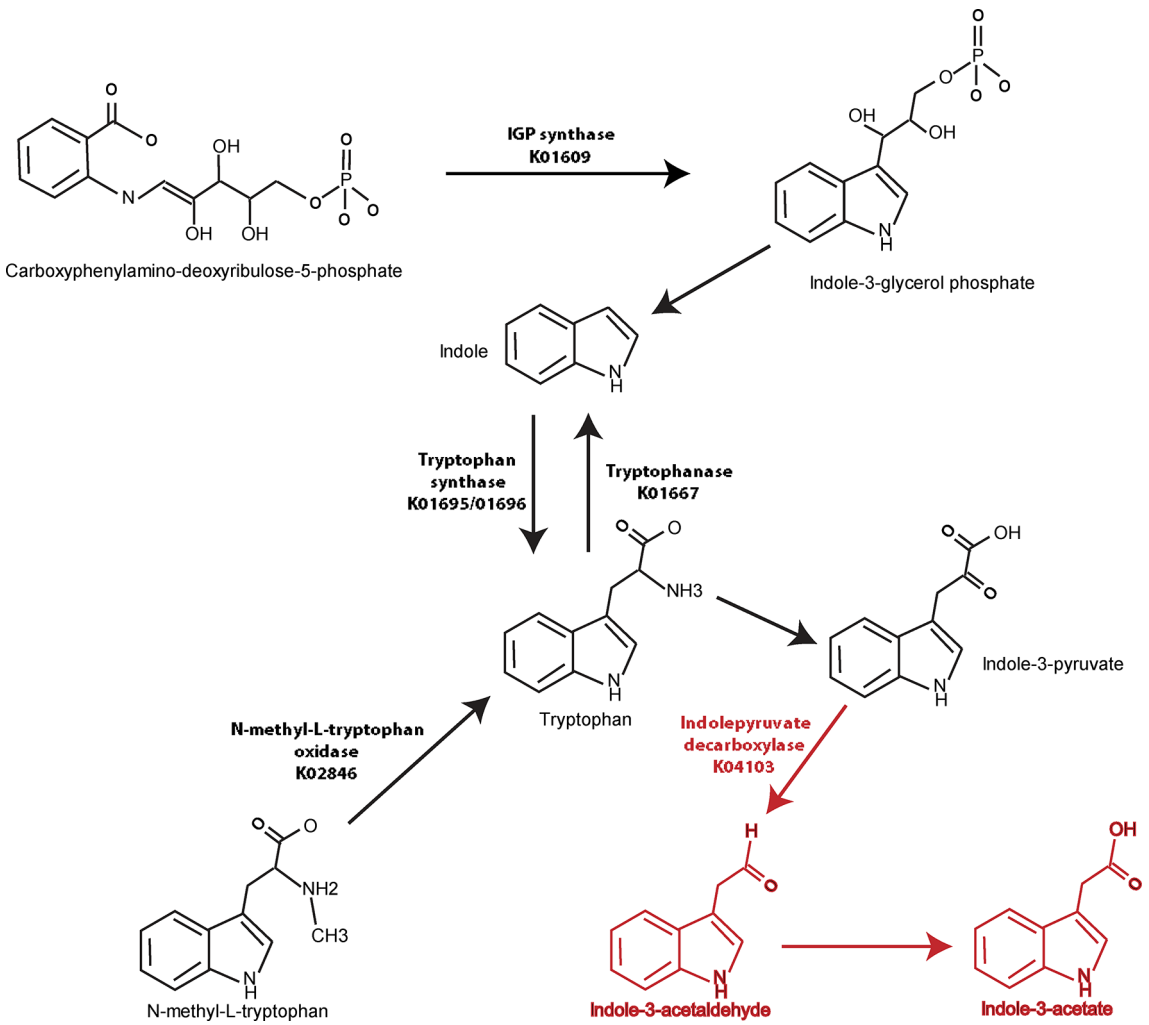

FIGURE 3 | Graphical demonstration of identified tryptophan pathway gene differences relative to generated metabolites in axSpA and HC. Genes encoding enzymes in the tryptophan synthesis/metabolism pathway were identified using metagenomics (**Table 2**) and are represented visually here. Numerous pathways towards tryptophan synthesis were identified as significantly higher in HC compared to axSpA and shown with black arrows and listed KO names. Indolepyruvate decarboxylase, which is responsible for tryptophan metabolism towards IAA and I3Ald, was found to be significantly increased in genetic abundance in axSpA compared to HC. This gene and the resulting metabolites that are increased in axSpA are identified in red.

published species in axSpA (**Supplemental Figure 6**) (4, 6, 7). Within our cohort, the most significantly associated species was *D. invisus*, which had a significant expansion in both the axSpA and CD-axSpA groups. *R. gnavus* had a decreased abundance in axSpA, and *A. muciniphilia* and *E. coli* were not statistically significant across groups but trended towards previously published results. Put together, these data suggest similarities within our cohort to those previously published, in spite of the limitation of TNFi use by our cohort.

Our evaluation of bacterial metagenomics between axSpA and HC identified a number of tryptophan metabolism pathways that are altered. In general, our data suggest the microbial community in HCs increased tryptophan synthesis (or decreased synthesis in the axSpA group), while the microbial community in axSpA increased tryptophan metabolism towards indoles (**Figure 3**). Analysis through HUMAnN 2.0, which evaluates global pathways rather than individual genes, also demonstrates increased tryptophan synthesis in HC relative to axSpA (**Table 1**). These metagenomics analyses are consistent with the identification of increased indole derivates through metabolomic screening in the gut tissue of patients with axSpA (**Figure 1**) (33). The combined observations could represent a functional difference of the axSpA microbiome. This is especially noteworthy given the lack of dysbiosis observed within the microbiome, implying that the shift in tryptophan metabolism is not specific to the particular dysbiosis in axSpA and perhaps more of a generalized community function as has previously been suggested (10).

While we focus our metagenomics conclusions on the tryptophan pathway, due to the consistency with our metabolomics analyses, we do observe similar metagenomics pathways in comparison with previously published results. For example, in agreement with other's findings of the TCA cycle and biotin synthesis being enriched in AS, and butanoate pathways and pyridoxal 5'-phosphate salvage pathways being enriched in controls (47, 50, 51). These studies focused on untreated AS, but when comparing untreated to TNFi treated AS cases, not only did the microbiome become less significantly different from controls, so did metagenomic findings (47). Intriguingly, the authors of this particular study found that aromatic amino acids, which includes tryptophan, synthesis was significantly reduced in untreated AS cases compared to both treated AS cases and controls (47). This may indicate that our findings, where arguably our AS subjects were not fully controlled based on BASDAI scores, have a smaller effect due to TNFi treatment that would be otherwise more pronounced in the absence of this confounder.

One key identified gene was indolepyruvate decarboxylase, which encodes for the enzyme that converts indole-3-pyruvate to IAA and I3Ald (52), and was increased in axSpA. Other genes encode for different aspects of tryptophan synthesis such as tryptophan synthase (alpha and beta chain) (53) (elevated in HC), indole-3-glycerol phosphate synthase (54) (elevated in HC), and *N*-methyl-L-tryptophan oxidase (55) (elevated in HC). Tryptophanase is involved in converting tryptophan to indole, which is considered a separate pathway directed away from IAA and I3Ald (56) (elevated in HC). Other findings are of unclear importance due to lack of current published knowledge such as indolepyruvate ferredoxin oxidoreductase, which converts indole-3-pyruvate to *S*-2-(indol-3-yl)acetyl-CoA (elevated in HC). The product of this reaction has unclear biologic function, but presumably can function in immune signaling by acting through the AhR. Tryptophanyl tRNA synthetase engages in protein synthesis through the propagation of protein elongation by adding tryptophan (57), and was found to be elevated in HC, which is consistent with our findings of tryptophan synthesis in HC.

Combining axSpA and CD-axSpA together as one group compared to HC weakened the trend towards increased tryptophan synthesis in the HC group but preserved tryptophan metabolism by indolepyruvate decarboxylase (**Supplemental Table 6**), suggesting that bowel inflammation could promote tryptophan synthesis but metabolism to indoles was specific to axSpA. In support of this conclusion, metagenomic differences in tryptophan metabolism between axSpA and CD-axSpA skewed significantly increased towards tryptophan synthesis in the setting of bowel inflammation, which was similar to CD vs HC (**Supplemental Tables 7** and **8**, respectively). Furthermore, CD and CD-axSpA were similar with regards to tryptophan synthesis (**Supplemental Tables 9** and **10**, respectively). When axSpA, CD-axSpA, or the two groups were combined, indolepyruvate decarboxylase was significantly represented compared to HC or CD, supporting the conclusion that this pathway of tryptophan metabolism is specific to axSpA.

LIMITATIONS

This study has a number of limitations to be addressed. First, the majority of subjects recruited in each of the three cohort groups had been on TNFi therapy. This limitation has been discussed above, and it is intriguing that our metagenomics data still correlates with alterations in the tryptophan metabolism pathway despite a lack of dysbiosis. Repeating this study on a cohort of newly diagnosed, untreated patients would be of interest to validate findings; however, a comparison CD-axSpA group would not be possible due to one or the other disease being diagnosed and treated prior to the development of the other disease.

Sample size in our cohort is somewhat small in general, making definitive statistical conclusions about the microbiome less universal, but our findings still hold true across the different disease states. Additionally, while our cohort purposefully included subjects that were HLA-B27 negative axSpA and females with axSpA, the numbers were underpowered to analyze separately. Other confounding variables such as disease activity, other medication and NSAID use (that are widespread in the general population), and diet, which is often not accurately assessed even in the setting of validated measures (58–60). Further studies that could separate out these groups would be of interest in understanding sex, genetic, medication and dietary differences, but including them in general gives a better picture of the AS disease state as a whole.

In addition, samples were collected as rectal swabs rather than fecal samples. This is still representative of the overall microbiome, but leads to issues such as decreased data recovery using shotgun metagenomics after removal of low abundance data (61). This leads to a perceived lower amount of species diversity as detailed above. Lastly, all subjects were recruited from a single center, so there is unclear geographic bias among the cohort population.

CONCLUSIONS

Using two separate 'omics approaches of metagenomics and metabolomics, we identified significant alterations in tryptophan metabolism with increased synthesis in HC and those with bowel inflammation and increased metabolism to indoles in the setting of axSpA. Within our study, we uniquely compare axSpA without bowel inflammation, CD, and overlapping CD-axSpA, allowing us to dissect the effects of bowel inflammation and axSpA in our observations. Although our cohort was likely influenced by the use of TNFi, subjects in the axSpA groups had elevated BASDAI scores, implying active disease. Despite the minimal evidence of dysbiosis across our groups, we still noted significant alterations in tryptophan metabolism by both metabolomic assessment and metagenomics analysis. Such findings potentially represent a dysbiotic community effect that has significant implications for host immune function and supports the use of multi 'omics approaches to identify possible pathways linking the microbiome to pathophysiologic relevance in disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Sequencing data is publicly assessable in the National Library of Medicine's Sequence Read Archive SUB8755981.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Colorado Multiple Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EHR and KAK conceptualized and designed the study. EHR, MEG, BPF, FIS, and AEF recruited the subjects. EHR, MEG, BPF, FIS, and AEF acquired the samples. AJB, AB, AS, and JAR processed and extracted the samples. AJB, AS, JAR, and KAK analyzed and interpreted the data. AJB and KAK drafted the manuscript with input from AS and JAR. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.587119/full#supplementary-material

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Leaky Gut Driven by Dysbiosis Augments Activation and Accumulation of Liver Macrophages *via* RIP3 Signaling Pathway in Autoimmune Hepatitis

Hongxia Zhang[1†], Man Liu[1†], Weilong Zhong[1], Yanping Zheng[1], Yanni Li[1], Liping Guo[1], Yujie Zhang[2], Ying Ran[1], Jingwen Zhao[1], Lu Zhou[1,3] and Bangmao Wang[1*]*

[1] Department of Gastroenterology and Hepatology, General Hospital, Tianjin Medical University, Tianjin, China, [2] Department of Pathology, General Hospital, Tianjin Medical University, Tianjin, China, [3] Department of Gastroenterology and Hepatology, People's Hospital of Hetian District, Hetian, China

The gut–liver axis has been increasingly recognized as a major autoimmunity modulator. However, the implications of intestinal barrier in the pathogenesis of autoimmune hepatitis (AIH) remain elusive. Here, we investigated the functional role of gut barrier and intestinal microbiota for hepatic innate immune response in AIH patients and murine models. In this study, we found that AIH patients displayed increased intestinal permeability and pronounced RIP3 activation of liver macrophages. In mice models, intestinal barrier dysfunction increased intestinal bacterial translocation, thus amplifying the hepatic RIP3-mediated innate immune response. Furthermore, GSK872 dampened RIP3 activation and ameliorated the activation and accumulation of liver macrophages *in vitro* and *in vivo* experiments. Strikingly, broad-spectrum antibiotic ablation significantly alleviated RIP3 activation and liver injury, highlighting the causal role of intestinal microbiota for disease progression. Our results provided a potentially novel mechanism of immune tolerance breakage in the liver *via* the gut-liver axis. In addition, we also explored the therapeutic and research potentials of regulating the intestinal microbiota for the therapy of AIH.

Keywords: autoimmune hepatitis, intestinal barrier, dysbiosis, macrophages, RIP3 signaling pathway, gut-liver axis

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****Correspondence:***
Bangmao Wang
gi.tmuh@sohu.com
Lu Zhou
lzhou01@tmu.edu.cn

[†]These authors have contributed equally to this work

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INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic immune-mediated inflammatory liver disease. Although genetic and environmental factors are involved in the pathogenesis of AIH, the underlying mechanisms remain unclear (1). In recent years, great importance has been attached to the role of intestinal barrier in the pathogenesis of diverse immune-mediated diseases (2–4). In particular, the liver is continuously exposed to gut-derived antigens through the portal vein, which influence its innate and adaptive immune responses (5, 6). It's known that intestinal barrier disruption can

trigger bacteria and bacterial products translocation, which consecutively activate immune cells to release various proinflammatory cytokines and chemokines in the liver (7, 8). Clinically, primary sclerosing cholangitis is a remarkable example of chronic biliary inflammation highly associated with inflammatory bowel disease, indicating that the gut-liver axis plays an important role on the pathogenesis (9, 10).

Macrophages represent a key cellular component of the liver essential for maintaining tissue homeostasis and ensuring rapid responses to hepatic injury (11). Researches have reported the key role for liver macrophages in AIH. H Grønbaek et al. studied 121 AIH patients in a cross-sectional design and demonstrated macrophage activation paralleling disease activity, severity and treatment response, suggesting a role for macrophage activation in AIH (12). Besides, Assis David N reported a distinct genetic and immunopathogenic basis for AIH at the macrophage migration inhibitory factor locus, which indicated that macrophages play a role in pathogenesis and as biomarkers of AIH (13, 14). Liver macrophages consist of ontogenically distinct populations termed as Kupffer cells and monocyte-derived macrophages (15). As macrophages accumulate gut-derived products such as lipopolysaccharide (LPS) and undergone activation, necrosis of macrophages and uncontrolled release of inflammatory cytokine and chemokine results in inflammation and fibrosis of liver tissues (16, 17). Thus, macrophage cell death has been considered to be a major contributor of immune-mediated liver injury (18). Receptor interacting protein kinase 3 (RIP3) has been increasingly recognized as a central player in necroptosis and RIP3 kinase activity supports the recruitment of mixed lineage kinase domain-like (MLKL) to trigger membrane leakage with the consequent release of inflammatory cytokines and chemokines (19–21). Recently, we reported that probiotics application in experimental autoimmune hepatitis (EAH) mice could improve the intestinal barrier and downregulate the RIP3 signaling of liver macrophages (22). Hence, we hypothesized that the activation of RIP3 signaling pathway may be a potential mechanism of gut-liver axis in AIH pathogenesis and thus can be a novel treatment target.

In this study, we demonstrated that intestinal barrier damage and RIP3-mediated activation of liver macrophages existed in AIH patients. As reveled by the tandem model of dextran sulfate sodium (DSS) - concanavalin A (Con A), the disruption of intestinal barrier prior to hepatitis aggravated the activation and accumulation of liver macrophages. This finding highlighted RIP3 as an important interface that mediated liver inflammation. Furthermore, the RIP3-mediated activation of liver macrophage in EAH mice was canceled by gut sterilization, suggesting that immune responses in the liver are potentially regulated by gut microbiota.

MATERIALS AND METHODS

Ethical Approval Statement

All experimental procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at Tianjin Medical University and followed the International Association of Veterinary Editors guidelines for the Care and Use of Laboratory Animal. The animal use protocol listed below has been reviewed and approved by the Animal Ethical and Welfare Committee of Tianjin Medical University, Approval No. IRB2020-WZ-119.

Participants

Sixty-eight patients with AIH including thirty-nine without cirrhosis (AIH-n) and twenty-nine with cirrhosis (AIH-c) and fifteen controls were included. The patients were recruited from the Gastroenterology Department at Tianjin Medical University General Hospital. AIH was diagnosed with the following criteria: (1) patients conformed with 1999 revised International Autoimmune Hepatitis Group score≥10 and/or (2) 2008 IAIHG simplified AIH score≥6 and/or (3)histological features indicative of AIH (23, 24). Patients data were collected prior to corticosteroid therapy. The control subjects (CTRL) were selected from the Health Management Center of Tianjin Medical University General Hospital and matched the patients with AIH in terms of age and gender. Inclusion criteria for the CTRL group were as follows: (1) normal ranges of liver function test, (2) an absence of hepatitis B/C virus antigen, (3) normal abdominal ultrasound tests, and (4) an absence of autoimmune diseases and family history. Blood was collected from the individuals. Feces were collected from six AIH-n patients. Intestinal mucosal biopsy specimens were collected from fourteen patients (six AIH-n patients and eight AIH-c patients) and six controls. Liver biopsy specimens were collected from six AIH-n patients and four patients with hepatic cyst.

Animal Experiments

Twenty-four female SPF C57BL/6 mice (6 weeks of age) were purchased from Beijing Animal Study Centre and reared under specific pathogen-free conditions in Animal Centre of the Tianjin Medical University. The mice were randomly divided into four groups (n=6 per group) including the CTRL group, DSS group, Con A group and DSS-Con A group. 1%DSS (MP Biomedicals) was dissolved in sterile distilled water ad libitum for 7 days to induce disruption of intestinal barrier integrity. Con A (15 mg/kg, Solarbio) was i.v. administered into the tail vein of mice 12 hours before liver resection (**Supplementary Figure 1A**). A RIP3 kinase inhibitor GSK872 (Merck) was diluted in 1 mg/mL dimethyl sulfoxide (DMSO). Another 12 C57BL/6 mice were randomized divided into two groups including DSS-Con A group and GSK872-pretreated group (n=6 per group) provided with 1% DSS water for 7 days and intraperitoneally treated with either GSK872 (1 mg/kg) or an equal volume of DMSO 1 h prior to Con A administration.

Another 26 female C57BL/6 mice were randomly divided into three groups (n=6 per group) including the CTRL group, EAH group and antibiotic mixture (Abx) group. The rest mice (n=8) were used to extract hepatic antigen S100 as previous description (25). All mice except the CTRL group were injected intraperitoneally with 0.5ml S100 emulsified in an equal volume of complete Freund's adjuvant (CFA, sigma, USA) on

day 7 and day 14 to induce EAH. The Abx group was pretreated with antibiotic mixture (0.5 g/L vancomycin, 1 g/L ampicillin, 1 g/L metronidazole, and 1 g/L neomycin; Sigma–Aldrich) for 2 weeks prior to S100 administration to deplete endogenous commensal microbiota. On day 28, all the animals were sacrificed under anesthesia (**Supplementary Figure 2A**).

Fecal Supernatants Extraction

Fecal samples from patients with AIH were mixed at equal weight. One gram of the mixed feces was diluted in 5 mL sterile PBS solution, then initial filtered, concentrated, homogenized, step by step filtered, centrifuged. The supernatant was collected and centrifuged at 5,000 × g for 10 min at 4°C, then the supernatant of feces from AIH patients (AIH-s) was collected and filter-sterilized through 0.22 μm filters (26).

Cell Line and Culture Conditions

Human Caco-2 cells (BNCC 338148) were cultured in Modified Eagle's Medium (MEM) (Gibco) supplemented with 20% fetal bovine serum and a penicillin-streptomycin solution. The cells were incubated in a humidified incubator containing 5% CO_2 at 37°C and were seeded in a 24-well plate at a density of 1×10^5 cells per well. In the stimulation experiment (AIH-s group), the cells were pre-treated with 10% AIH-s for 24h, and the control groups were treated 10% inactive AIH-s or PBS for 24h. Mouse macrophage cell line RAW264.7 was plated in Dulbecco modified eagle medium (Gibco) supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Gibco). The cells were cultured under same conditions as above. RAW264.7 cells were seeded in a 12-well plate at a density of 1×10^5 cells per well. In LPS experiments (LPS group), the cells were treated with LPS (3 mg/mL, Solarbio Biotech) for 24 h. In GSK872 experiments (LPS-GSK872 group), the cells were treated with LPS (3 μg/mL) and GSK872 (3 μM) for 24 h.

In Vivo Permeability Assay

Intestinal permeability was determined through fluorescein isothiocyanate (FITC)-dextran assay. FITC-D (4kDa, Sigma-Aldrich) was dissolved in normal saline infusion (50 mg/mL) and administered to mice through gavage at 6 mg/10 g body weight. Whole blood was collected 4h after FITC-D administration by using heparinized microhematocrit capillary tubes *via* eye bleed. Plasma was extracted from the blood through centrifugation at 4°C for 10 min at 3,000 rpm. Fluorescence intensity was analyzed using a plate reader. FITC-D concentration of each mouse was detected based on the FITC-D standard curve.

