DANGER SIGNALS TRIGGERING IMMUNE RESPONSE AND INFLAMMATION

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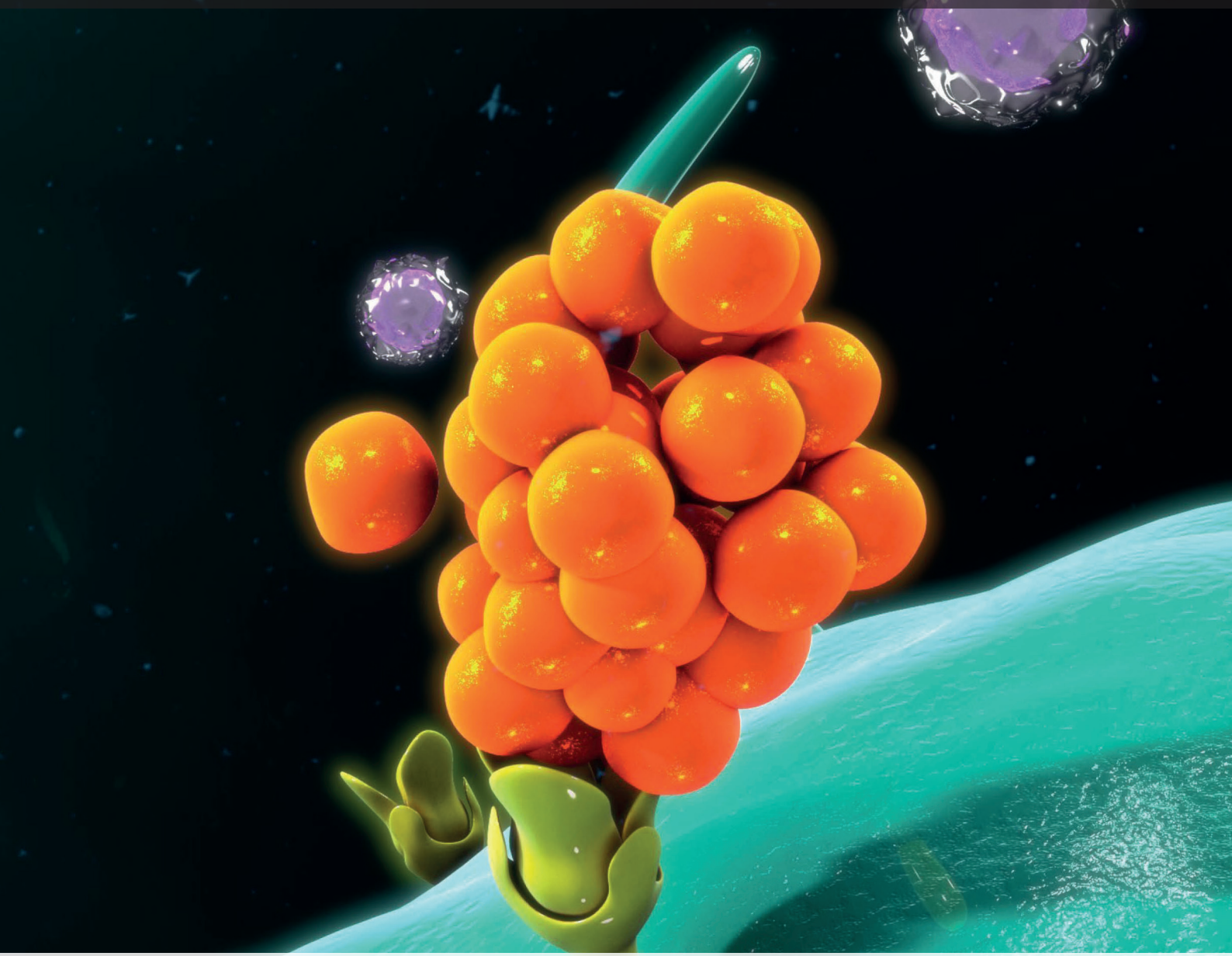