Enzyme-linked Immunosorbent Assay (ELISA) and Biochemical Analysis

The blood was centrifuged at 3000 rpm for 10 min, and the plasma was then stored at −80°C. LPS, D-lactic acid (DLA), and diamine oxidase (DAO) plasma concentrations were quantified with ELISA kits (SenBeiJia Biotech) in accordance with the manufacturer's instructions. Zonulin plasma concentrations were quantified using ELISA kits (Elabscience). Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were tested by using the automated chemistry analyzer from the clinical laboratory of the Tianjin Medical University General Hospital.

Histology and Immunohistochemistry (IHC)

The liver and intestinal tissues of patients and mice were collected and fixed in 4% paraformaldehyde. The paraffin-embedded liver and intestinal tissues were sectioned at approximately 5 μm and stained with hematoxylin and eosin (HE) following the standard HE protocol. Pathological changes in the liver and intestinal tissues were evaluated by two independent and experienced pathologists. Intestinal tissue sections from patients were stained with primary anti- zonula occludens-1 (ZO-1) antibody (ab96587, Abcam, Cambridge, MA, USA) or anti-Occludin antibody (ab216327, Abcam, Cambridge, MA, USA) at 4°C overnight and incubated with second antibody for 30 min at 37°C. The staining index was calculated by multiplying percentage positive cells rating by intensity rating in every field for quantitative analysis.

Immunofluorescence

Caco-2 cell monolayers were fixed in cold methanol for 5 min at -20°C. Monolayers were then washed and blocked with bovine serum albumin (BSA) for 30 min at room temperature. Cells were then incubated with either anti-ZO-1 (1:50, Abcam, USA) or anti-Occludin (1:150, Abcam, USA) overnight at 4°C. Cells were then incubated with the secondary antibody for 1 h at room temperature. Cell nuclei were stained with DAPI. Double immunofluorescence analyses for liver macrophages were performed with 4 mm-thick frozen sections. Slides were fixed with acetone, blocked with 5% bovine serum albumin, and incubated with primary antibodies against CD68 (ab955, Abcam, Cambridge, MA, USA) and MAC387 (ab92507, Abcam, Cambridge, MA, USA) at 4°C overnight. The slices were restored to room temperature the next day, incubated with the corresponding secondary antibodies for 1h at 37°C, underwent DAPI reaction, sealed, and observed under a fluorescence microscope.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from liver tissues with TRIzol (Thermo Scientific Inc.), followed by cDNA reverse transcription using the FastKing RT kit (TIANGEN). Real-time-PCR was performed using SYBR® Select Master Mix (Thermo Scientific Inc.). Oligonucleotide primers for target genes are listed in **Table 1** and **Table 2**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an endogenous control. The relative mRNA expression levels of the target gene were evaluated by calculating the fold-changes normalized to the GAPDH for each sample using $2^{-\Delta\Delta Ct}$ methods. All cDNA samples were analyzed in triplicate.

Western Blotting

The liver and intestinal tissues were dissolved in RIPA, PMSF, and protease inhibitors. After homogenization, the protein concentrations were determined using bicinchoninic acid

TABLE 1 | The Oligonucleotide primers used in Realtime-PCR analysis.

Human gene	Primer sequences (5′- 3′)
GAPDH	Forward primer: CCCTTCATTGACCTCAACTACATGG
	Reverse primer: CATGGTGGTGAAGACGCCAG
TNF-α	Forward primer: ACTCCAGGCGGTGCCTATG
	Reverse primer: GAGCGTGGTGGCCCCT
IL-6	Forward primer: CCAGTTGCCTTCTTGGGACT
	Reverse primer: GGTCTGTTGGGAGTGGTATCC
IL-1β	Forward primer: GTGGCTGTGGAGAAGCTGTG
	Reverse primer: GAAGGTCCACGGGAAAGACAC
CCL2	Forward primer: TTTTCCCCTAGCTTTCCC
	Reverse primer: GCAATTTCCCCAAGTCTCT
CCR2	Forward primer: AGGGCTGTATCACATCGG
	Reverse primer: ACTTGTCACCACCCCAAA
RIP3	Forward primer: TCCAGGGAGGTCAAGGC
	Reverse primer: ACAAGGAGCCGTTCTCCA
MLKL	Forward primer: TTCACCCATAAGCCAAGGAG
	Reverse primer: GGATCTCCTGCATGCATTTT

TABLE 2 | The Oligonucleotide primers used in Realtime-PCR analysis.

Murine gene	Primer sequences (5′- 3′)
GAPDH	Forward primer: TGTGTCCGTCGTGGATCTGA
	Reverse primer: CCTGCTTCACCACCTTCTTGA
ZO-1	Forward primer: GGGCCATCTCAACTCCTGTA
	Reverse primer: AGAAGGGCTGACGGGTAAAT
Occludin	Forward primer: ACTATGCGGAAAGAGTTGACAG
	Reverse primer: GTCATCCACACTCAAGGTCAG
TNF-α	Forward primer: ACTCCAGGCGGTGCCTATG
	Reverse primer: GAGCGTGGTGGCCCCT
IL-6	Forward primer: CCAGTTGCCTTCTTGGGACT
	Reverse primer: GGTCTGTTGGGAGTGGTATCC
IL-1β	Forward primer: GTGGCTGTGGAGAAGCTGTG
	Reverse primer: GAAGGTCCACGGGAAAGACAC
CCL2	Forward primer: ACCTTTTCCACAACCACCT
	Reverse primer: GCATCACAGTCCGAGTCA
CCR2	Forward primer: AAGGGTCACAGGATTAGGAAG
	Reverse primer: ATGGTTCAGTCACGGCATA
RIP3	Forward primer: GAAGACACGGCACTCCTTGGTA
	Reverse primer: CTTGAGGCAGTAGTTCTTGGTGG
MLKL	Forward primer: CCTTGCTTGCTTGCTTTT
	Reverse primer: TTTCCTTGAGTTTGAGCCA

protein assay (Thermo Scientific Inc.). Proteins were separated using SDS-polyacrylamide gel electrophoresis system and then blotted onto a polyvinylidene fluoride membrane (Invitrogen, USA). Primary anti- RIP3(ab62344, Abcam, Cambridge, MA, USA), anti-MLKL (ab196436, Abcam, Cambridge, MA, USA), anti-TNF-α (ab183218, Abcam, Cambridge, MA, USA), anti-IL-6 (ab229381, Abcam, Cambridge, MA, USA), anti-ZO-1 (Abcam, USA), anti-Occludin (Abcam, USA), and anti-GAPDH (CST) antibody were then applied, and anti-GAPDH antibody was employed as the loading control. After incubation with horseradish peroxidase-conjugated secondary antibodies, the chemiluminescent signal was detected. Band intensity was determined by image processor program (Image J).

Cell Isolation and Flow Cytometry Analysis

MAbs specific for CD45, CD11b, and F4/80 were obtained from BD Biosciences. Single-cell suspensions of lymphocyte were harvested from mouse liver. The cells were suspended in buffer, incubated with the above antibody for 30 min, and examined on a FACSCalibur flow cytometer (Becton Dickinson, USA). RAW264.7 cells seeded in a 12-well plate at a density of 1×10^5 cells per well were stained with Annexin V/PI (BD Pharmingen, San Diego, CA, USA) to detect the apoptosis state in accordance with the manufacturer specifications. Data were analyzed using FlowJo 7.6 software.

RIP3 siRNA Knockdown

Transient genetic silencing of RIP3 was performed by reverse transfection of RAW264.7 cells with 20–30 Nm Silencer® Select siRNAs (Life Technologies, Inc.) using Lipofectamine® RNAiMax reagent (Life Technologies, Inc.), and Opti-MEM® medium (Life Technologies, Inc.). Negative nontarget siRNA was used as control. Knockdown efficiency was confirmed by Western blot analysis and qRT-PCR.

Intestinal Microbiota Analysis

16S rRNA gene sequencing procedure was performed by GENEWIZ, lnc. (Suzhou, China). Total fecal bacteria DNA extractions were acquired by QIAamp ® Fast DNA Stool Mini Kit (QIAamp, Germany). The microbial 16S V3-V4 region was amplified with indexes and adaptors-linked universal primers (341F: ACTCCTACGGGAGGCAGCA G,806R: GGACTACHVGGGTWTCTAAT). PCR was performed using KAPA HiFi Hotstart PCR kit high fidelity enzyme in triplicate. Amplicon libraries were quantified by Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, US) and then sequenced on Illumina HiSeq platform (Illumina, San Diego, US) for paired end reads of 250 bp. After the singletons were discarded and the chimeras were removed, the tags were clustered into operational taxonomic units (OTUs) using USEARCH (v7.0.1090) at 97% similarity. A representative sequence of each OTU was subjected to taxonomy-based analysis using the RDP database. Heatmap was created using R. Cluster analysis. Alpha and beta diversities were analyzed using QIIME. The relative abundance of bacteria was expressed as the percentage.

Statistical Analysis

Data were presented as mean ± SD. Comparisons among different groups were performed by unpaired one-way ANOVA or Student's t-test using SPSS 22.0. $p<0.05$ was considered statistically significant.

RESULTS

Increased Intestinal Permeability and Loss of Epithelial Barrier Integrity in AIH Patients

The main clinical and demographic features of enrolled patients and controls are shown in **Supplementary Table 1**. Plasma LPS, DLA, and DAO tests revealed an increased intestinal permeability in AIH-n group compared with that in the CTRL group (**Figure 1A**). To further assess the integrity of the intestinal barrier in these patients, we detected the structural

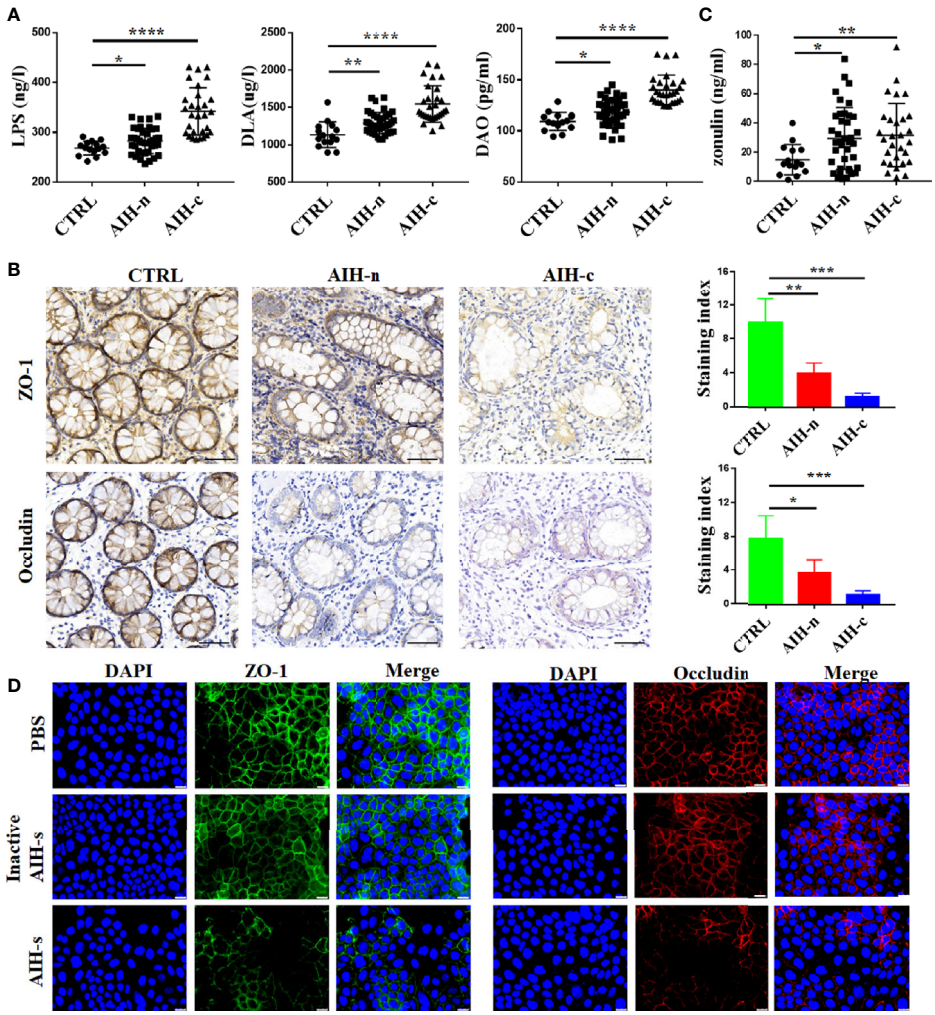

FIGURE 1 | Increased intestinal permeability and loss of epithelial barrier integrity in AIH patients. **(A)** The plasma concentrations of LPS, DLA and DAO in CTRL group (n=15), AIH-n group (n=39) and AIH-c group (n=29). **(B)** Expression of ZO-1 and Occludin in the colon were assessed by immunostaining. **(C)** The plasma concentration of zonulin in the three groups. **(D)** Representative immunostaining of ZO-1 and Occludin in Caco-2 cells. Scale bars: 50μm. The data were presented as means ± SD (Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

proteins including ZO-1 and Occludin in the ileocecal junction through IHC staining (**Figure 1B**). The staining index of ZO-1 and Occludin in AIH-n group was significantly decreased compared with that in the CTRL group. Zonulin is the only physiological modulator of intercellular tight junctions involved in the trafficking of macromolecules and therefore in tolerance/immune response balance (27). Hence, the plasma zonulin levels were evaluated and was found to be significantly increased in the two AIH groups compared with that in the CTRL group (**Figure 1C**), indicating that breakage of the intestinal barrier integrity is an early event in the pathogenesis of AIH.

Previous data have demonstrated that treatment-naïve AIH patients had compositional and functional alterations of gut microbiome (28). To investigate the effects of gut dysbiosis on intestinal barrier function, we used a vitro model in which Caco-2 epithelial cell monolayers treated with the supernatant of feces from AIH patients (AIH-s). As shown in **Figure 1D**, the expressions of ZO-1 and Occludin were dramatically decreased in the AIH-s group compared to the control groups (inactive AIH-s group and PBS group).

Activation and Infiltration of Macrophages in Liver Tissue of AIH Patients

Liver macrophages, which consist of resident macrophages (Kupffer cells) and monocytes-derived macrophages, maintain liver immune homeostasis. Therefore, macrophage heterogeneity and activation status in the liver tissues of AIH patients were studied. Double immunofluorescence analyses for CD68 and MAC387 revealed that the number of resident and infiltrating macrophages significantly increased in the liver tissues of AIH-n

group (**Figure 2A**). Furthermore, intracellular staining of inflammatory cytokines such as TNF-α and IL-6 co-stained with CD68 showed that the liver macrophages in AIH patients are significantly activated and a majority of the inflammatory signature derives from liver macrophages (**Figures 2B, C**). Besides, the mRNA expression of related inflammatory cytokines and chemokines in liver tissues was investigated (**Figure 2D**). TNF-α, IL-6, and IL-1β expression was significantly increased in the AIH-n group. CCL2 expression was also increased, and that of CCR2 was not significantly different.

RIP3 has been increasingly recognized as a key inflammatory signal adapter that mediates programmed necroptosis and the consequent release of inflammatory cytokines and chemokines (29, 30). Therefore, the activation of RIP3 and MLKL (the direct downstream effector of RIP3) in the liver tissues of AIH-n group was further explored. As shown in **Figure 2E**, the AIH-n group had significantly higher mRNA expression of RIP3 and MLKL than the hepatic cyst group. Concordantly, the protein expression of RIP3 and MLKL was also increased in the AIH-n group (**Figure 2F**).

Breakage of Intestinal Barrier Augments Activation and Infiltration of Liver Macrophages

Clinical data indicated an increase in the intestinal permeability and alterations of liver immune homeostasis in AIH-n patients. Hence, we hypothesized that the enteropathy and breakage of the intestinal barrier integrity are not epiphenomena but could play a pathogenic role in AIH by regulating liver inflammation. For hypothesis testing, the tandem model of DSS-Con A was employed. As shown in **Supplementary Figure 1B**, the body weight of mice in each group did not differ significantly ($p > 0.05$). In addition, the liver index and spleen index were significantly increased in the Con A group compared with those in the CTRL group, but no difference was observed between the Con A and DSS-Con A groups (**Supplementary Figure 1C, D**). Furthermore, the gut barrier integrity of the tandem model was assessed. The results showed that the DSS-Con A group had significantly higher plasma concentration of FITC-dextran compared with the Con A group (**Figure 3A**). The mRNA and protein expression levels of ZO-1 and Occludin were significantly decreased in the gut mucosa of DSS-Con A group compared with those of Con A group (**Figures 3B, C**).

Next, the inflammation and immunity of liver tissues were evaluated, and our data showed that the breakage of the intestinal barrier aggravated the Con A-mediated liver inflammation in the portal area (**Figure 4A**). The plasma transaminase levels of DSS-Con A group significantly increased compared with those of Con A group (**Figure 4B**). Moreover, the mice in the DSS-Con A group had a severe inflammatory cytokine milieu with higher mRNA expression of TNF-α, IL-6, and IL-1β in the liver tissues compared with that in Con A group, and the CCL2 expression was also

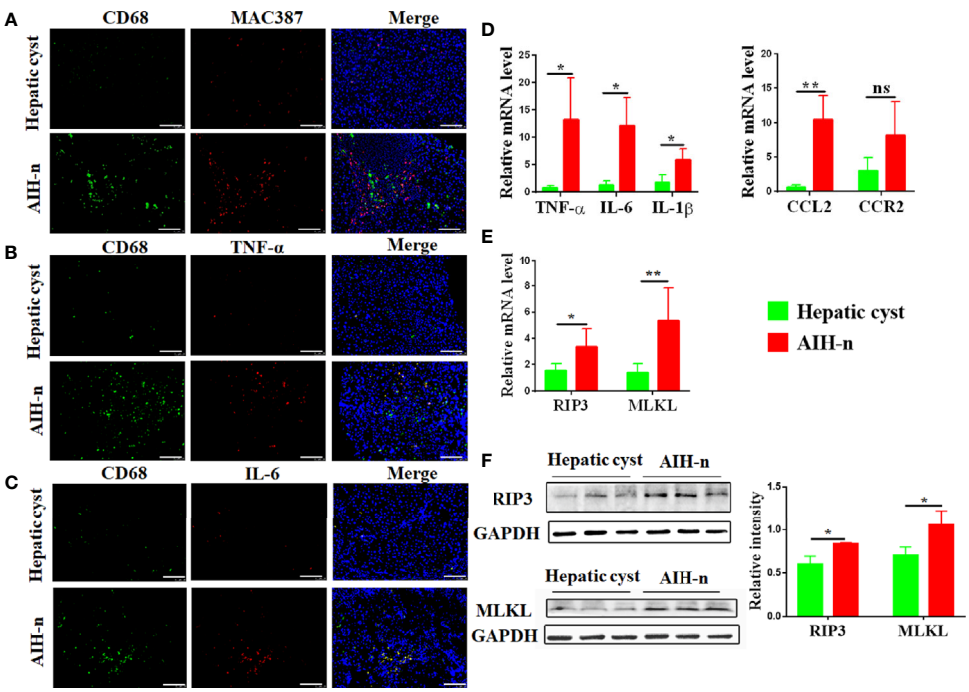

FIGURE 2 | Activation and infiltrating of macrophages in the liver tissue of AIH patients. **(A–C)** Double-immunofluorescence staining for CD68 and MAC387 **(A)** CD68 and TNF-α **(B)** CD68 and IL-6 **(C)** in liver tissues of patients with AIH and hepatic cyst. **(D)** RT-qPCR analysis of TNF-α, IL-6, IL-1β, CCL2 and CCR2 on tissue homogenates from the liver of the two groups. **(E)** RT-qPCR analysis of RIP3 and MLKL in the liver of the two groups. **(F)** Protein levels of RIP3 and MLKL in the liver of the two groups were detected and the relative intensity was quantified. Scale bars: 100μm. The data were presented as means ± SD (Student's t-test, *$p < 0.05$, **$p < 0.01$, ns: $p < 0.05$).

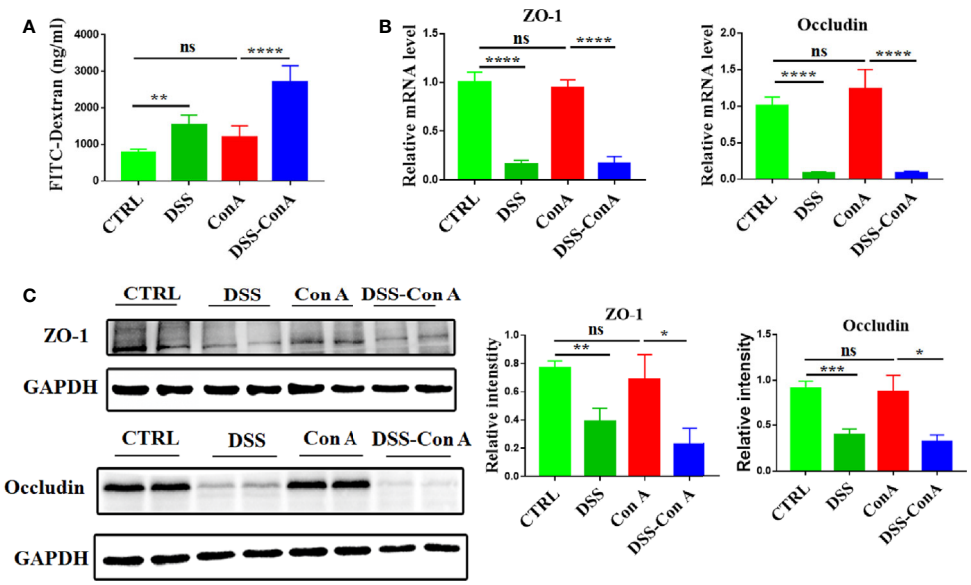

FIGURE 3 | Breakage of intestinal barrier integrity by 1%DSS. **(A)** FITC-dextran *in vivo* permeability assay in the four groups. **(B)** RT-qPCR analysis of ZO-1 and Occludin on tissue homogenates from the colon of the four groups. **(C)** Protein levels of ZO-1 and Occludin in the colon of the four groups were detected and the relative intensity was quantified. (n=6). The data were presented as means ± SD (Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: p < 0.05).

significantly increased in DSS-Con A group (**Supplementary Figure 3A, B**). In addition, immunofluorescence staining of inflammatory cytokines in F4/80$^+$ cells indicated that the liver macrophages in DSS-Con A group had a more severe inflammatory signature compared with that in Con A group (**Figure 4C**). The CD45$^+$ F4/80$^+$ CD11b$^+$ population of liver mononuclear cells were analyzed *via* flow cytometry to examine the state of the resident and infiltrating macrophages in the liver following the intestinal barrier breakage. The number of CD45$^+$ F4/80^{hi} CD11b^{lo} liver resident Kupffer cells was significantly decreased, whereas that of CD45$^+$ F4/80^{lo} CD11b^{hi} infiltrating macrophages significantly increased in Con A group compared with those of CTRL group. However, no difference was found between Con A and DSS-Con A groups. The ratio of infiltrating macrophages to Kupffer cells significantly increased in DSS-Con A group compared with that in Con A group (**Figure 4D**). These results suggested that the intestinal barrier breakage contributes to Con A-mediated liver injury by promoting the activation and infiltration of liver macrophages.

Intestinal Barrier Disruption Aggravates Activation of RIP3 Signaling Pathway of Liver Tissue

The mechanism underlying the augmented activation and accumulation of liver macrophage was studied under this tandem model. The protein expression of RIP3 and MLKL in the liver tissue was significantly upregulated in DSS-Con A group compared with that in Con A group (**Figure 5A**). The relative mRNA expression of RIP3 and MLKL also increased (**Supplementary Figure 3C**). As shown in **Figure 5B**, p-RIP3-positive cells and p-MLKL-positive cells were markedly increased in the liver tissues from DSS-Con A group, particularly in macrophages. Further, the mice were treated with GSK872 to investigate whether the inhibition of RIP3 signaling pathway can ameliorate the activation and infiltration of liver macrophages in the DSS-Con A group. The liver of GSK872-pretreated group showed markedly diminished RIP3 and MLKL expression (**Figure 5C**) and significantly inhibited mRNA expression levels of inflammatory cytokines and chemokines including TNF-α, IL-6, IL-1β, and CCL2 (**Figures 5D, E**). GSK872 also inhibited the infiltration of CD45$^+$ F4/80^{lo} CD11b^{hi} macrophages, leading to a significantly decreased ratio of infiltrating macrophages to Kupffer cells (**Figure 5F**). Accordingly, the GSK872-pretreated group had significantly reduced inflammation in the portal area of liver tissues and decreased plasma transaminases levels compared with the DSS-Con A group (**Figures 5G, H**). Besides, the annexin V/PI apoptosis assay for further assessment of cell death showed that breakage of the intestinal barrier significantly induced the late apoptosis of liver macrophages and GSK872 markedly decreased the early and late apoptosis rates (**Supplementary Figure 4**). All together, these results indicated that the pronounced activation and infiltration of liver macrophages in the DSS-Con A group are regulated by RIP3 signaling pathway.

RIP3 Signaling Pathway Regulates the Expression of Macrophage-related Cytokines and Chemokines in RAW264.7 Cell Lines

The expression levels of macrophage-related cytokines and chemokines were analyzed by activating or inhibiting the RIP3 signaling pathway *in vitro* to further explore the effect of RIP3

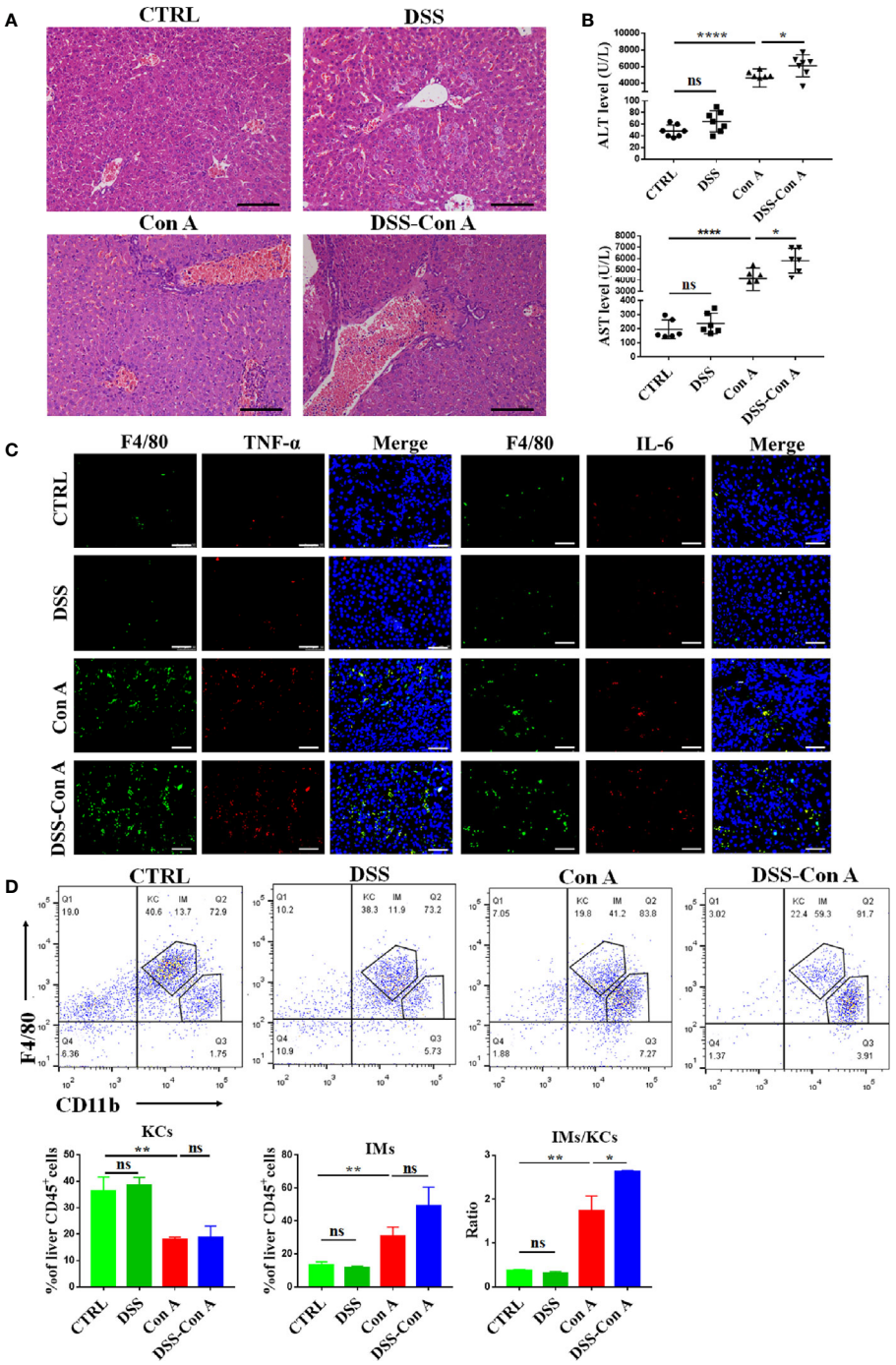

FIGURE 4 | Breakage of the intestinal barrier aggravated liver injury and infiltration of liver macrophages. **(A)** HE staining of liver tissues in four groups. **(B)** The plasma concentrations of ALT and AST in four groups. **(C)** Double-immunofluorescence staining for F4/80 and TNF-α, F4/80 and IL-6 of liver tissues in four groups. **(D)** Representative flow cytometry plots and percentage of $CD45^{+}F4/80^{hi}CD11b^{lo}$ Kupffer cells and $CD45^{+}F4/80^{lo}CD11b^{hi}$ infiltrating macrophages in mononuclear cells from liver of the four groups. (n=6). Scale bars: 50μm. The data were presented as means ± SD (Student's t-test, *p < 0.05, **p < 0.01, ****p < 0.0001, ns: p < 0.05).

signaling pathway on liver macrophages. The results showed that the relative mRNA expression of RIP3 and MLKL was upregulated after LPS stimulation in RAW264.7 cells (**Supplementary Figure 5A**). The protein level of RIP3 and MLKL also significantly increased in the LPS group (**Figure 6A**). Upon the activation of RIP3 signaling pathway by LPS, the relative mRNA expression levels of cytokines and chemokines including TNF-α, IL-6, IL-1β, CCL2, and CCR2 as well as the

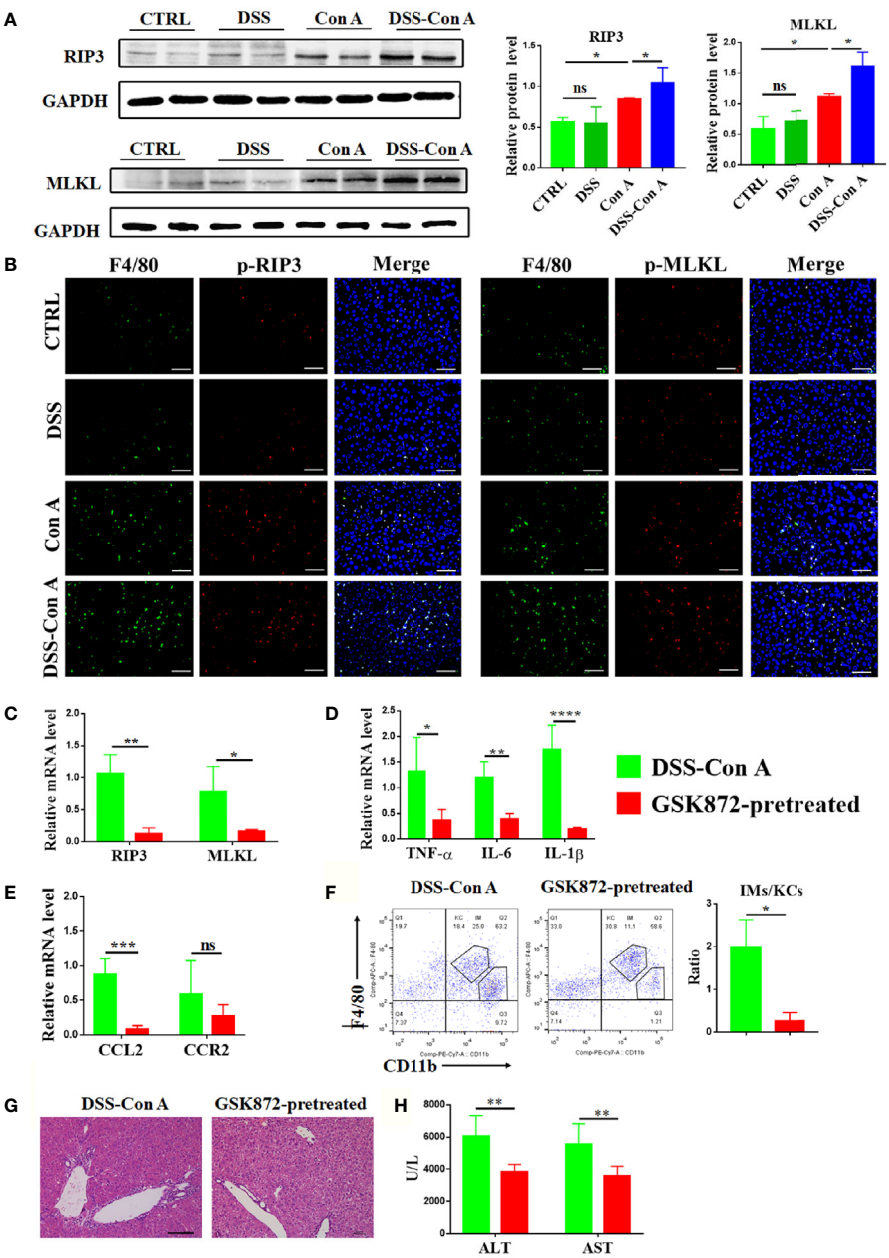

FIGURE 5 | Breakage of intestinal barrier aggravated the activation of RIP3 signaling pathway of the liver tissue. **(A)** Protein levels of RIP3 and MLKL in the liver of the four groups were detected and the relative intensity was quantified. **(B)** Representative double-immunofluorescence staining for F4/80 and p-RIP3, F4/80 and p-MLKL of liver tissues in four groups. **(C)** RT-qPCR analysis of RIP3 and MLKL on liver tissue of DSS-ConA group and GSK872-pretreated group. **(D)** RT-qPCR analysis of TNF-α, IL-6, and IL-1β on liver tissue of the two groups. **(E)** RT-qPCR analysis of CCL2 and CCR2 on liver tissue of the two groups. **(F)** Representative flow cytometry plots and percentage of $CD45^{+}F4/80^{hi}$ $CD11b^{lo}$ Kupffer cells and $CD45^{+}$ $F4/80^{lo}$ $CD11b^{hi}$ infiltrating macrophages in mononuclear cells from liver of the two groups. **(G)** HE staining of the liver tissue from the two groups. **(H)** The plasma concentrations of ALT and AST of the two groups. (n=6). Scale bars: 50μm. The data were presented as means ± SD (Student's t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: $p < 0.05$).

protein expression of key cytokines such as TNF-α and IL-6 were also significantly increased (**Supplementary Figure 5B, C**). By contrast, the relative mRNA expression of cytokines and chemokines were down-regulated when the RIP3 signaling pathway was inhibited with GSK872 (**Figures 6B–D**). The protein level of TNF-α and IL-6 in the LPS-GSK872 group also decreased but the difference was not statistically significant (**Supplementary Figure 5C**). Besides, the annexin V/PI apoptosis assay showed that LPS significantly induced the early and late apoptosis of RAW264.7 cells and GSK872 markedly

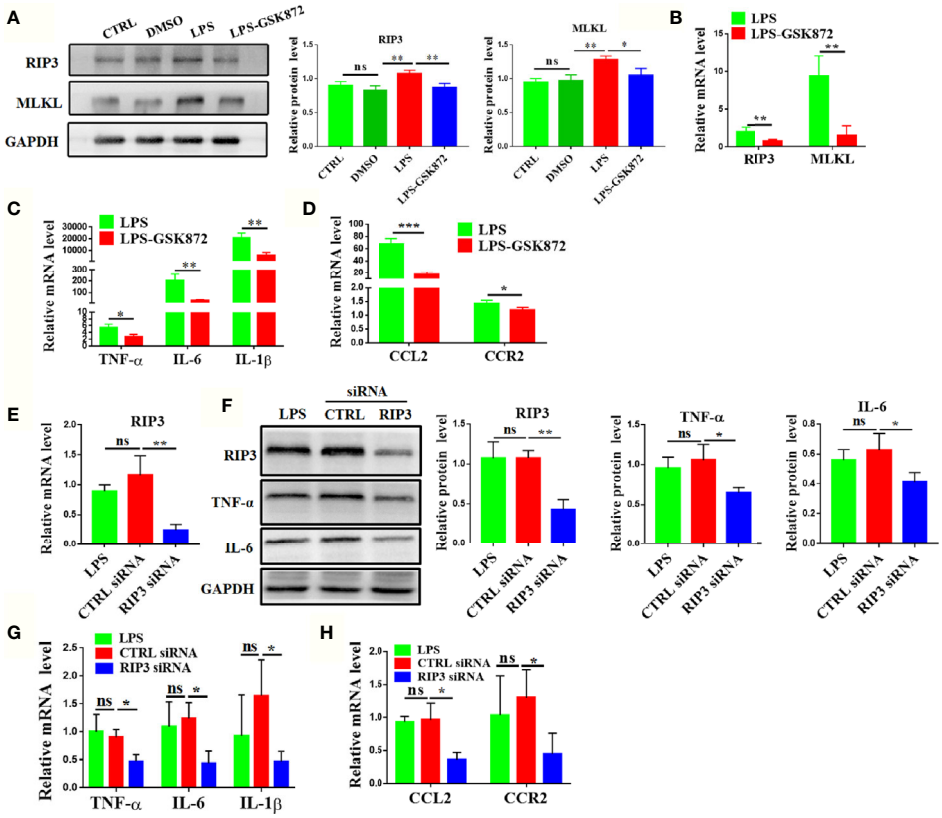

FIGURE 6 | RIP3 signaling pathway regulates the expression of macrophage-related cytokines and chemokines in RAW264.7 cell lines. **(A, B)** Relative expression of RIP3 and MLKL was inhibited by GSK872. **(C, D)** Relative expression of TNF-α, IL-6, and IL-1β **(C)**, CCL2 and CCR2(D) was down-regulated by GSK872. **(E, F)** Relative expression of RIP3 was inhibited by RIP3 siRNA and key cytokines such as TNF-α and IL-6 were detected and the relative intensity was quantified. **(G, H)** Relative expression of TNF-α, IL-6, and IL-1β **(G)**, CCL2 and CCR2(H) was down-regulated by RIP3 siRNA. The data were presented as means ± SD of three independent experiments (Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001, ns: p > 0.05).

decreased the late apoptosis rate (**Supplementary Figure 6**). Furthermore, RIP3 was efficiently knocked down by RIP3 siRNA when compared with CTRL siRNA (**Figures 6E, F**). Accordingly, the protein level of key cytokines such as TNF-α and IL-6 also significantly decreased in the RIP3 siRNA group (**Figure 6F**) and the relative mRNA expression of macrophage-related cytokines and chemokines including TNF-α, IL-6, IL-1β, CCL2, and CCR2 also significantly decreased in the RIP3 siRNA group (**Figures 6G, H**). All these results emphasized that the RIP3 signaling pathway can be activated by intestinal LPS to regulate the activation and accumulation of macrophages.

RIP3-mediated Activation and Infiltration of Liver Macrophages Requires Gut Commensal Microbiota

Disruption of the intestinal barrier lead to bacterial translocation, which consecutively activates immune cells to release various proinflammatory cytokines and chemokines (31). To test whether the gut commensal bacteria are required for RIP3 activation, EAH mouse model displaying dysbiosis in fecal microbiomes was used. **Figures 7A, B** shows that as measured by observed index and fisher index, the EAH group had significantly decreased alpha-diversity compared with the CTRL group. Principal component analysis based on weighted UniFrac distances revealed a different structure between the two groups (**Figure 7C**). The gut microbiota of all the samples in the two groups were dominated by three major phyla: Bacteroidetes, Firmicutes, and Proteobacteria (**Figure 7D**). Compared with those of the CTRL group, higher abundance of Bacteroidetes and lower abundance of Firmicutes and Proteobacteria were found in the EAH group. This phenomenon resulted in a decreased Firmicutes/Bacteroidetes (F/B) ratio (0.19) in the EAH group compared with that in the CTRL group (1.32). Genus-level analysis revealed that the mice in the EAH group had increased relative abundance of potential pathogenic bacteria, such as Bacteroides and Prevotellaceae_UCG-001, and a relatively low abundance of Lactobacillus and Ruminiclostridium (**Figure 7E**). Gut microbiota was also compared between the two groups through linear discriminant analysis effect size (LEfSe) to identify the specific microbiota linked to EAH. Prevotellaceae, which is associated with

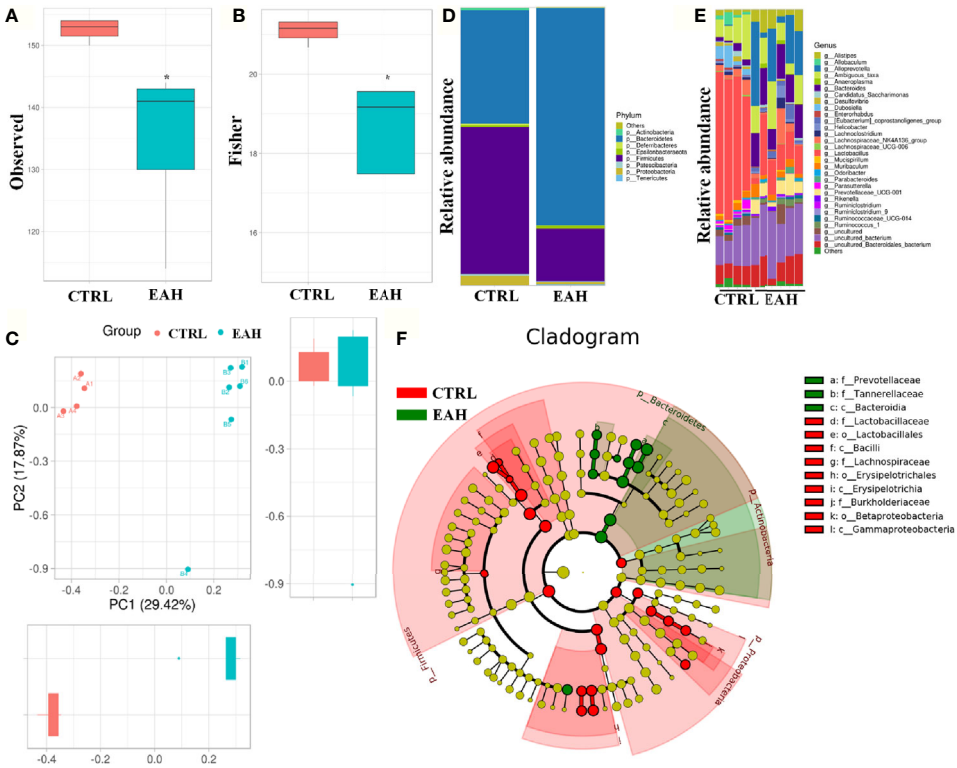

FIGURE 7 | Alteration of the gut microbiota composition in EAH mice. **(A, B)** Observed **(A)** and Fisher **(B)** diversity indexes of the gut microbiota in CTRL group (n=4) and EAH group (n=6). **(C)** ANOSIM based on weighted UniFrac distances. **(D)** Bar charts of the gut microbiota composition at the phylum level in CTRL group and EAH group. **(E)** Bar charts of the gut microbiota composition at the genus level in each mouse. **(F)** Cladogram generated from the LEfSe. (*p < 0.05).

autoimmune diseases such as rheumatoid arthritis (32), were highly abundant in the EAH group (**Figure 7F**).

To analyze the role of gut commensal microbiota in liver immunity, the mice were treated with broad-spectrum antibiotic mixture prior to EAH induction. As shown in **Supplementary Figure 2B**, the mice in the EAH group had lower body weight than those in the CTRL group after 4 weeks, whereas no significant weight loss was found in the Abx group. In addition, the liver index and spleen index were significantly increased in the EAH group compared with those in the CTRL group, whereas no significant increase was observed in the Abx group (**Supplementary Figure 2C, D**). OTU comparison among the three groups revealed lower abundance of OTUs in the Abx group compared with those in the CTRL and EAH groups (28 OTUs vs. 176 OTUs vs. 181 OTUs). Among the 28 OTUs in the Abx group, 25 were shared by the three groups (**Figures 8A, B**). Analysis of Chao1 index and Shannon index indicated that the community richness and diversity also significantly decreased in the Abx group relative to those in the other two groups (**Figure 8C**). Remarkably, none of the mice in Abx group had developed hepatitis, and the transaminase level of the mice in the Abx group nearly returned to normal (**Figures 8D, E**). The intestinal permeability of these mice was also evaluated, and the results showed that the plasma FITC-D and LPS levels of Abx-treated mice were significantly decreased compared with those of the EAH mice (**Figure 8F**). The RIP3 signaling pathway was significantly inhibited with lower expression of inflammation cytokines and chemokines in liver tissues of the Abx group compared with those of the EAH group (**Figures 8G–I**). Besides, we evaluated the changes of liver macrophages by immunofluorescence and the results revealed that the mice in EAH group had increased number of macrophages in the liver tissue and the macrophages was significantly activated than that in the CTRL group, however, the broad-spectrum antibiotic mixture significantly alleviated the accumulation and activation of liver macrophages as shown in **Supplementary Figure 7**.

DISCUSSION

It's known that alterations in the gut microbiota have been related with most autoimmune diseases, but in most cases, it remains unclear whether these changes are a cause or effect of the disease or merely a reflection of epidemiological differences between groups. The gut–liver axis has clinical importance as a potential therapeutic target in a wide range of chronic liver diseases (33–35). Recent evidence suggests that the intestinal environment specifically, modifications of the microbiome

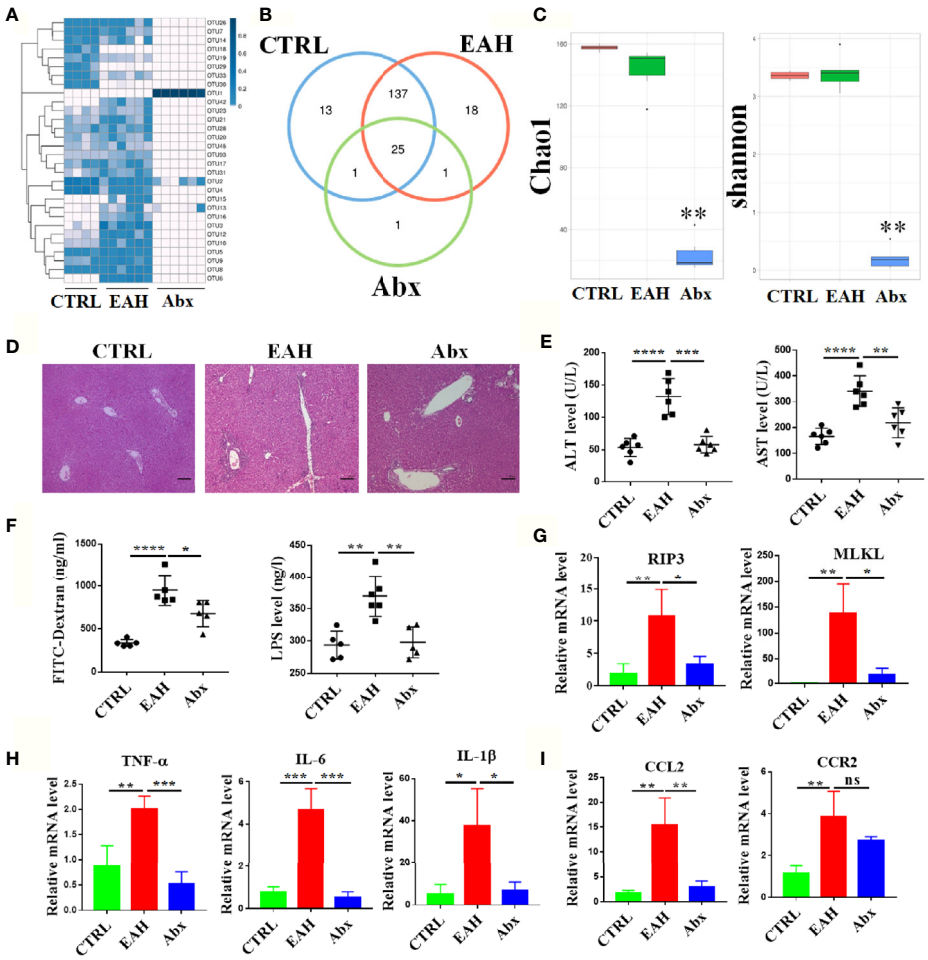

FIGURE 8 | RIP3-mediated activation and infiltration of liver macrophages requires gut commensal microbiota. **(A, B)** Bacterial OUT heatmap **(A)** and Venn diagrams **(B)** in the CTRL group (n=4), EAH group (n=6) and Abx group (n=6). **(C)** The community richness and diversity of the gut microbiota were evaluated with Chao1 and Shannon indexes. **(D)** HE staining of the liver tissue from three groups. **(E)** The plasma concentrations of ALT and AST of three groups. **(F)**The plasma concentrations of FITC-D and LPS of three groups. **(G–I)** RT-qPCR analysis of RIP3 and MLKL **(G)** TNF-α, IL-6, and IL-1β **(H)** CCL2 and CCR2 **(I)** in three groups. Scale bars: 100μm. The data were presented as means ± SD (Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: p > 0.05).

profile, regulate the pathogenesis of AIH by inducing intestinal inflammation and increasing gut permeability (36). In this present study, we try to explore the causality between the leaky gut/dysbiosis and AIH, demonstrating that loss of gut barrier integrity breaks liver immune homeostasis and augments liver injury.

The gut barrier is a fundamental gatekeeper to prevent translocation of bacterial components and the intestinal epithelial barrier (IEB) are crucial to prevent the passage of commensal bacteria and pathogens, from the lumen into the systemic circulation (37). The IEB is a single layer of epithelial cells held together by a complex junctional system composed of tight junctions, adherent junctions, and desmosomes (38). Recent studies indicated that leaky gut and increased intestinal permeability contributed to disease initiation and progression (39–41). Alterations of gut barrier integrity are found in patients affected by extraintestinal autoimmune diseases, but a direct causal link between enteropathy and triggering of autoimmunity is yet to be established. It was reported that, in mice models of T1D, loss of gut barrier integrity can lead to activation of islet-reactive T cells within the intestinal mucosa (42). Remarkably, Vieira et al. found that translocation of a gut pathobiont, Enterococcus gallinarum, to the liver and other systemic tissues triggers autoimmune responses in a genetic background predisposing to autoimmunity (43). In the present study, we demonstrated that the expression of tight junction proteins and the intestinal permeability is altered in AIH patients. Those alterations were detected at an early stage in AIH progression that is concomitant with the activation of the liver macrophages. Dysbiosis and reduction of tight junction proteins could ultimately lead to intestinal bacterial translocation with increased serum LPS levels and broken liver immune homeostasis that we observed in AIH patients and EAH mice.

The presence of loss of gut barrier integrity in patients and mice models of autoimmune diseases, such as T1D and systemic lupus erythematosus, has been known for long time but a causal link between the intestinal alterations and induction autoimmunity was never established (44). Our data demonstrate that loss of gut barrier integrity and modifications of the structural proteins prior to hepatitis, break liver immune tolerance, thus augment liver injury. Different triggering events have been reported such as viral infections or any factor that perturbs the liver environment leading to inflammation, tissue damage, and the release of sequestered liver antigen resulting in the stimulation of autoreactive immune cells (34). Here, we show that breakage of the gut barrier is one of those events that unleashes liver autoimmunity and provokes liver injury. The liver is particularly enriched in macrophages, which protect against infection, control host–microbiota mutualism, and maintain liver homeostasis (15, 45). Our data showed that intestinal barrier disruption increased activation and accumulation of liver macrophages thus aggravated liver injury. In the present study, we analyzed subsets of liver immune cells *via* flow cytometry and found the ratio of $CD45^+$ $F4/80^{hi}$ $CD11b^{lo}$ infiltrating macrophages to $CD45^+$ $F4/80^{lo}$ $CD11b^{hi}$ Kupffer cells significantly increased in DSS-Con A group.

RIP3 is an essential part of the cellular machinery that executes "programmed" or "regulated" necrosis. Bacterial products such as LPS can activate the RIP3 signaling pathway (46, 47). Upon activation, the necrosome complex phosphorylates MLKL, leading to fatal permeabilization of the plasma membrane that exerts pro-inflammatory functions (21). In agreement with the induced gene expression of necrosome-related genes, Western blot analyses of liver specimen revealed the elevated levels of RIP3 protein in DSS-Con A mice and EAH mice. Whether RIP3 regulates the activation and infiltration of liver macrophage in DSS-Con A mice remains unclear. Thus, these mice were treated with GSK872 to dampen the RIP3 activation and thus significantly ameliorated the activation and accumulations of liver macrophages. The same results were observed *in vitro*. Our data are in accordance with a recent report showing that the RIP3 expression is up-regulated in liver tissues and macrophages of humans and mice with liver fibrosis; in addition, the absence of RIP3 in macrophages could alleviate inflammation and macrophage or neutrophil accumulation in mice after carbon tetrachloride or bile duct ligation treatment (48). It's worth noting that the link between the RIP3 pathway and AIH is not definitively clear without gene knock-out (KO). However, it's reported that RIP3 regulates stem cells generation through modulating cell cycle progression genes and RIP3 KO displayed lower expression of cell cycle genes and a slower proliferation rate compared to wild type (49). Besides, Patrick-Simon Welz et al. demonstrated that genetic deficiency in RIP3 prevented the development of spontaneous pathology in both the small intestine and colon of mice, which may prevent us from studying the role of the intestinal barrier in AIH (50). For the above reasons, we used GSK872, a RIP3 specific inhibitor *in vivo* and *in vitro* experiments to illustrate the role of RIP3 pathway in AIH. Our data suggested that the loss of intestinal barrier augmented liver macrophage activation and infiltration and the RIP3 signaling pathway might be the underlying molecular mechanism of intestinal barrier disruption on AIH pathogenesis.

The gut microbiota has a strong impact in AIH pathogenesis as demonstrated both in humans and mice models, but it is still unclear how commensal bacteria modulate liver autoimmunity. In fact, while in other autoimmune diseases such as rheumatoid arthritis, autoimmune encephalomyelitis and multiple sclerosis, the gut microbiota plays a clear triggering role (51, 52). In AIH, key epitopes that might trigger the disease might be sought among environmental agents especially within the intestinal microbiome (1, 53). Our data showed that gut inflammation by itself in EAH mice depleted of endogenous microbiota is not capable to activate liver autoimmunity, which suggests that the commensal gut microbiota is required for the activation of innate immune response. The intestinal microbiota maintains gut barrier integrity, shapes the mucosal immune system and balances host defense with microbial metabolites, components, and attachment to host cells (54). While the role of gut microbiota in liver immunity is still controversial. In some cases, it plays a beneficial effect, for example, in SPF and gnotobiotic mice, gut microbiota and commensal D-lactate programs Kupffer cells to capture and kill circulating pathogens (55). On the contrary, the gut microbiota contributes to a mouse model of spontaneous bile duct inflammation and GF mice develop a milder biliary affection, thus suggesting that commensal strains are important to trigger liver autoimmunity (56).

Our study showed that the intestinal barrier and gut microbiota in patients with AIH and murine models were destroyed. Diminished intestinal barrier function contributed to the activation and accumulation of liver macrophages *via* RIP3 signaling pathway. This phenomenon further aggravated the immune response in the inflamed liver. Our results revealed the novel mechanism of immune tolerance breakage in the liver *via* the gut–liver axis. In addition, therapeutic and research potentials of regulating the intestinal microbiota for AIH therapy were explored.

DATA AVAILABILITY STATEMENT

We have uploaded the 16S rRNA data into the SRA database successfully. The SRA records will be accessible with the following link: https://www.ncbi.nlm.nih.gov/sra/PRJNA678135.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Animal Ethical and Welfare Committee of Tianjin Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Animal Ethical and Welfare Committee of Tianjin Medical University.

AUTHOR CONTRIBUTIONS

LZ and BW designed the study. HZ, ML, WZ and YZ performed the experiments. YL, LG, YZ, YR, WC and JZ analyzed the results. HZ and LZ wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.624360/full#supplementary-material

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Innate Immune Modulation Induced by EBV Lytic Infection Promotes Endothelial Cell Inflammation and Vascular Injury in Scleroderma

Antonella Farina[1†], *Edoardo Rosato*[2†], *Michael York*[3], *Benjamin E. Gewurz*[4,5,6], *Maria Trojanowska*[3] *and Giuseppina Alessandra Farina*[3*]

[1] Department of Experimental Medicine, Sapienza University, Rome, Italy, [2] Department of Clinical Medicine, Sapienza University, Rome, Italy, [3] Division of Rheumatology, Boston University School of Medicine, Boston, MA, United States, [4] Division of Infectious Diseases, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, United States, [5] Program in Virology, Harvard Medical School, Boston, MA, United States, [6] Broad Institute of Harvard and MIT, Cambridge, MA, United States

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***Correspondence:**
Giuseppina Alessandra Farina
farina@bu.edu

[†]These authors have contributed equally to this work

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Microvascular injury is considered an initial event in the pathogenesis of scleroderma and endothelial cells are suspected of being the target of the autoimmune process seen in the disease. EBV has long been proposed as a trigger for autoimmune diseases, including scleroderma. Nevertheless, its contribution to the pathogenic process remains poorly understood. In this study, we report that EBV lytic antigens are detected in scleroderma dermal vessels, suggesting that endothelial cells might represent a target for EBV infection in scleroderma skin. We show that EBV DNA load is remarkably increased in peripheral blood, plasma and circulating monocytes from scleroderma patients compared to healthy EBV carriers, and that monocytes represent the prominent subsets of EBV-infected cells in scleroderma. Given that monocytes have the capacity to adhere to the endothelium, we then investigated whether monocyte-associated EBV could infect primary human endothelial cells. We demonstrated that endothelial cells are infectable by EBV, using human monocytes bound to recombinant EBV as a shuttle, even though cell-free virus failed to infect them. We show that EBV induces activation of TLR9 innate immune response and markers of vascular injury in infected endothelial cells and that up-regulation is associated with the expression of EBV lytic genes in infected cells. EBV innate immune modulation suggests a novel mechanism mediating inflammation, by which EBV triggers endothelial cell and vascular injury in scleroderma. In addition, our data point to up-regulation of EBV DNA loads as potential biomarker in developing vasculopathy in scleroderma. These findings provide the framework for the development of novel therapeutic interventions to shift the scleroderma treatment paradigm towards antiviral therapies.

Keywords: scleroderma/SSc, Epstein-Barr Virus, endothelial cells, TLR9 innate immune response, vascular injury, digital ulcers, EBV DNA load, monocytes

INTRODUCTION

Systemic sclerosis (Scleroderma, SSc) is a rare heterogeneous autoimmune disease characterized by immune abnormalities, vascular damage and fibrosis (1–4). There is evidence supporting the presence of vascular injury and remodeling in many tissues, including the skin in the early phase of SSc disease, suggesting that endothelial cell injury might be the first pathogenetic event in the development of SSc (4, 5). As a consequence, the progressive vascular dysfunction drives some of the most characteristic clinical features of SSc, including Raynaud's phenomenon (RP), ischemic digital ulcers, pulmonary arterial hypertension and SSc renal crisis (6, 7). Vascular abnormalities could also precede the onset of fibrosis in the majority of SSc patients, further supporting that endothelial cell dysfunction and microvascular damage might play a key role in the pathogenesis of the disease (5, 8). Despite the importance of the vascular involvement in the pathogenesis of SSc, many aspects of SSc vasculopathy including the nature of the injury and the fate of injured endothelial cells (ECs) remain poorly understood (1, 4, 8).

EBV is commonly associated with autoimmune disorders, including SSc (9–25). One of the most convincing cases is the epidemiological association between EBV seropositivity and two of autoimmune diseases, systemic lupus erythematosus (SLE) and multiple sclerosis (21, 26–28). Importantly, a recent study has demonstrated that EBNA2, an EBV/latent protein colocalizes with autoimmune risk loci in B cells of several autoimmune diseases including SLE, strongly suggesting that EBV contributes to the origin of the genetic risk in these disorders (29). Evidences linking EBV and SSc have been also reported, since EBV latent antigens and high titers of EBV antibodies have been detected more often in SSc patients (30–32). Interestingly, it has been shown that B cells from healthy donor upon EBV transformation were able to produce anti-topoisomerase antibodies (Scl-70) strongly supporting the notion that production of auto-antibodies might be directly related to EBV infection in SSc (33). Despite the extensive range of evidence for a causal link between EBV and autoimmune diseases, to date there is no known mechanism that explains how EBV may contribute to the pathogenesis of these diseases.

The role of EBV`s antigens in triggering autoimmunity has been extensively studied, however, whether the active form of EBV infection (lytic EBV replication) is potentially involved in the pathogenesis of these diseases it is poorly understood (34). Recent evidence suggests EBV lytic reactivation and production of infectious EBV may be pathogenic in several autoimmune disorders, including SSc, while the presence of lytic EBV is found at low levels in healthy populations persistently infected by EBV (10, 12, 35–37). This suggests that viral reactivation occurs more frequently in individuals with a perturbed immune condition (10, 28, 38). In this regard, higher EBV loads in peripheral blood associated with an aberrant serological response to EBV lytic antigens, have been found in patients with autoimmune disorders (30, 34, 39). EBV genome load has been found highly increased in blood from SLE patients, independently of treatment with immunosuppressive agents (9–11) and increased viral activation has been associated with the occurrence of disease activity and flares, supporting the linkage between EBV replication and exacerbation of the disease (10, 40). Recently, we have reported evidence of EBV encoded mRNA and lytic cycle proteins in the majority of fibroblasts and anti-inflammatory (M2) macrophages in SSc (41). We also found that EBV-lytic genes, and proteins were present in SSc monocytes, while small, EBV-encoded RNAs (EBERs) associated with all stages of EBV infection have been detected in dermal ECs of SSc patients, suggesting that monocytes and ECs might represent a target of EBV in SSc (35, 41). We have also demonstrated that the active form of EBV infection drives innate immune inflammation through the induction of the TLR8 inflammatory pathway in infected monocytes, and that SSc monocytes carrying infectious EBV exhibited a robust induction of the IFN signature, as well as altered TLR8 expression compared to healthy donors (HDs) (35). These results showed for the first time that infectious EBV is exclusively present in a subset of SSc monocytes, but not in monocytes from HDs and EBV reactivation triggers a broad spectrum of host genes and cellular pathways including the innate immune responses in the infected cells.

Consistent with our previous finding that SSc monocytes carry EBV active infection and supported by the notion that monocytes have the ability to adhere to the endothelium "*in vivo*" (42), we chose to interrogate the interaction of monocyte-associated EBV with endothelial cells as the mechanism by which the virus induces dysfunctional inflammation and vascular injury in SSc.

In this study, we report that EBV DNA load is remarkably increased in peripheral blood and plasma from SSc patients compared to healthy donor EBV carriers. We also show that B cells and monocytes represent the prominent subsets of EBV-infected cells in SSc, while EBV DNA load is significantly lower or undetected in monocytes from SLE patients as well as in B cells and monocytes from healthy EBV carriers. We demonstrate that human primary dermal endothelial cells are infected with EBV using human monocytes bound to EBV recombinant virus as a cellular shuttle to transfer EBV particles to target cells, while cell-free virus failed to infect the endothelial cells. We show that up-regulation of innate immune mediators, such as TLR9, IRF5, IRF7, IFN-inducible genes MX2 and CXCL10, and several markers of vascular injury are induced by EBV in infected endothelial cells. Finally, to substantiate the association of EBV loads with clinical signs of vascular disease, we show that SSc patients with high level of EBV loads in blood develop an increased number of digital ulcers and marked reduction in skin perfusion, compared to SSc patients with undetectable levels of EBV DNA load with less or no signs of active vascular disease.

Altogether, these results provide new insight into the mechanism employed by EBV in inducing vascular damage through the activation of innate immune inflammatory response and markers of vascular injury in infected endothelial cells. Detection of EBV loads in whole blood and in monocytes can potentially be used as biomarker to evaluate the risk of endothelial cell dysfunction and vascular damage in a cohort of SSc patients with an active EBV infection.

MATERIALS AND METHODS

Study Approval

Experiments were approved by the Institutional Review Board of Sapienza University of Rome (Comitato Etico N° 3377/25-09-14, Rome, Italy) and Boston University (Boston, USA) and performed in accordance with NIH guidelines.

Study Subjects

All study subjects met the criteria for SSc as defined previously (43). All subjects gave written informed consent. Subjects selected for this study, diffuse cutaneous SSc (SSc) patients: blood (n=65) and skin tissue (n=10), systemic lupus erythematosus (SLE) blood (n=10) and normal healthy donors (HD) blood (n=55) skin tissue (n=10), are summarized in **Supplemental Table 1**. All the patients and HDs included in the study were positive for EBV serology. 71 of 75 SSc patients and 10 SLE patients included in the study were naïve for immunosuppressive therapy (IT) or did not receive any immunosuppressive therapy for a time > 6 months. Four SSc patients were on some form of standard treatment (Mycophenolate or Steroids). Blood samples of these patients were included in the detection of EBV DNA from whole blood. Healthy donors were defined by lacking any current or prior history of malignancy, autoimmune disease, or recurrent/chronic infections. Data such as sex, age, treatment status, disease activity, clinical manifestations, and laboratory parameters were extracted from the medical records of all patients used for these studies and are displayed in **Supplemental Table 1**.

Monocyte Isolation

Blood was collected from EBV-seropositive HD, SSc and SLE patients in CPT tubes designed for one-step cell separation (Becton Dickinson), and PBMCs were isolated as described previously (44). After positive selection of CD19 cells (CD19+) using magnetic bead isolation (CD19+ selection EasySep, StemCell), monocytes were negatively selected using the Human Monocyte Enrichment Kit without CD16 Depletion (EasySep, StemCell) as described previously.

Quantitative PCR to Quantify EBV DNA

DNA was extracted from Whole Blood (WB), plasma and 1–3 × 10^6 circulating B-cells and Monocytes, using QIAmp DNA blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturers' protocol. DNA was eluted off the column in an equivalent volume of H_20 and stored at -20°C. Previously designed primers and probes that detect a 70 bp region of the EBV BALF5 gene were used (45). EBV DNA was quantified using StepOne™ Real-Time PCR system (Applied Biosystems). Power SYBR Green chemistry (Applied Biosystems) was used for all reactions according to the protocol provided by Applied Biosystems, as described previously. To generate a standard curve, we used an EBV (B95-8 Strain) quantitated viral load control (Advanced Biotechnologies Inc (ABI), Columbia, MD, USA). Each sample was tested in triplicate, and the mean of the two values was shown as the copy number of the sample. The viral loads were log-transformed and then calculated based on EBV genome copies/ml and/or 1–3 × 10^6 circulating B-cells and Monocytes. The limit of detection by qPCR was 50 copies/mL WB, plasma or per 1-3 X 10^6 cells.

Nucleic Acid Extraction, RNA Preparation and Real-Time Polymerase Chain Reaction (q-PCR)

Total RNA from monocytes was extracted using an miRNAsy kit according to the manufacturer`s protocol (Quiagen, Valencia, CA) and processed as reported previously (41). The synthesized cDNAs were used as templates for quantitative real-time PCR. Primer sets designed to detect EBV genes and innate immune mediator genes were used as described previously (35, 41, 46–48). Expression of mRNA for the indicated genes was detected using SYBRGreen chemistry amplification (Applied Biosystems, Life Technologies, Grand Island, NY) as previously described (41). To assure specificity of the primer sets, amplicons generated from the PCR reaction were analyzed for specific melting temperatures by using the melting curve software. All real time-PCR was carried out using StepOnePlus Sequence Detector (Applied Biosystems, Life Technologies, Grand Island, NY). The change in the relative expression of each gene was calculated using δδCt formula choosing the same healthy human subject as the control for all relative expression analyses (47). Target and control reactions were run on separate wells of the same q-PCR plate (47).

Tissue Samples

Skin biopsies were obtained from forearm of 10 diffuse SSc patients and 10 healthy donors (HDs). All biopsy specimens were collected under patient consent and approval of the Boston University Medical Campus and "Sapienza" University, Rome, Institutional Review Board. Demographics and clinical characteristics of these cohorts are defined in **Supplemental Table 1**.

Histology

All analyses used conventional formalin-fixed, paraffin-embedded sections. Tissue sections were deparaffinised and two-color immunohistochemistry was performed as described previously (35, 49). A double-staining protocol was used on paraffin-embedded slides. After dewaxing, heat antigen retrieval was performerd in Tris/EDTA pH 9.0 for 20 minutes. Blocking was achieved using 3% H_2O_2 followed by BloxAll (Vector Labs, Burlingame, CA) or 2% horse serum. Antibodies (Abs) were separately titrated for the two modalities. Primary Abs were mouse anti-human CD31 (mAbs C31.3 + JC/70A; Novus Biologicals, Littleton, CO), mouse anti-Zebra mAb (AZ69, Argene Varilhes, France). Appropriate Vector ImmPress Polymers (ImmPress AP or ImmPress P anti-mouse IgG Polymerdetection kit) were used to detect primary antibodies, followed by development with either HighDef Blue (Enzo, Farmingdale, NY, USA, alkaline phosphatase), or DAB (DakoCytomation) [brown, horseradish peroxidase (HRP)].

Tissue Culture

Human dermal microvascular endothelial cells (HDMECs, ECs) were isolated from foreskin biopsies, grown on collagen-coated 2 well glass chamber slides in Endothelial Cell Basal Medium-2 (EBM-2) (Lonza, Walkersville, MD) and characterized as described previously (50). EBV-p2089 is a recombinant virus, generated by inserting EBV (B95-8) genome into a Bacterial Artificial Chromosome (BAC) and produced in a cell line (2089/

293) stably transduced with genes for hygromycin resistance and green fluorescent protein (GFP) under selection (kind gift of Dr. Henri-Jacques Delecluse, German Cancer Research Center, Heidelberg, Germany) as described previously (51, 52). All cells were cultured in the presence of penicillin/streptomycin and fetal bovine serum. For continuous culture of EBV-p2089/293, hygromycin (100 μg/ml) were added to the cultures.

Monocyte-Endothelial Cells Transfer Infection

Virus preparations: preparations were made from 293 cells carrying recombinant B95.8 EBV genomes (EBV/p2089), as previously described (51–53). Briefly, 293 cells carrying EBV/p2089 were transfected with wt-Zta plasmid (pCMV-Zta) (kind gift of Dr. George Miller, Yale University) to stimulate virus production (54). After 6 days post transfection, supernatants were collected and EBV-p2089 concentration quantitated by qPCR. EBV genome was also assayed by immunofluorescence as reported previously (51–53, 55).

Monocyte EBV/p2089 binding assay: freshly isolated monocytes from healthy donors (10^6 cells/mL) were irradiated with high dose of UV (2500 μjoule/cm^2). Subsequently, 10^5 UV irradiated monocytes were re-suspended in virus preparations (500000 virus DNA copies/mL in RPMI medium 1640) (EBV/p2089 loaded monocytes) or in medium without virus preparations (mock loaded monocytes) for 4 hours (hrs) at 4°C. Cell viability was evaluated in EBV/p2089 loaded monocytes and mock loaded monocytes at baseline and 4-48 hrs post UV-irradiation treatment by trypan blue dye exclusion procedure. EBV/p2089 binding to monocyte surfaces was quantitated by qPCR amplifying within the BALF5 gene.

Transfer infection: 10^5 virus loaded monocytes or mock loaded monocytes were added to 0.5/mL wells that had been seeded 48 hrs earlier with 3 x 10^5 endothelial cells. Each transfer infection or mock infection has been made in triplicates and each experiment was repeated five times with different monocyte healthy donors and endothelial cells lines. After co-culture for up to 24 hrs, supernatants were removed from endothelial cells cultures by washing and replaced with fresh medium for 24 hrs. Transfer infection was assayed at 24 and 48 hrs after the initiation of co-culture by counting the percentage of GFP-positive cells in the cultures. After co-culture for up to 48 hrs post infection (PI), cells were harvested and processed for DNA, RNA and immunostaining analysis. Viral genome in endothelial cells was quantitated by qPCR with a BALF5 gene probe. EBV/p2089-GFP in endothelial cell cultures was detected using a FluoView FV10i confocal microscope system (Olympus, Center Valley, PA) at 488 (green) and 405 nm (blue). Original magnification 60x.

Immunocytochemistry (ICC) and Immunofluorescence (IF)

For ICC analysis, cells were grown on Nunc glass 2 well chamber slides, fixed and permeabilized with ice-cold acetone/methanol (1:1) at -20°C for 3`. No heat antigen retrieval was performed. Blocking was achieved using 3% BSA. A double immunocytostaining was performed after blocking and endothelial cells stained with rabbit anti-human von-Willebrand-Factor (VWF) polyclonal abs (DakoCytomation), mouse anti-Zebra mAb as described above and mouse anti-human CD163 mAb (EDHu-1, AbD Serotec, Raleigh, NC). Appropriate Vector ImmPress Polymers (rabbit, and mouse) were used to detect primary antibodies, followed by development with either Vector AMEC [red, horseradish peroxidase (HRP)], HighDef Blue (Enzo, HRP or alkaline phosphatase), or DAB (DakoCytomation) [brown, horseradish peroxidase (HRP)].

For IF, cells were fixed with 100% acetone. ECs were stained sequentially with mouse anti-LMP1 monoclonal abs (CS.1-4, DakoCytomation), Cy3-conjugated-labeled donkey anti-mouse IgG, quenching with mouse IgG (Jackson IR, West Grove, PA), followed by counterstaining with DAPI (Vectashield, Vector Laboratories, Burlingame, CA). Original magnification as indicated.

Nailfold Videocapilloscopy (NVC)

NVC was performed with a videocapillaroscope (Pinnacle Studio Version 8) equipped with a 500 × optical probe. Based on Cutolo et al., 'SSc patterns' were described as early, active and late (56). At the same time, whole blood was collected from SSc patients and EBV DNA extracted as described above.

Laser Speckle Contrast Analysis (LASCA)

LASCA was performed after resting the subject in a temperature-controlled room at 24 ± 1°C for 20 min. According to previous studies, peripheral blood perfusion (PBP) of hand dorsum was measured by LASCA (Pericam, Perimed, Sweden). The scanner was placed perpendicularly 15 cm away from the hands according to the manufacturer's instructions. Two-dimensional images (measurement area 12 × 12 cm) were acquired at the highest time and spatial resolution. PBP was expressed by arbitrary perfusion units (pU). All values are calculated as mean of both hands (57, 58).

Statistical Analysis

For qPCR EBV DNA quantification and mRNA expression results data are expressed as the mean ± SEM. Statistical comparisons between groups were tested by two-tailed *t* test. Significance was taken at $P \leq 0.05$. For the NVC and LASCA the results are expressed as median and interquartile range (IQR). SPSS version 25.0 software was used for statistical analysis. The tShapiro–Wilk test was used to evaluate normal distribution of data. Group comparisons were made by Mann-Whitney test. Spearman's rank correlation coefficient was used to test for associations between numerical variables. The chi-square test or Fisher's exact test, as appropriate, were used to compare categorical variables. P-values < 0.05 were considered significant.

RESULTS

EBV DNA Loads Is Increased in Multiple Blood Components of Patients With Scleroderma

Evidence of high EBV viral loads have been reported in peripheral blood from patients with autoimmune diseases

(30, 34, 39). Specifically, EBV lytic genes in B cells from patients with SLE and increased viral activation have been associated with the occurrence of disease activity and flares in these patients (10, 40). Association of higher levels of cell-associated viral genomes in circulating blood cells have also been found in patients with rheumatoid arthritis (RA), where it has been shown that viral replication correlated with enhanced EBV-specific immune responses in RA, further supporting the linkage between EBV replication and exacerbation of autoimmune diseases (10, 12, 40). Supported by the finding that EBV replication occurs in SSc (35), we sought to quantify circulating EBV DNA load in SSc patients. EBV DNA load was significantly increased in 30 of 50 (60%) SSc patients, while it was detectable in 11 of 45 (24%) healthy donor (HD) EBV carriers in whole blood (WB). Mean EBV DNA copies/mL were 2973.3 for SSc vs 154 in HDs 1mL of WB (**Figure 1A**). We also found significantly high levels of EBV loads in plasma from 9 SSc patients (50%), while EBV was undetected in plasma from all HD EBV carriers (**Figure 1B**). Interestingly, EBV loads in WB and those in plasma correlate with each other to some extent, though viral DNA was detectable in WB, but not in plasma in some patients (**Figure 1C**).

Given that EBV DNA can exist in different forms, such as cell-free EBV-DNA in plasma or cell-associated EBV in WB, we next investigated whether monocytes and/or B cells might also carry the viral genome in SSc. 10 SSc patients with active diffuse cutaneous disease and 10 HD EBV carriers were selected to quantify the amount of EBV DNA load in freshly isolated B cells and monocytes. We found strikingly increased EBV DNA loads in monocytes and B cells from SSc patients, but not in monocytes from healthy EBV carriers (**Figure 1D**). We also found a low viral DNA level in B cells from HDs, likely from latent EBV infection. Given that EBV loads are increased in SLE patients, a disease with overlapping autoantibody specificities and sometimes overlapping clinical manifestations with SSc (59–61), we sought to quantify the amount of cellular-EBV in B cells and monocytes from SLE patients. We found that EBV

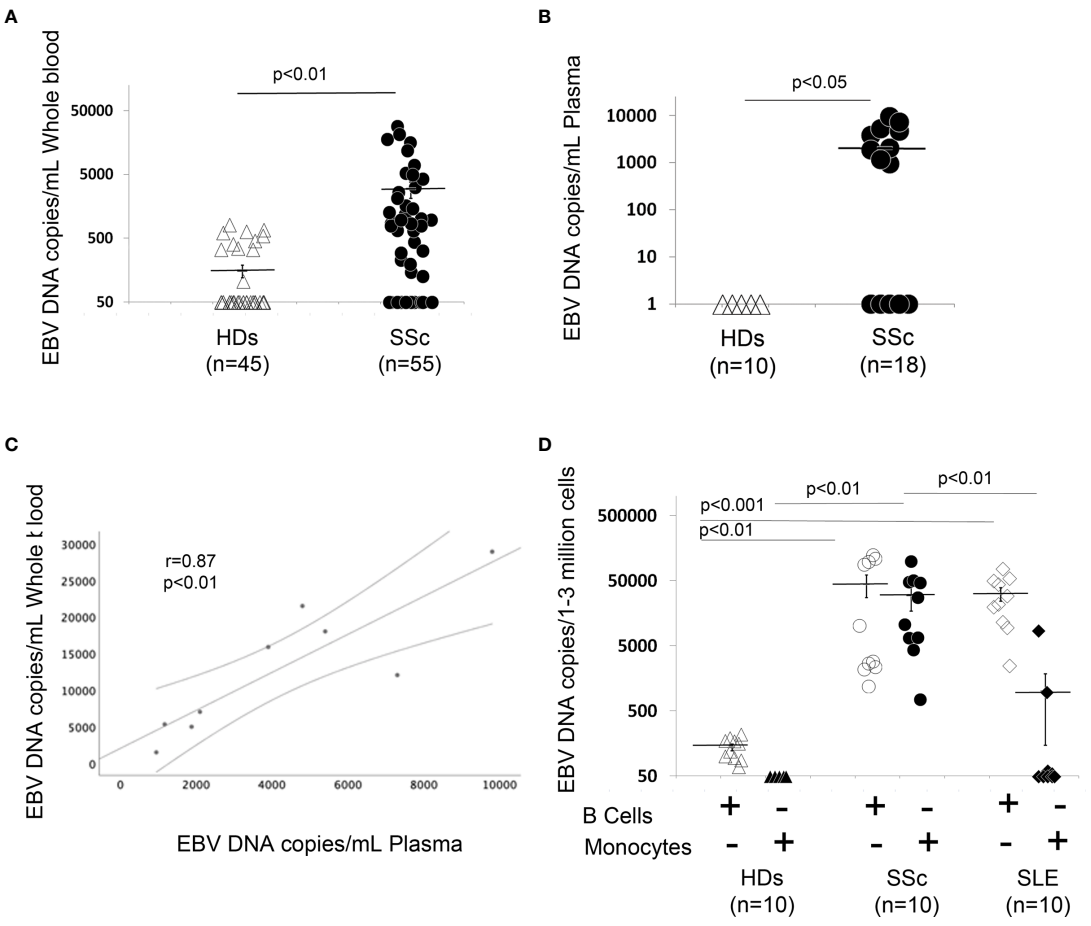

FIGURE 1 | EBV DNA load is increased in blood, plasma, B cells and monocytes from patients with SSc. **(A, B)** EBV DNA was extracted from whole blood **(A)** or plasma **(B)**. **(C)** SSc patients with high copies of EBV DNA in blood show high copies of EBV in plasma (Spearman r=0.81, p<0.01). **(D)** EBV DNA was extracted from B cells and monocytes. EBV DNA was quantified by qPCR. EBV quantitative curve was generated using ABI's Viral load control (Advanced Biotechnologies, Columbia, MD, USA). qPCR analysis was performed using primers and probe designated to amplify the EBV polymerase BALF5, and measured using SYBR Green chemistry. Shown here are copies of viral DNA calculated by standard curve. All the samples were tested in triplicates. The average of copies number is represented by horizontal line ± SE. p-values calculated using Student T-Test 2 tails.

DNA load is significantly increased in SLE B cells compared to the HDs, while a slightly increased EBV DNA load was detected in monocytes from 3 (30%) SLE patients (**Figure 1D**). These results suggest that monocytes might be a specific target of EBV replication in SSc, but not in patients with SLE.

ZTA/EBV Lytic Antigen Is Expressed in the Vessels of SSc Skin

Our previous study showed that EBV RNAs, mostly represented by EBERs are also present in the endothelial cells in the skin of SSc patients (41). To further explore whether lytic EBV antigens are present in dermal ECs, the abundance of EBV-encoded immediate early lytic transcription factor ZTA (also called Zebra or BZLF1) was evaluated in the vessels from SSc and HDs skin biopsies. Dermal vessels were identified using the CD31 marker, a specific antigen for ECs. Immunohistochemical staining of SSc skin showed that ZTA/EBV+ cells co-localized with CD31+ cells in two of ten SSc skin biopsies (20%), while it was undetected in CD31+ endothelial cells from eight SSc and ten HD skin samples (**Figures 2A–C**). Interestingly, ZTA staining was mostly detected in damaged or apoptotic cells of the vessels that appeared to be destroyed (**Figure 2A**, lower images), suggesting that EBV lytic-infection damages nuclei in the infected cells (62). Zta positive cells and negative for CD31 antigen were also detected in eight of ten SSc skin samples (**Figure 2B**), indicating that non-endothelial cells, possibly fibroblasts and monocytes, were infected with EBV, as we reported previously (35, 41).

EBV Infects Human Dermal Microvascular Endothelial Cells *In Vitro*

As ECs are negative for the EBV CD21/CR2 receptor employed by EBV to infect B cells and T-cells (63–65), we used monocytes bound to recombinant EBV/p2089 (EBV/p2089 loaded monocytes) as the vehicle to infect human dermal primary microvascular endothelial cells (HDMECs) (EBV-monocyte transfer-infection) (55, 66, 67), as previously shown in fibroblasts (41). EBV/p2089 is a recombinant virus equipped with the full spectrum of viral infection programs that allows efficient infection of various primary human cells and a green fluorescence protein (GFP) marker (51, 52, 67). Based on the EBV/p2089 encoded GFP marker, this system provides an efficient way to track recombinant EBV infection and EBV genome dependent gene expression in infected cells. Given that monocytes might persist longer in most cellular cultures, potentially contaminating endothelial cell purity, lethally irradiated cells have been used as vehicle to infect ECs with EBV (66), Therefore, we pretreated monocytes with UV irradiation before exposing them to EBV/p2089 preparations. Viability of EBV/p2089 loaded monocytes and mock loaded monocytes was less than 5% in both condition (**Figures 3A, B**), suggesting that almost all monocytes die in cultures 48 hrs post UV irradiation treatment. Monocytes not exposed to UV treatment survived 7 days in cultures (data not shown). After exposing irradiated monocytes to EBV/p2089, we next asked whether virus binding to monocytes reaches quantifiable levels in irradiated cells. Viral genome load was highly increased in virus loaded monocytes (**Figure 4A**), suggesting that EBV/p2089 is

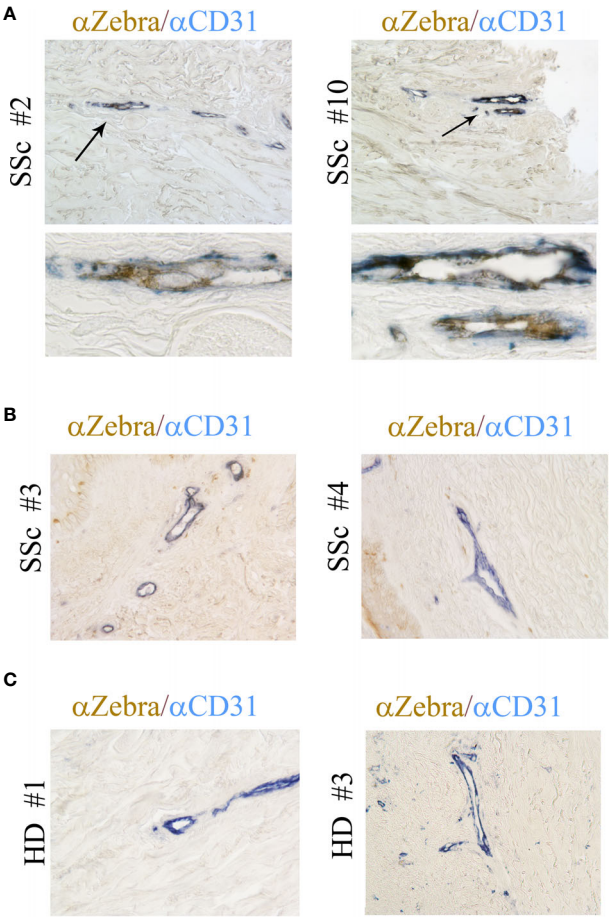

FIGURE 2 | ZTA/Zebra-EBV lytic antigen is expressed in the vessels from SSc. **(A–C)** Dual Immunostaining for ZTA/EBV (brown) and CD31 endothelial cell antigen (blue) in skin sections from four representatives SSc patients and two representative healthy donors. **(A)** Arrows indicate magnification of double positive staining in SSc biopsies. Upper image original magnification 100x; lower image: magnification 1000x. **(B, C)** Original magnification 100x.

capable of efficiently binding to irradiated monocytes. We then posed the question whether virus loaded monocytes, even though dying, remain capable of mediating virus transfer to endothelial cells. Significantly increased load of EBV/p2089 was detected in infected endothelial cells (**Figure 4B**) and EBV/p2089-GFP-fluorescent signal was localized in infected cells (**Figure 5**). An estimated 40% ECs showed perinuclear and cytoplasmic/cytoskeletal GFP-fluorescence at 48 hours post infection (PI) (**Figure 5A**). Overall, these results suggest that efficient rates of infection can be achieved by using virus loaded irradiated-monocytes in mediating transfer infection to endothelial cells.

To further characterize the EBV infection program in endothelial cells, we measured expression of latency, immediate early, early, and late lytic-genes were tested. We found expression of ZTA in a population of EBV/p2089 infected cells positive for Von Willebrand Factor (VWF), an endothelial cell antigen, while EBV/late lytic gene BLLF1 which encodes gp350 was not detected (**Figure 5B** and **Figure 6A**).

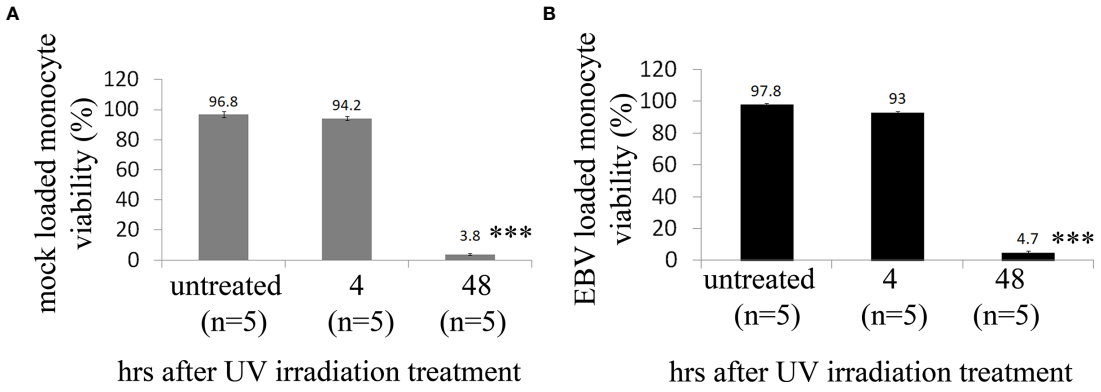

FIGURE 3 | Cell viability of human monocytes after exposure of UV irradiation treatment. **(A, B)** Freshly isolated human monocytes from healthy donors (HDs) were irradiated with high dose of UV and then re-suspended in virus preparations (EBV/p2089 loaded monocytes) or in medium without virus preparations (mock loaded monocytes). Cell viability was evaluated by trypan blue dye exclusion procedure. Bars represent mean ± S.E.M. from five different HDs. p-values ($p<0.0001$) calculated using two-tailed T-test. ***$p<0.0001$.

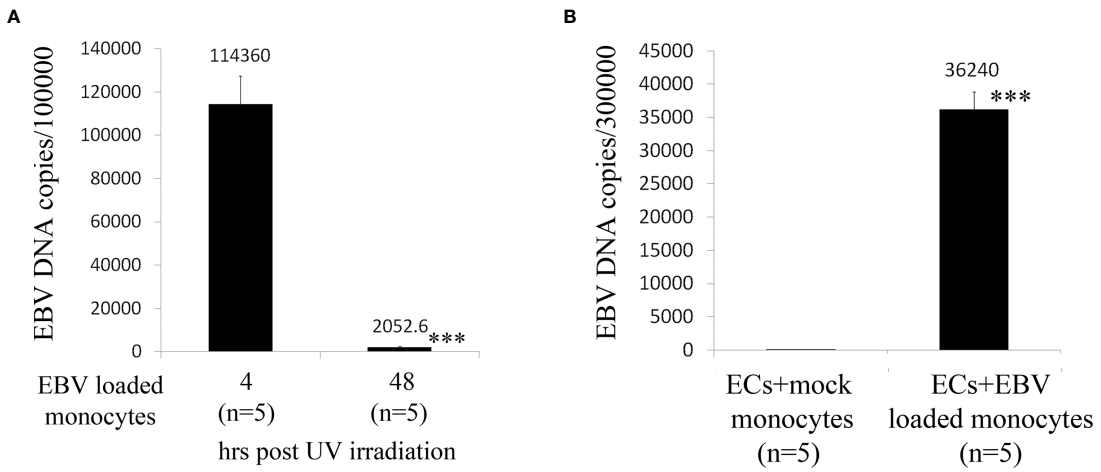

FIGURE 4 | Assay for virus genomic copies in monocytes and endothelial cells. **(A)** Irradiated monocytes were exposed to EBV preparations at known concentration (EBV/p2089 loaded monocytes) or re-suspended in medium without virus preparation (mock loaded monocytes). **(B)** EBV/p2089 loaded monocytes and mock loaded monocytes were co-cultured with human dermal microvascular endothelial cells (HDMECs) grown on collagen-coated chamber slides. After co-culture for up to 48 hrs post infection (PI), cells were harvested and processed for DNA assay. **(A, B)** Virus DNA copies per exposed cells were quantified by qPCR amplifying within the BALF5 gene and measured using SYBR Green chemistry. Shown here are copies of viral DNA calculated by standard curve. All the samples were tested in triplicates. The average of copies number is represented by horizontal line ± SE. p-values calculated using Student T-Test 2 tails. ***$p<0.0001$.

These results suggest that EBV replication was abortive in ECs. Expression of EBV Latent Membrane Protein 1 (LMP1) was also detected in a distinct population of ECs, indicating that EBV may establish latent infection in a subset of ECs (**Figure 6B**), though LMP1 can also be expressed as a lytic antigen (68, 69).

To further evaluate the purity of the EC population after EBV/p2089-monocyte transfer, monocyte markers were evaluated by qPCR, using mRNA extracted from EC cultures. CD14 and CD163 mRNA expression were undetectable in mock infected and monocyte transfer-infected-endothelial cells (data not shown). Accordingly, we did not detect CD163 monocyte surface marker expression in the endothelial cell cultures, further confirming the absence of monocytes in these cultures (**Figure 6C**). Cell free EBV/p2089-virus failed to infect endothelial cell lines.

EBV Induces TLR9 Innate Immune Responses and Markers of Vascular Injury in Infected ECs

While evidence supports activation of the innate immune response in mediating inflammation in SSc (2, 70, 71), the mechanisms by which the immune deregulation can affect the endothelium in SSc is still unclear. Given the prominent contribution of EBV lytic infection in inducing activation of the innate immune response in infected monocytes and fibroblasts

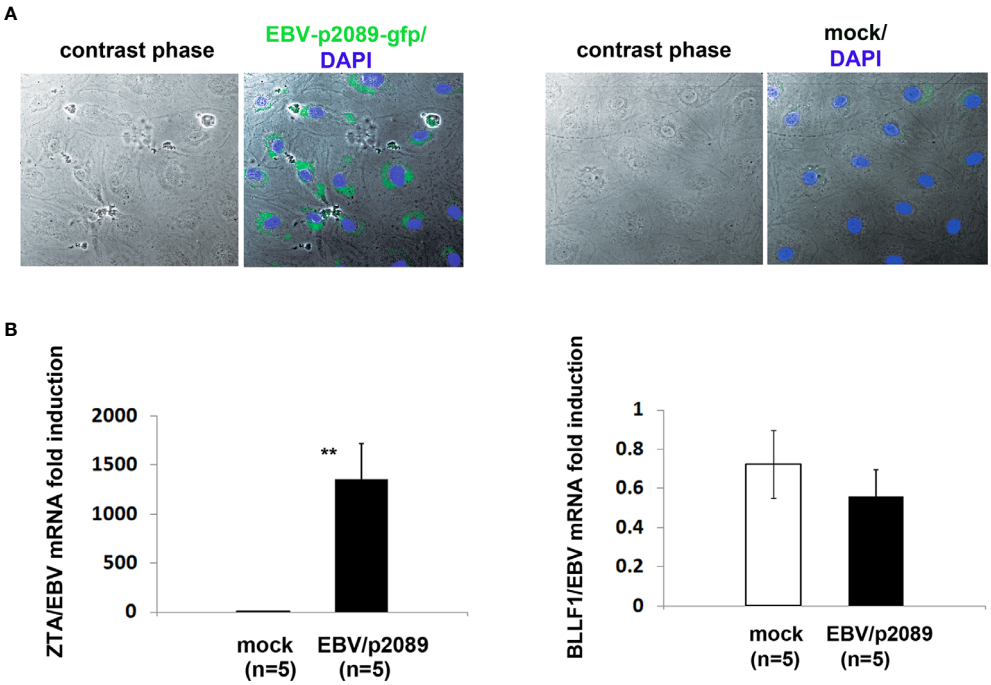

FIGURE 5 | EBV/p2089 infection of human dermal microvascular endothelial cells *in vitro*. Monocytes from healthy donors previously exposed to EBV-p2089 were co-cultured with human dermal microvascular endothelial cells (HDMECs) grown on collagen-coated chamber slides. In parallel, mock infected cultures were established by co-culturing HDMECs with monocytes not exposed to EBV-p2089. After 48h monocytes and EBV-p2089 free virus were removed from endothelial cells. **(A)** One representative of five experiments. Immunofluorescence staining of HDMECs infected with EBV-p2089. Cells were fixed with 100% acetone and mounted using Vectashield mounting medium with DAPI. EBV-p2089-GFP in endothelial cell cultures was detected using a FluoView FV10i confocal microscope system at 488 (green) and 405 nm (blue). Original magnification 600x. **(B)** Expression of EBV lytic genes by qPCR in EBV-p2089 infected endothelial cells. Data are expressed as the fold-change normalized to mRNA expression in a single sample of mock infection. Bars represent mean ± S.E.M. **p<0.001.

(35, 41), we sought to evaluate whether EBV might also induce a similar innate immune response in EBV/p2089 infected endothelial cells. Based on the previous reports describing pro-inflammatory genes, IFNα and markers of vascular inflammation such as ET-1 mRNA (EDN1) as increased in SSc vessels and skin (46, 72), we choose to assess the expression of these genes in endothelial cells infected with EBV/p2089- compared to mock infected cells. Expression of TLR9 mRNA was significantly induced in EBV/p2089-infected-ECs (**Figure 7A**), as were mRNAs encoding IRF7, IRF5 and selected Interferon-stimulated-genes (ISGs), such as MX1 and CXCL10 (**Figures 7B–E**). TLR3, TLR4 and TLR7 mRNA expression was not detected in EBV-p2089 infected cells (data not shown), suggesting that EBV-induced innate immune response is mediated by TLR9 in infected ECs. Interestingly, genes which were previously identified as markers of vascular dysfunction in SSc, such as EDN1, thrombospondin 1 (THBS1), and heparan sulfate proteoglycan 2 (HSPG2) (46, 72, 73) were also induced by EBV in infected endothelial cells (**Figures 7F–H**). No increase of TLRs, IRFs or ISGs was observed in mock infected endothelial cell cultures (**Figures 7A–H**). Altogether, these results suggest that EBV induces activation of the TLR9 innate immune response in infected endothelial cells possibly contributing to endothelial cell dysfunctional activation and injury in SSc.

Increased EBV DNA Load Correlates With Clinical Patterns of Vascular Injury in SSc Patients

The presence of vascular injury occurs in many tissues, including the skin, in the early phase of SSc (4). Vasospasm, characterized by Raynaud`s phenomenon, and a marked decrease in the number of capillaries in clinically involved and uninvolved skin has been reported, suggesting that endothelial cell death and defective angiogenesis might be responsible for the EC loss and vessel rarefaction in SSc (74). These pathological consequences lead to the presence of ischemic digital ulcers (DUs) with a marked decreased perfusion in the affected organs and the skin of SSc patients (75). Given that EBV induced an IFN response in infected ECs, and IFNs promote EC damage and loss (76), we next interrogated whether SSc patients with elevated peripheral blood EBV loads might present clinical signs of vascular injury. Clinical examination of the number of DUs was analyzed in 41 SSc patients. Remarkably, SSc patients with new active DUs showed significantly higher levels of EBV loads than patients without DUs (**Figure 8A**). Moreover, SSc patients with a past history of digital ulcers show increased levels of viral loads compared to SSc patients with no history of DUs and low or undetectable level of EBV loads (**Figure 8B**). Given that specific capillary abnormalities occur early in the disease and can be

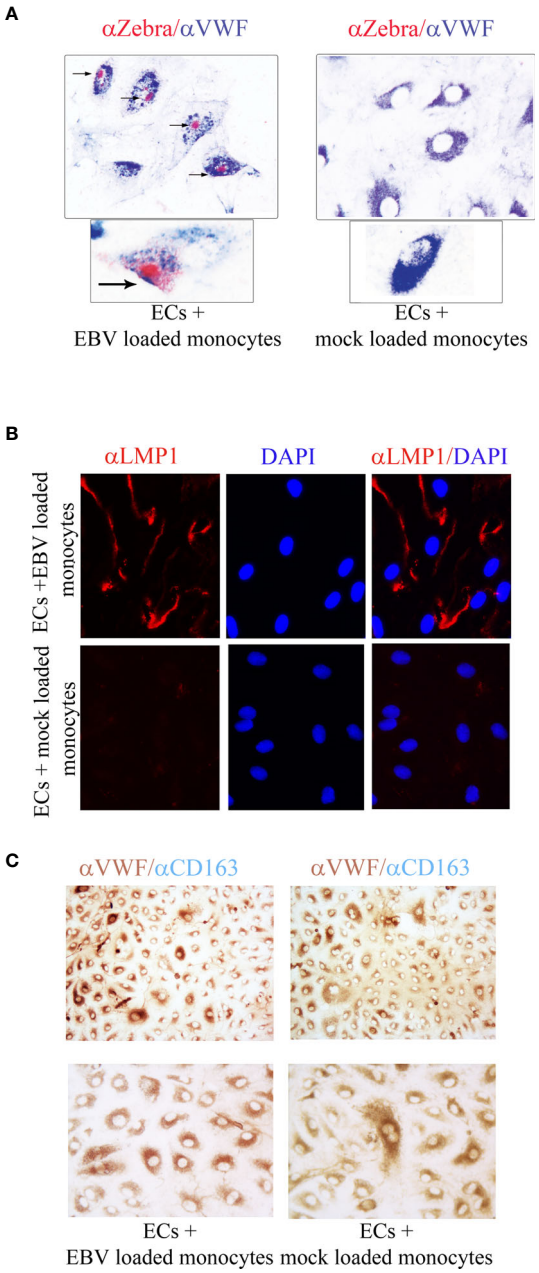

FIGURE 6 | ZTA/EBV lytic and LMP1/EBV antigens are expressed in human dermal microvascular endothelial cells infected with EBVp2089. EBV/p2089 loaded monocytes were co-cultured with endothelial cells. After 48 hrs monocytes and free cell EBV-p2089 were removed. **(A)** Dual immunocytochemistry (ICC) staining showing Von Willebrand Factor (VWF) antigen in the cytoplasm and Zta protein in the nuclei of a subset of infected endothelial cells. Original magnification 400x, box area: original magnification 1000x. **(B)** Immunofluorescence of endothelial cells stained with LMP1/EBV latent antigen (red) as indicated. Diaminidino-2-phenylindole (DAPI) was used as counterstaining for the nuclei. Original magnification 400x. **(C)** Dual ICC staining showing positive cells for Von Willebrand Factor (VWF) antigen in the cytoplasm (brown) and negative for CD163 mononcyte surface antigen (blue). Upper image Original magnification 100x, lower image original magnification 400x. Representative of 5 experiments.

detected by nailfold videocapillaroscopy (NVC) (77, 78), 41 SSc patients with diffuse cutaneous disease were analyzed to identify distinct scleroderma-specific patterns. NVC showed that SSc patients with active and late patterns have significantly increased EBV DNA loads, while patients with the early capillaroscopic pattern show a low or undetectable levels of EBV loads in the peripheral blood (**Figure 8C**). Further confirming association of EBV infection with clinical signs of SSc vasculopathy, significant reduction in skin perfusion has been found in patients with higher level of EBV loads compared to SSc patients with undetectable level of viral loads (**Figures 9A, B**). Altogether, these results suggest that lytic EBV antigens with increased virus production associates with clinical signs of vascular injury and altered perfusion in the context of SSc.

DISCUSSION

Several mechanisms have been described to explain how EBV triggers autoimmune disease, such as antigen cross-reactivity with self-nuclei protein and/or bystander activation of autoreactive cells (34, 39, 79–82). In this study, we report a novel feature employed by EBV that triggers the TLR9 antiviral response and markers of vascular injury in infected endothelial cells. We also demonstrate for the first time that human monocytes bound to EBV recombinant virus are capable to transfer EBV to the endothelial cells, suggesting that circulating EBV infected monocytes, directly and indirectly might contribute to the vascular injury in SSc. While EBV has not previously been associated with monocyte infection, it is notable that the gamma-herpesvirus MHV 68 infects dendritic cells and macrophages (83).

Evidence of EBV infection in ECs has been reported (66, 84). It has been shown that EBV could infect vascular ECs both in human tissues and in cultures. EBER-positive cells have been reported in the ECs from patients with systemic granulomatous arteritis, an autoimmune disease characterized by vasculitis of the large vessels (85). Additional evidence of EBV infecting ECs were reported in primary human-umbilical cord-derived ECs (HUVECs) exposed to EBV-immortalized lymphoblastoid cell lines (LCL). It has been found that ECs, when infected with EBV, express genes related to EBV latency programs, such as EBNA1 and produced high level of IL-6 (66). Further confirmation of EC infectability by EBV comes from a study of multiple sclerosis (MS), another autoimmune disease where EBV has been implicated in the pathogenesis (84). Specifically, it has been shown that EBV infects human brain microvascular ECs (HBMECs) leading to EC activation and increased production of CCL-5 (RANTES) and the adhesion molecule, ICAM-1 in infected cells, suggesting that infected microvascular cells release inflammatory cytokines upon EBV infection (84). Here we report a novel system that successfully infects human dermal microvascular endothelial cells (HDMECs) *in vitro*, providing evidence that EBV is able to infect ECs using monocytes as a vehicle for infection (86, 87). It is likely that EBV uses alternative strategies to infect ECs that bypass the absence of CD21, similar to the described transmission of EBV to human epithelial cells and fibroblasts (41, 52, 88).

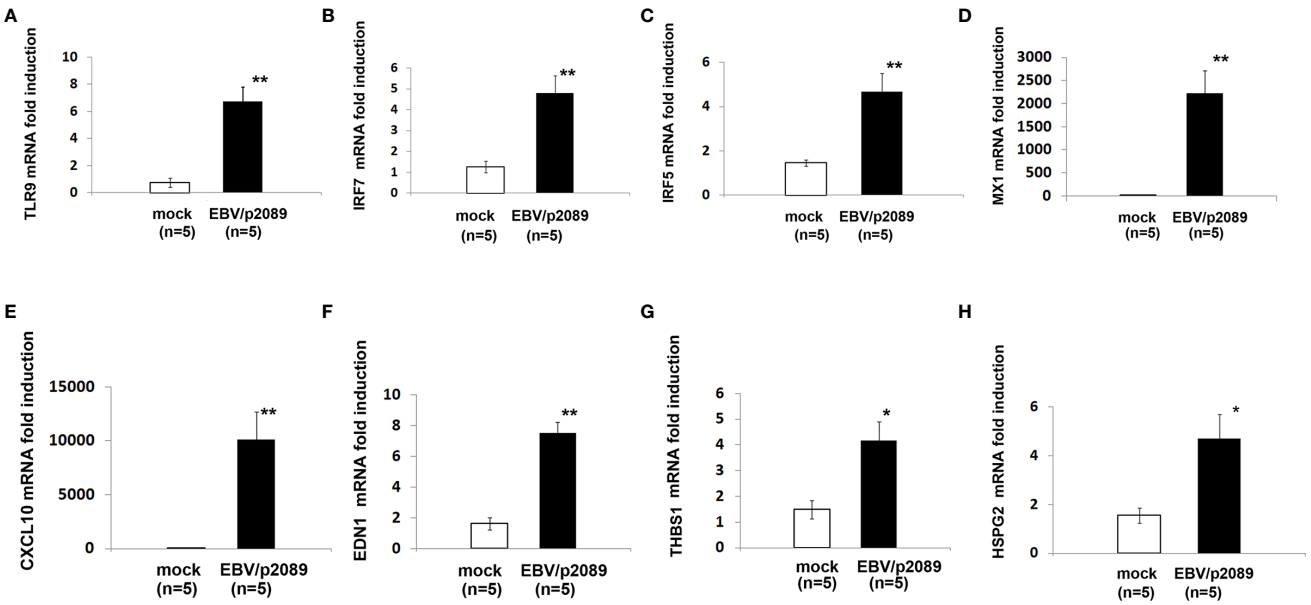

FIGURE 7 | EBV activates innate antiviral response and markers of vascular injury in infected endothelial cells. Monocytes from healthy donors (HDs) bound to EBV-p2089 were co-cultured with human dermal microvascular endothelial cells (HDMECs). Monocytes not exposed to EBV-p2089 were co-cultured with HDMECs as mock-infection. After 42h monocytes EBV-p2089 cell-free virus were removed from endothelial cell cultures and total RNA extracted after. **(A–H)** mRNA expression of indicated genes in EBVp2089-infected and mock-infected endothelial cells, evaluated by qPCR. Fold-changes shown on the graph are normalized to mRNA expression by one mock infected cell line. Bars represent mean ± S.E.M. from 5 separate endothelial cell lines. p-values calculated using two-tailed T-test. *p<0.05, **p<0.001.

An interesting aspect of our study is that we found expression of EBV-BZLF1/ZTA immediate early lytic- (**Figure 5B** and **Figure 6A**) and early lytic BFRF1 genes (data not shown) in infected cells, while EBV/late lytic genes were not detected, suggesting that EBV replication is incomplete, which implies that some but not all lytic genes are expressed in infected cells (89). Although we were not able to determine at what stage the viral cycle becomes abortive, it is possible that viral replication is interrupted at the early stage of the lytic infection, as it has seen in fibroblasts infected with EBV (41). Whether lytic DNA replication, which is driven by EBV early genes and is required for late gene expression, remains to be investigated.

Activation of the TLR9 signaling has been implicated in the pathogenesis of several autoimmune diseases including SSc (90–93). Elevation of TLR9 expression and increased TLR9 signature has been found in the skin of SSc patients compared to control skin (90). Furthermore, recent studies showed the importance of TLR9 activation pathway in inducing pro-fibrotic profile responses, involving autocrine TGF-β production, in human normal dermal fibroblasts stimulated with the TLR9 ligand CpG (90). Here, in our study, we show that EBV up-regulates TLR9 mRNA and TLR9 innate immune mediators in infected ECs, suggesting that EBV DNA is implicated in the activation of the TLR9 pathway and that viral DNA might be recognized by TLR9 in ECs. Given that the linear form of unmethylated EBV ds-DNA, which is produced by EBV lytic replication can be detected by TLR9, while the methylated EBV DNA, which is abundant in particular latency states remains invisible to TLR9 (94–97), it is possible that activation of TLR9 is induced by un-methylated EBV ds-DNA in infected ECs and that viral nucleic acids might represent the TLR9 ligand in SSc dermal endothelial cells.

In agreement with the previous finding that interferon alpha is up-regulated in SSc ECs, we found that EBV mediates TLR9 inflammatory response by inducing expression of the IRF innate immune mediators and IFN inducible genes MX1 and CXCL10. Since type I IFNs are potent antiangiogenic cytokines known to promote endothelial death and inhibit endothelial migration (76, 98–101), it is conceivable that EBV infection through the activation of the TLR9 innate immune inflammation and type I IFN contributes to the endothelial cell loss and vasculopathy in SSc. Moreover, given that more than one EBV lytic gene can destroy nuclear membranes during lytic infection (62, 102), it is also possible that expression of EBV early lytic genes in infected cells may directly cause EC apoptosis.

We also found that genes such as endothelin 1, thrombospondin 1 and heparan sulfate proteoglycan 2 (HSPG2), which are not known to be activated by TLR9 inflammatory response, are induced by EBV in infected endothelial cells. One explanation could be that TLR9 activates distinct gene profiles depending on the infected cell type, in this case the endothelial cells, or pathways different from TLR9 might be activated by EBV in infected endothelial cells. Given that these genes are of particular interest as they have been associated with vascular activation and dysfunction as well as fibrosis in SSc and several other diseases, further studies might be required to clarify this important aspect.

In this study we report for the first time that EBV DNA load is highly increased in SSc blood and plasma. Although the origin of

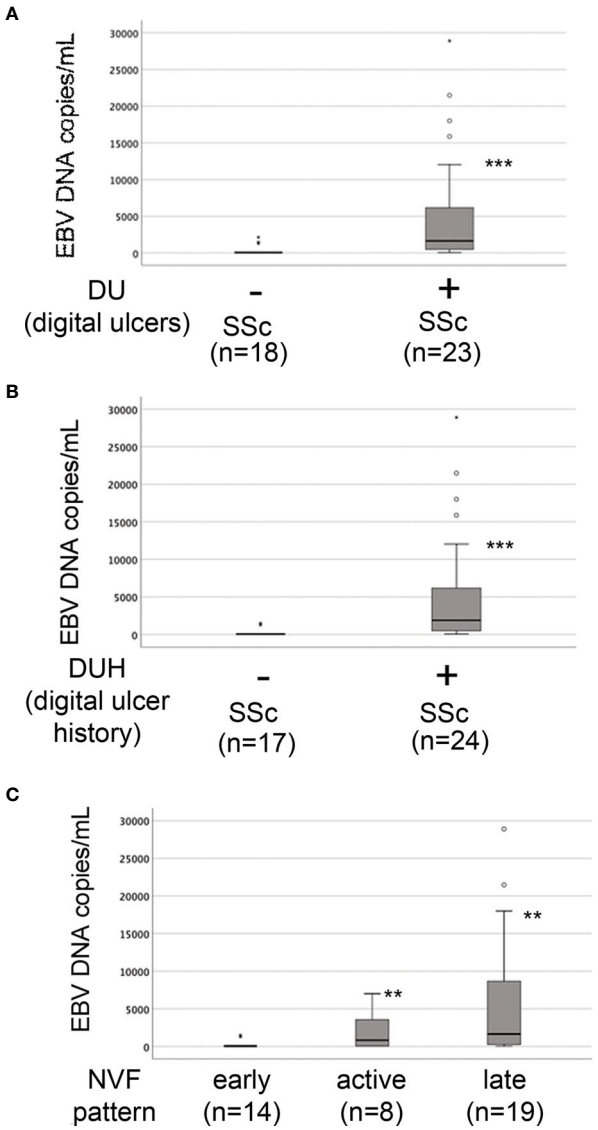

FIGURE 8 | Increased EBV loads correlate with an increased number of digital ulcers and patterns of microvascular damage in SSc. EBV DNA was extracted from whole blood and quantified by qPCR. EBV quantitative curve was generated using ABI's Viral load control. qPCR analysis was performed using primers and probe designated to amplified the EBV polymerase BALF5, and measured using SYBR Green chemistry. Shown here are copies of viral DNA calculated by standard curve. **(A, B)** SSc patients with active digital ulcers and past history of digital ulcers showing higher level of EBV DNA loads compared to SSc patients with no or fewer digital ulcers showing low or undetectable level of EBV DNA loads. **(C)** SSc patients with increased level of EBV DNA loads show active and late patterns detected by nailfold videocapillaroscopic compared to patients with low or undetectable level of viral loads in the blood. Mann-Whitney test was used for statistical analysis. **p<0.001, ***p<0.0001.

EBV DNA in the circulation is not clear, it is possible that it may be derived from apoptotic cells as detected at early stage in post-transplant lymphoproliferative disease (PTLD), in patients with nasopharyngeal carcinoma and Hodgkin's disease (103). It is generally accepted that cells harboring EBV DNA are likely to be B cells (104), but occasionally T cells, natural killer cells, monocytes, and immature dendritic cells can be infected as well (105–107). Consistent with this observation, we report that EBV DNA load is largely located in B cells and circulating monocytes from patients with SSc. The finding that SSc monocytes carry EBV DNA loads is in agreement with our previous observation that circulating monocytes express EBV immediate early, early and late lytic genes (35), suggesting that SSc monocytes might be capable to produce and release virions in SSc.

Although the mechanism responsible for the increase of the EBV DNA load in autoimmune diseases and SSc remains to be established, it is possible that impaired immune function could lead to an increase in EBV DNA replication in SSc patients, rendering these patients long-term viral carriers. Given the numerous HLA polymorphisms in the HLA-class-I/II-genes strongly associating with the risk of developing autoimmune diseases including SSc (2, 59, 108), it is also possible that SSc genetic susceptibility associated with the genetic variability in EBV strain might predispose SSc patients to an uncontrolled, persistent active EBV lytic infection (89, 109–113). An important aspect of this study is that SSc monocytes carry high levels of EBV DNA while monocytes from SLE patients do not. One explanation could be that the presence of pro-fibrotic phenotype that underlies SSc monocytes might facilitate EBV reactivation and/or viral persistence in SSc patients. Given that TGFβ is important to induce SSc pro-fibrotic phenotype and TGFβ is also important for EBV replication, since it induces Zta and could potentially reactivate EBV *in vivo* (36, 114, 115), it possible that pro-fibrotic phenotype promotes EBV reactivation in SSc monocytes.

Our data show that EBV DNA loads are increased in the blood from SSc, with a frequency of 60% in SSc patients. Notably, almost all, with the exception of 4, of the SSc patients enrolled in the study, were not in a state of immunosuppression therapy, indicating that the increase in EBV DNA replication occurs spontaneously and independently of immunosuppressant therapy in SSc.

Our findings that SSc with high EBV DNA copies are associated with severe clinical signs of vascular injury, while SSc patients with lower or undetectable levels of EBV DNA show milder or no signs of vessel damage, suggest that EBV loads alone might represent a useful tool for monitoring vascular damage in SSc. As the few patients that showed lower or undetectable level of circulating EBV DNA loads did not have signs of active vascular injury, it is possible that those patients with lower viral DNA show less risk to develop vascular diseases. As an alternative explanation, it could be that variability in EBV DNA loads might reflect different organ involvement or represent different stage of the disease in SSc. Noteworthy, we observed that four SSc patients with highly increased viral loads and severe signs of vascular injury were affected by pulmonary arterial hypertension (PAH) and SSc renal crisis (three and one SSc patients, respectively), suggesting that the vascular system of these organs might also be a target of EBV infection. Therefore,

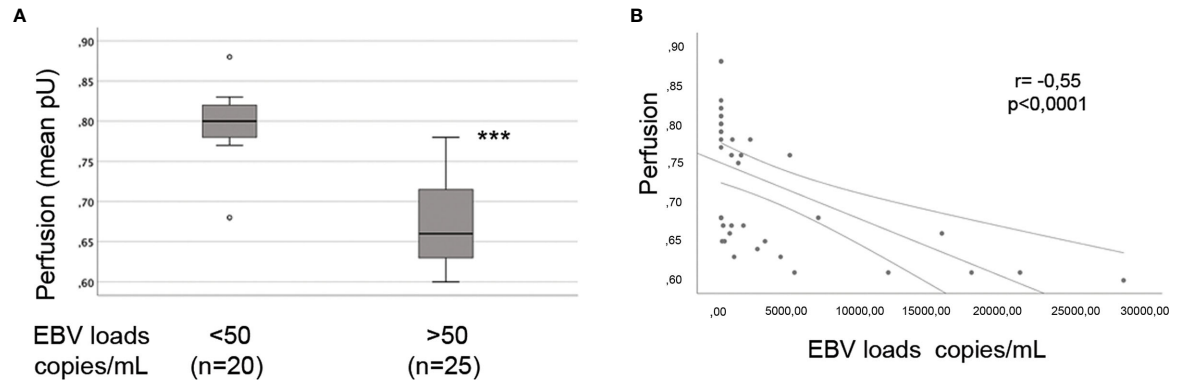

FIGURE 9 | Increased EBV loads inversely correlate with hand perfusion in SSc patients. EBV DNA was extracted from whole blood and quantified by qPCR. EBV quantitative curve was generated using ABI's Viral load control. qPCR analysis was performed using primers and probe designated to amplified the EBV polymerase BALF5, and measured using SYBR Green chemistry. Shown here are copies of viral DNA calculated by standard curve. **(A)** SSc patients with significantly higher level of EBV DNA loads show reduction in the hand perfusion compared to SSc patients with low or undetectable level of EBV DNA loads. Mann-Whitney test was used for statistical analysis **(B)** Inverse correlation between increase of EBV DNA copies and reduction in blood perfusion in SSc patients (Spearman r=0.55, p<0.0001). ***p<0.0001.

monitoring for dynamic changes in EBV loads might be important in identifying those at risk for developing vascular disease and provides more relevant information for adapting therapy. Further studies will be required to evaluate this important aspect.

A limitation of the current study is that it was not possible to correlate viral load in monocytes with clinical signs of vascular injury. For this retrospective study, much clinical data was not available for the SSc patients with increased EBV viral loads in monocytes. Future studies involving evaluation of EBV load in monocytes from SSc patients, with and without vascular damage, may provide further evidence of the role of EBV as a crucial co-factor for the development of SSc vasculopathy.

In summary, microvascular injury occurs in the early stage of SSc, and widespread change of the microvasculature is a cardinal feature of SSc. Thus, understanding the endothelial cell injury induced by lytic EBV has the potential to address the concept that an active viral infection drives endothelial cells dysfunction and vessel injury in SSc. In addition, our data point to up-regulation of EBV DNA loads as a potential biomarker for developing vascular injury in SSc. Our results provide the framework to support the development and testing of antiviral therapeutic interventions in SSc treatment paradigms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Boston University IRB committee and Sapienza University ethic committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors participated in the preparation of the manuscript in a significant way. Study design: GF, AF, ER BG. Acquisition of clinical specimens: ER, MY. Experiments performed: AF, ER, GF. Analysis and interpretation data: AF, ER, BG, GF. Statistical analysis: ER, GF. Manuscript preparation: AF, ER, BG, MT, GF. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.651013/full#supplementary-material

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Immune Response to *Enterococcus gallinarum* in Lupus Patients Is Associated With a Subset of Lupus-Associated Autoantibodies

Harini Bagavant[1]*, *Antonina M. Araszkiewicz*[1], *Jessica K. Ingram*[1], *Katarzyna Cizio*[1], *Joan T. Merrill*[1], *Cristina Arriens*[1], *Joel M. Guthridge*[1], *Judith A. James*[1,2] *and Umesh S. Deshmukh*[1]

[1] Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, Oklahoma City, OK, United States, [2] Departments of Medicine and Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, United States

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Stefania Gallucci,
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***Correspondence:**
Harini Bagavant
harini-bagavant@omrf.org

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Interactions between gut microbes and the immune system influence autoimmune disorders like systemic lupus erythematosus (SLE). Recently, *Enterococcus gallinarum*, a gram-positive commensal gut bacterium, was implicated as a candidate pathobiont in SLE. The present study was undertaken to evaluate the influence of *E. gallinarum* exposure on clinical parameters of SLE. Since circulating IgG antibodies to whole bacteria have been established as a surrogate marker for bacterial exposure, anti-*E. gallinarum* IgG antibodies were measured in banked serum samples from SLE patients and healthy controls in the Oklahoma Cohort for Rheumatic Diseases. The associations between anti-*E. gallinarum* antibody titers and clinical indicators of lupus were studied. Antibodies to human RNA were studied in a subset of patients. Our results show that sera from both patients and healthy controls had IgG and IgA antibodies reactive with *E. gallinarum*. The antibody titers between the two groups were not different. However, SLE patients with Ribosomal P autoantibodies had higher anti-*E. gallinarum* IgG titers compared to healthy controls. In addition to anti-Ribosomal P, higher anti-*E. gallinarum* titers were also significantly associated with the presence of anti-dsDNA and anti-Sm autoantibodies. In the subset of patients with anti-Ribosomal P and anti-dsDNA, the anti-*E. gallinarum* titers correlated significantly with antibodies to human RNA. Our data show that both healthy individuals and SLE patients were sero-reactive to *E. gallinarum.* In SLE patients, the immune response to *E. gallinarum* was associated with antibody response to a specific subset of lupus autoantigens. These findings provide additional evidence that *E. gallinarum* may be a pathobiont for SLE in susceptible individuals.

Keywords: lupus, gut microbes, ribosomal P, microbiome, bacterial antibodies, autoantibodies, anti-RNA

INTRODUCTION

Regulated interactions between the immune system and microbes at mucosal surfaces play a critical role in maintaining immune homeostasis (1, 2). Under dysbiotic conditions, disruption of these interactions can manifest as a loss of immune tolerance and the development of autoimmunity. In SLE, patients show gut microbial changes with reduced microbial diversity and alterations in fecal and serum metabolites (3). Analyses from different patient cohorts show changes in *Firmicutes*/*Baciteroides* ratios, increase in *Lactobaccillaceae*, and expansion of specific bacteria like *Ruminococcus gnavus* in the gut (4–8). In some studies, antibodies to these gut bacteria are associated with increased autoantibody titers and lupus disease activity. Further, inflammatory processes influence the local gut micro-environment and have the potential to modulate the microbial composition on the mucosal surface (9). Thus, a continual interaction between local and systemic autoimmunity, gut mucosa, and microbiota may regulate disease evolution.

In addition to the gut, the bacterial community in the oral environment can also influence SLE. Indeed, bacterial species of oral microbiota origin are observed in the gut of SLE patients (10) Commensal oral bacteria like *Capnocytophaga* have the potential of stimulating lupus-antigen reactive T cells and autoantibodies through molecular mimicry (11, 12). In SLE patients, antibodies to specific periodontal pathogens like *A. actinomycetemcomitans* and *P. gingivalis* are associated with higher disease activity (13). IgG antibody titers against a bacterial strain indicate prior or ongoing exposure to that strain (14, 15). Thus, it is plausible that dysbiosis at different mucosal surfaces and the exposure of the immune system to specific commensal and/or pathogenic bacteria contribute to inflammatory responses and exacerbation of SLE.

The influence of gut bacteria on SLE pathogenesis have been successfully investigated in mouse models and specific bacterial strains that may be relevant in human disease have been identified (6, 7, 16). However, extrapolating the findings from inbred mouse strains to a highly diverse human population, in a heterogenous disease like SLE, remains a significant challenge. Recently, *Enterococcus gallinarum*, a gram-positive commensal bacteria present in the gut of lupus-prone (NZW x BXSB) F1 mice, has emerged as a candidate pathobiont for triggering SLE (16). Mono-colonization of the gut mucosa with *E. gallinarum* modulated adhesion molecules on the mucosal epithelium and allowed the migration of bacteria through the mucosa into the liver and systemic circulation in mice. *E. gallinarum* was also isolated from fecal samples and liver biopsies from patients with autoimmune hepatic disease and lupus patients with hepatic involvement. These patients showed a strong correlation between circulating antibodies to the bacterial RNA and human RNA, suggesting a causal relationship between the hepatic entry of *E. gallinarum* and SLE. However, this exciting observation was done in a limited number of SLE patients. In addition, whether *E. gallinarum* influences the clinical features of SLE in patients was unclear. To address these issues, we measured the levels of IgG and IgA antibodies to *E. gallinarum* (anti-*Eg)* in banked serum samples from a diverse and well-characterized cohort of SLE patients. Antibody responses to *E. gallinarum* were used as a surrogate marker of exposure to this bacteria, and the association between anti-*Eg* titers and clinical indicators of SLE was studied.

MATERIALS AND METHODS

Study Design

The research was performed in accordance with the Helsinki Declaration and approved by the Oklahoma Medical Research Foundation Institutional Review Board. Banked serum samples and clinical data from SLE patients seen between May 2002 and October 2014 were obtained from the Oklahoma Rheumatic Disease Research Core Center (ORDRCC). The patients who met ≥4 of the 1997 modified American College of Rheumatology Classification Criteria for SLE (17, 18) were evaluated for disease activity and serum autoantibody profiles. The demographics of the patients (n=303) in this study are shown in **Supplementary Table 1**. Serum autoantibodies were measured using multiplex fluorescent bead-based assays. The antigens studied were dsDNA, chromatin, Ro/SSA, La/SSB, Sm, smRNP, RNP, RNP-A, RNP-68, Centromere B, Scl-70, and Ribosomal P. The antibody levels were quantified based on the fluorescence intensity for each specificity. The positive cut-off for the anti-dsDNA was set at 10 IU/mL (range 0- >300) and for all other specificities was 1.2 IU/mL (range 0- >8) per manufacturer's recommendations. Clinical assessments of SLE were performed using the hybrid SELENA- SLE Disease Activity Index (SLEDAI) (19) and the British Isles Lupus Assessment Group (BILAG-2004) Index (20). Serum samples from de-identified healthy volunteers (n=66) were studied for antibodies to *E. gallinarum*, *E. faecalis*, and human RNA.

Detection of Antibody to *Enterococci*

Enterococcus gallinarum (ATCC#BAA-748) and *Enterococcus faecalis* (ATCC#19433, Type strain) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These strains are utilized extensively as control/reference strains, and their use will allow for comparisons with studies performed by other investigators in future. The bacteria were cultured in Brain Heart Infusion broth, harvested, washed extensively with PBS, and stored as pellets in single-use aliquots at -80°C. An ELISA-based assay was used to measure antibodies to formalin-fixed whole bacteria as previously described (21). All sera from SLE patients and healthy controls were tested at a 1:500 dilution for anti-bacterial IgG and 1:100 dilution for anti-*E. gallinarum* IgA antibody titers. Serial dilutions from a pooled serum sample were included in each assay as a calibrator. A standard curve was constructed, and the titers of anti-bacterial antibody were calculated for each sample and expressed as units/mL.

Detection of Antibodies to RNA

Human RNA was purified from THP1 (ATCC#TIB-202), a human monocytic cell line, propagated in RPMI-1640 with 10% bovine calf serum. RNA was extracted from THP1 cells using the RNeasy Mini Kit (Qiagen, Germantown, MD). Genomic DNA contaminants in the human RNA were eliminated by RNase-free DNase1 digestion using manufacturer's protocols (Qiagen, Germantown, MD), followed by purification using RNeasy Mini columns (Qiagen, Germantown, MD).

Synthetic double-stranded RNA (poly I:C) HMW was purchased from Invivogen (San Diego, CA).

IgG antibodies to RNA [human RNA, and poly (I:C)] were measured using an ELISA. RNA (5μg/mL) dissolved in PBS with 1 mM EDTA was coated on DNA-BIND ELISA plates (Corning, Glendale, AZ) overnight at 4°C. After blocking, the plates were incubated with serum samples (1:100 dilution) for 2 hours. Bound antibodies were detected with HRP-conjugated goat anti-human IgG (Southern Biotechnology, Birmingham, AL) and enzyme activity determined by tetramethylbenzidine substrate (Bio-Rad Laboratories, Hercules, CA). The reaction was stopped with 2.5N sulfuric acid, and the absorbance was read at 450nm.

Statistical Analysis

Graph Pad Prism 9.0 software (GraphPad Software, San Diego, CA) was used for statistical analyses. Anti-bacterial antibody titers were $\log_{10}$ transformed. Normality tests were performed on each dataset, and non-parametric tests were used for non-Gaussian distributions. Antibody titers between two groups were compared using a t-test for normal distributions or Mann-Whitney test for non-Gaussian distributions. Antibody titers between multiple groups were compared using a one-way ANOVA test, and Sidak's multiple comparisons post-test determined adjusted p values. For non-Gaussian distributions, antibody reactivity in multiple groups was compared by the Kruskal-Wallis test, followed by Dunn's multiple comparison post-test. Correlations were determined by Pearson's method for normal distributions and Spearman's method for non-Gaussian distributions. Proportions were compared by the Chi-square test. A p-value of <0.05 was considered significant. Post-hoc power calculations were performed using https://epitools.ausvet.com.au/.

RESULTS

Higher Titers of Anti-*Eg* IgG Are Associated With Ribosomal P, dsDNA, and Sm Autoantibodies in SLE Patients

IgG antibody titers to formalin-fixed whole *E. gallinarum* bacteria were measured in sera from lupus patients (n=303) and healthy donors (n=66). Anti-*Eg* IgG were detected in all the sera tested, and the titers were not significantly different between the two groups (**Figure 1A**). The anti-*Eg* IgG titers between SLE patients based on self-reported race/ethnicities were also not different (**Supplementary Figure 1A**). Since *E. gallinarum* is associated with the gut mucosa, serum IgA antibody titers were also measured. No significant differences were seen in anti-*Eg* IgA titers between SLE patients and healthy donors or between patients in different racial/ethnic groups (**Figure 1B** and **Supplementary Figure 1B**). Anti-*Eg* IgG or anti-*Eg* IgA titers were not different between male and female patients (data not shown). No correlation was noted between age and anti-*Eg* IgG titers. However, anti-*Eg* IgA titers showed a statistically significant inverse correlation with age (Spearman r= -0.1941; p=0.0013). The anti-*Eg* IgG and IgA titers in the SLE patients showed a statistically significant, albeit modest, correlation (**Figure 1C**). The finding that anti-*Eg* IgG and IgA titers are not different suggests a comparable exposure to *E. gallinarum* in all groups.

Patients were stratified into groups based on the presence or absence of autoantibodies to different lupus-associated antigens. The anti-*Eg* titers between each autoantibody-positive and -negative group were compared (**Table 1**). As shown in **Figure 2**, higher anti-*Eg* IgG titers were associated with antibodies to Ribosomal P (p=0.0059), dsDNA (p=0.0093), and Sm (p=0.0315).

A comparison between patients positive for anti-Ribosomal P, anti-dsDNA, or anti-Sm antibodies with healthy controls showed that anti-Ribosomal P reactivity in patients was consistently associated with higher anti-*Eg* IgG titers (adjusted p value = 0.0178; **Figure 2B**). Compared to healthy controls, higher anti-*Eg* IgG was also seen in patients with anti-dsDNA or anti-Sm following pair-wise analyses (**Supplementary Table 2**).

Statistical significance was not reached in comparisons of anti-*Eg* IgG titers between the other autoantibody-positive and -negative groups (**Supplementary Figure 2**) or between autoantibody positive patients and healthy controls (data not shown).

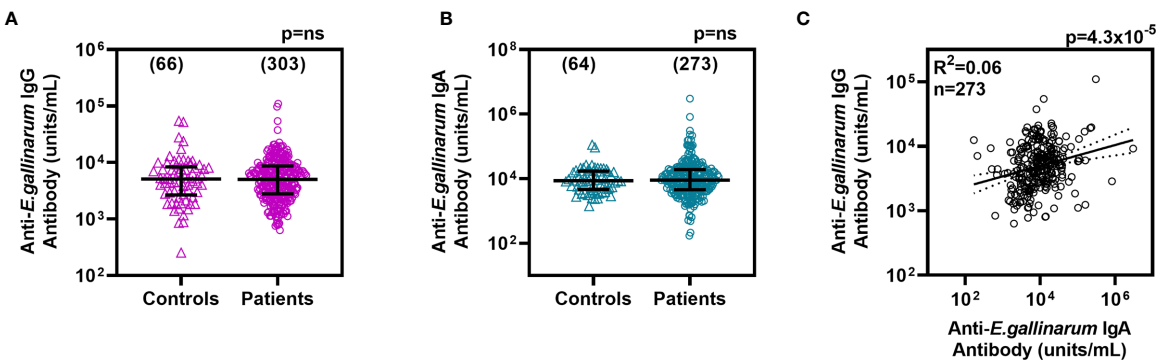

FIGURE 1 | IgG anti-*Eg* **(A)** and IgA anti-*Eg* **(B)** titers in sera from healthy controls and lupus patients. Antibody titers are plotted as units/mL and the lines show median ± interquartile ranges. Correlation between IgG and IgA anti-*Eg* titers in lupus patients **(C)**. Each data point represents one serum sample and the number of samples studied are shown in parentheses. Antibody levels were compared by Mann-Whitney test and the correlation coefficient was determined by Pearson's method. ns, not significant.

TABLE 1 | Association between lupus autoantibodies and anti-bacterial IgG titers in SLE patients.

Autoantibody specificity		anti-*E. gallinarum* IgG			anti-*E. faecalis* IgG			anti-*S. gordonii* IgG		
		Median[@]	IQR*	p value[#]	Median	IQR	p value	Median	IQR	p value
Ribosomal P	Neg	4732	5319	***0.0059***	6823	7462	0.1419	9311	8072	>0.9999
	Pos	7745	6381		11066	15642		10666	4987	
dsDNA	Neg	4688	5097	***0.0093***	6546	7228	***0.0001***	9099	7276	0.1568
	Pos	7015	7184		11776	16919		12823	8371	
Sm	Neg	4699	5066	***0.0315***	6310	6997	***0.0004***	9099	7589	0.6758
	Pos	7047	7766		11749	14055		10789	6987	
chromatin	Neg	4688	5137	0.0694	6252	6974	***0.0021***	9226	6701	>0.9999
	Pos	6124	6775		9954	13121		10447	7539	
SSA	Neg	4909	5336	0.7944	7129	7363	>0.9999	9727	7899	>0.9999
	Pos	5929	5920		7870	10458		8750	9456	
SSB	Neg	5012	5537	0.8708	7396	8432	>0.9999	9705	8260	>0.9999
	Pos	5861	7253		5702	8419		8318	6387	
SmRNP	Neg	4819	5368	0.0694	6026	6439	***<0.0001***	9099	6924	0.2978
	Pos	5834	6642		10889	12498		10789	8304	
RNP	Neg	4909	5433	0.111	6310	7317	***0.0018***	9099	7477	0.4678
	Pos	6209	6868		10889	13194		10789	8183	
RNP A	Neg	4909	5389	0.086	6397	7316	***0.0034***	9162	7571	0.7906
	Pos	6368	7101		10889	13312		10568	7738	
RNP 68	Neg	4977	5828	0.7646	6653	7158	***0.0073***	9247	7606	>0.9999
	Pos	6209	5262		12445	11464		12078	8895	
Centromere B	Neg	7047	7766	0.7947	7261	7678	>0.9999	9311	7917	>0.9999
	Pos	6531	10186		7295	12884		11429	9627	
Scl 70	Neg	5000	5608	0.1727	7295	7983	>0.9999	9397	7866	>0.9999
	Pos	13032	12903		7063	12541		14655	10246	

[@]*Antibody Units/ml; *IQR, interquartile range; [#]adjusted p value.*
Bold and underlined values indicate statistical significance (p<0.05).

SLE patient categorization based on disease activity measures, including SLEDAI scores or BILAG indices, or clinical subsets failed to correlate with anti-*Eg* IgG titers. Similarly, the anti-*Eg* IgA titers failed to show association with the presence or absence of autoantibody specificity (**Supplementary Figure 3**), disease activity measures or clinical subsets (data not shown).

Higher Antibody Titers to Gut Commensal Bacteria *E. Faecalis* and *S. Gordonii* Are Not Associated With the Presence of Anti-Ribosomal P Antibodies

To determine whether exposure to other Enterococci also shows associations with lupus autoantibodies, we measured IgG antibodies to *E. faecalis,* a commensal bacterium represented in the gut microbiome. Anti-*E. faecalis* IgG titers were not significantly different between healthy donors and SLE patients (**Supplementary Figure 4A**). Further, anti-*Eg*, and anti-*E. faecalis* IgG titers in patients showed a significant correlation (**Supplementary Figure 4B**), suggesting comparable exposure to the immune system and the possibility of cross-reactive antibodies.

Further analysis showed that anti-*E. faecalis* IgG titers were significantly higher in patients positive for antibodies to dsDNA, Sm, chromatin, and RNP autoantigens. However, the anti-*E. faecalis* titers between anti-Ribosomal P positive and negative patients failed to reach statistical significance (**Table 1**). A *post hoc* analysis showed that in this experiment, sample sizes gave >80% power to detect a significant difference in a two-tailed statistical test with a confidence level of 0.95. Thus, the negative result was likely not due to insufficient power, suggesting that the anti-Ribosomal P positivity and anti-*Eg* association is specific compared to *E. faecalis*-directed immune responses.

We have previously reported that in SLE patients, high titer antibodies to pathogenic periodontal but not commensal bacteria are associated with increased disease activity indices (13). *Streptococcus gordonii* is a gram-positive commensal bacterium present in dental plaque and also found in the gut mucosa. Anti-*S. gordonii* IgG titers failed to show significant associations with any of the lupus autoantibody specificities (**Table 1**).

Reactivity to Ribosomal P and dsDNA Links Anti-Human RNA and Anti-*Eg* Antibodies in SLE Patients

A close association was reported between anti-Ribosomal P and anti-dsDNA in SLE patients (22, 23) and is replicated in our SLE patients (65% of anti-Ribosomal P positive patients are also anti-dsDNA positive). However, since ribosomes are closely bound to RNA, we postulated that the lack of immunoregulation in SLE patients would favor the presence of antibodies to human RNA in anti-Ribosomal P positive patients. To test this hypothesis, we purified RNA from a human monocytic cell line as a substrate to measure anti-human RNA in SLE patients who were Ribosomal P antibody positive (n=26) or randomly selected Ribosomal P negative (n=33). Patients positive for anti-Ribosomal P had higher anti-human RNA titers than anti-Ribosomal P negative patients (**Figure 3A**). Further, anti-human RNA titers in anti-Ribosomal P positive patients showed a modest but significant

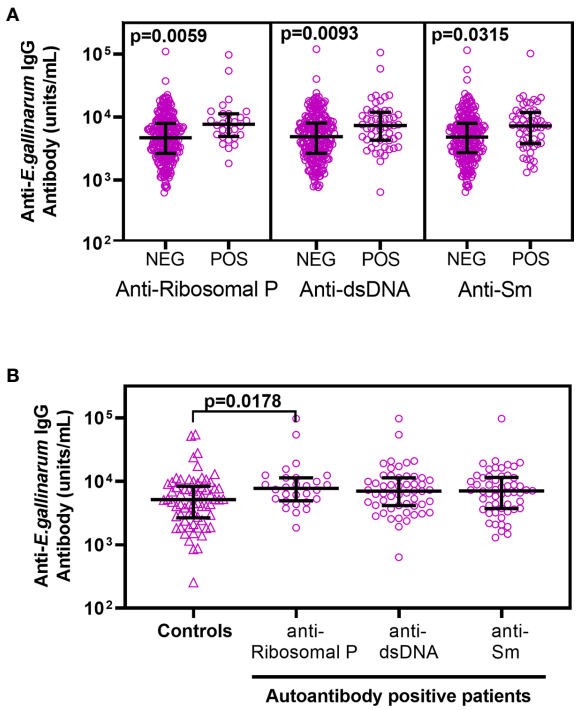

FIGURE 2 | Lupus patients positive for anti-Ribosomal P, anti-dsDNA, and anti-Sm show significantly higher anti-*Eg* IgG titers **(A)**. SLE patients were stratified into autoantibody positive and autoantibody negative groups based on their reactivity to each antigen. The anti-*Eg* IgG titers were compared between the different groups using ANOVA, followed by Sidak's multiple comparison's post-test. The data from autoantibodies that failed to show significant association with anti-*Eg* IgG titers are shown in **Supplementary Figure 2**. A comparison of anti-*Eg* IgG titers in healthy controls with patients positive for Ribosomal P, anti-dsDNA, and anti-Sm using ANOVA followed by Sidak's multiple comparison post-test **(B)**. Adjusted p values < 0.05 reaching statistical significance are shown.

correlation with anti-*Eg* IgG antibody (Spearman r= 0.422, p=0.0319) (**Figure 3B**).

Anti-RNA antibodies in SLE patients also react with viral dsRNA and synthetic dsRNA (24). To investigate whether anti-RNA reactivity was skewed by RNA binding protein contaminants co-purified in the human RNA preparation, the same sera were screened for antibodies to synthetic dsRNA (poly I:C) coated on an ELISA plate. Anti-dsRNA reactivity was higher in Ribosomal P antibody-positive patients (**Supplementary Figure 5A**). The anti-human RNA and anti-dsRNA titers showed a strong correlation, Spearman r=0.782, p=2.57x10^{-13}, n=59 (**Supplementary Figure 5B**), thereby confirming the reactivity to the nucleotide backbone.

Higher anti-RNA antibody titers are associated with higher disease activity (**Supplementary Table 3**) and a diversified autoantibody repertoire. Therefore, the association of anti-human RNA with anti-*Eg* titers might not be unique to Ribosomal P positivity. To investigate whether other autoantibody specificities also showed a similar relationship, patients were stratified into autoantibody-positive and -negative groups, and the correlation between anti-*Eg* and anti-human RNA titers in each group was studied (**Supplementary Table 4**). In addition to anti-Ribosomal P, anti-human RNA titers were also higher in patients positive for anti-dsDNA (**Figure 3C**). Further, anti-*Eg* IgG also showed modest but significant correlations with anti-human RNA titers in anti-dsDNA positive patients (r=0.492, p=0.0146). (**Figure 3D** and **Supplementary Table 4**).

DISCUSSION

The present study was prompted by a novel report describing the possible role of the pathobiont *E. gallinarum* in SLE pathogenesis (16). Since the Manfredo-Vieira et al. study was done in a limited number of lupus patients (n=15), we sought to investigate the role of *E. gallinarum* in a larger cohort of SLE patients (n=303). Furthermore, we also expanded the investigation into evaluating the association between *E. gallinarum* and multiple autoantibody specificities and SLE clinical parameters.

Using banked serum samples from a well-characterized cohort of SLE patients, our study demonstrates that IgG and IgA antibodies to *E. gallinarum* were present in lupus patients and healthy controls. Despite the differences in the numbers and characteristics of the patient populations, ELISA methodologies, and the specific bacterial strains, both studies showed comparable IgG and IgA anti-*Eg* titers between healthy controls and SLE patients. In our analysis, although anti-*Eg* titers did not correlate with either of the two disease activity indices (SLEDAI and BILAG), higher titers of anti-*Eg* IgG in patients were significantly associated with the presence of autoantibodies to Ribosomal P proteins, dsDNA, and Sm.

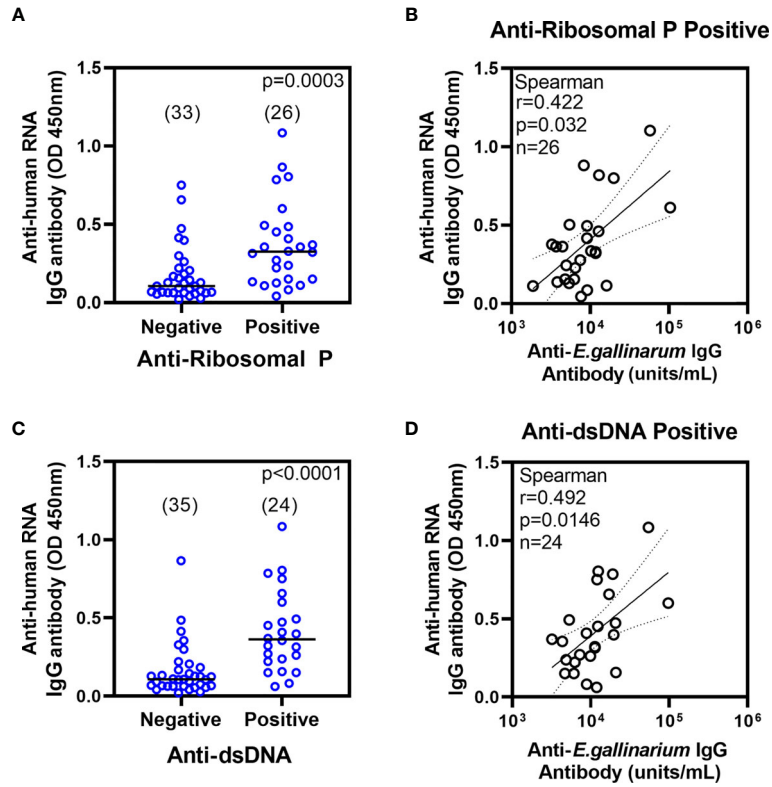

FIGURE 3 | Anti-human RNA IgG antibodies in anti-Ribosomal P **(A)** and anti-dsDNA **(C)** negative and positive patients. All samples were tested at a 1:100 serum dilution and results are shown as absorbance at 450nm. Antibody levels were compared using non-parametric Mann-Whitney test. Number of samples are shown in parentheses. Correlation between anti-human RNA IgG and anti-*Eg* IgG titers in patients positive for anti-Ribosomal P **(B)** and anti-dsDNA **(D)**. OD, optical density.

In addition, only anti-*Eg*, but not anti-*E. faecalis* or anti-*S. gordonii* IgG antibody titers showed the strongest association with anti-Ribosomal P. Considered collectively, both studies suggest an involvement of *E. gallinarum*, and potentially other closely related enterococci, in SLE pathogenesis (3, 16). The analysis of gut microbiome in SLE patients from Guangzhou Province in China showed enrichment of the genus *Enterococcus* (3). Interestingly at species level, while this study reported an increase in *bacterium Te59R* (closely related *via* the 16S rRNA sequence to *Enterococcus faecium)*, it did not mention the detection of *E. gallinarum* in SLE patients. Whether lack of *E. gallinarum* reporting in this study is due to differences in patient demographics or/and methodology needs to be investigated in future.

Ribosomal P proteins are three highly conserved phosphorylated proteins on the 60s subunit of ribosomes and are a target for autoantibodies (25). Ribosomal P autoantibodies occur in a minority of lupus patients and in patients with autoimmune hepatitis (25, 26). In the present cohort, anti-Ribosomal P reactivity was seen in only 8.6% of the patients. Although anti-Ribosomal P antibodies are most frequently reported with neuropsychiatric lupus (27–29), they also identify a subgroup of patients at high risk of hepatic involvement. Studies by Stafford, Reichlin and colleagues showed that anti-Ribosomal P antibodies, if present, in healthy adults and children are masked and only detected following affinity purification on ribosome coated columns (30, 31). Thus, it is important to note that anti-Ribosomal P reactivity is highly specific for disease states, predominantly SLE, and is not detectable in sera from healthy individuals as reported in multiple studies (22, 32–35).

Ribosomal P protein is expressed on the cell membrane and can bind to sera from lupus patients (36). Ribosomal P antibodies can penetrate live hepatoma cells and block protein synthesis leading to cellular injury (37). Furthermore, we also noted higher anti-human RNA antibody titers in patients positive for anti-Ribosomal P. Considering that *E. gallinarum* was detected in liver biopsies from lupus patients and anti-*Eg* IgG was unique in its association with antibodies to Ribosomal P, it can be surmised that *E. gallinarum* mediated hepatic and/or systemic inflammation may contribute to anti-Ribosomal P autoimmune responses in some SLE patients. Whether this occurs through molecular mimicry or intermolecular epitope spreading will be tested in future studies by longitudinal analysis of serum samples from lupus patients and by developing experimental mouse model systems.

We have previously reported associations between the lupus autoantibodies and higher titers to the dental plaque bacteria *A. actinomycetemcomitans* and *P. gingivalis* implicated in periodontal disease (13). It is interesting to note that the antibodies to these oral pathogens were not different in patients with or without Ribosomal P reactivity. *A. actinomycetemcomitans* and

P. gingivalis secrete virulence factors, invade the periodontal tissues, migrate to distant organs and cause inflammation (38, 39). In contrast, *E. gallinarum* is a commensal gut resident bacterium that can translocate to the liver. Taken together, these results suggest that the mechanism(s) of how periodontal and gut bacteria influence lupus might be different.

Some limitations of the present study include the unavailability of stool samples for microbiome analysis, a lack of patient medication history, and the absence of demographic data on the healthy controls. However, this study reinforces previous reports by our group and others (40, 41) that in retrospective studies of large and diverse patient cohorts, evaluating serum antibodies to pathogenic and commensal bacteria is a valuable tool to investigate the interaction between the microbial environment and autoimmunity. These data provide a rationale for performing metagenomic analyses of mucosal microbial communities in diverse SLE patient cohorts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Oklahoma Medical Research Foundation Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HB designed and performed experiments, analysed the data, and wrote the manuscript. AA, JI, and KC performed experiments and analysed data. JM, CA, and JJ contributed to study design, acquisition of clinical data, data analysis, and writing of the manuscript. JG contributed to study design, critical review of data analysis, and writing of the manuscript. UD conceived of the idea, designed experiments, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.635072/full#supplementary-material

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