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DANGER SIGNALS TRIGGERING IMMUNE RESPONSE AND INFLAMMATION

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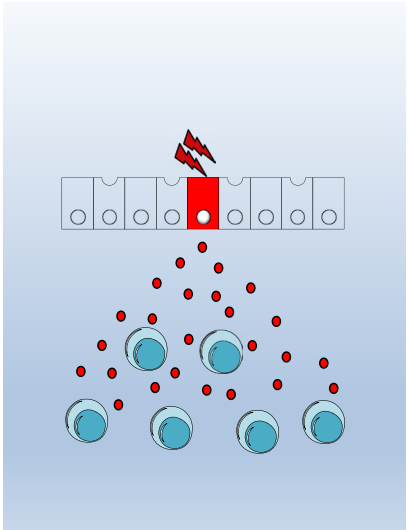

Following tissue damage, pathogen-associated molecular patterns (PAMPs) or damage-associated molecular pattern molecules (DAMPs), also called alarmins are released. Immune cells including neutrophils, macrophages, dendritic cells, B cells, innate lymphoid cells, NK cells, T effector cells, regulatory T cells, CD8 T cells will detect the "danger" and mediate antigen-independent immune responses.

Image by Sophie Paczesny.

Cover image: sciencepics/Shutterstock.com

The immune system detects "danger" through a series of what we call pathogen-associated molecular patterns (PAMPs) or damage-associated molecular pattern molecules (DAMPs), working in concert with both positive and negative signals derived from other tissues. PAMPs are molecules associated with groups of pathogens that are small molecular motifs conserved within a class of microbes. They are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors. A vast array of different types of molecules can serve as PAMPs, including glycans and glycoconjugates. Bacterial lipopolysaccharides (LPSs), endotoxins found on the cell membranes of Gram-negative bacteria, are considered to be the prototypical class of PAMPs. LPSs are specifically recognized by TLR4, a recognition receptor of the innate immune system. Other PAMPs include bacterial flagellin (recognized by TLR5), lipoteichoic acid from Gram-positive bacteria, peptidoglycan, and nucleic acid variants normally associated with viruses, such as double-stranded RNA, recognized by TLR3 or unmethylated CpG motifs, recognized by TLR9. DAMPs, also known as alarmins, are molecules released by stressed cells undergoing necrosis that act as endogenous danger signals to promote and exacerbate the immune and inflammatory response. DAMPs vary greatly depending on the type of cell (epithelial, mesenchymal, etc.) and injured tissue. Some endogenous danger signals include heat-shock

mesenchymal, etc.) and injured tissue. Some endogenous danger signals include heat-shock proteins, HMGB1 (high-mobility group box 1), reactive oxygen intermediates, extracellular matrix breakdown products such as hyaluronan fragments, neuromediators, and cytokines like the interferons (IFNs). Non-protein DAMPs include ATP, uric acid, heparin sulfate, and DNA. Furthermore, accumulating evidence supports correlation between alarmins and changes in the microbiome. Increased serum or plasma levels of these DAMPs have been associated with many inflammatory diseases, including gastric and intestinal inflammatory diseases, graft-versus-host disease (GVHD), sepsis and multiple organ failure, allergies particularly in the lungs, atherosclerosis, age-associated insulin resistance, arthritis, lupus, neuro-inflammation/degeneration and more recently in tumors, which is particularly interesting with the emergence of immunotherapies. Therapeutic strategies are being developed to modulate the expression of these DAMPs for the treatment of these diseases.

A vast number of reviews have already been published in this area; thus, in an effort to not duplicate what has already been written, we will focus on recent discoveries particularly in disease models that are epidemic in Western society: intestinal chronic inflammatory diseases including GVHD and its relationship with the microbiome, chronic infectious diseases, allergies, autoimmune diseases, neuroinflammation and cancers. We will also focus on the basic cellular roles of macrophages, T cells and B cells.

This research topic brings together sixteen articles that provide novel insights into the mechanisms of action of DAMPS/alarmins and their regulation and subsequent immunologically driven responses.

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EDITORIAL
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Editorial: Danger Signals Triggering Immune Response and Inflammation

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Editorial on the Research Topic

Danger Signals Triggering Immune Response and Inflammation

The immune system detects "danger" through a series of what we call pathogen-associated molecular patterns (PAMPs) or damage-associated molecular pattern molecules (DAMPs), working in concert with both positive and negative signals derived from other tissues. PAMPs are molecules associated with groups of pathogens that are small molecular motifs conserved within a class of microbes. They are recognized by toll-like receptors (TLRs) and other pattern-recognition receptors. A vast array of different types of molecules can serve as PAMPs, including glycans and glycoconjugates. Bacterial lipopolysaccharides (LPSs), endotoxins found on the cell membranes of Gram-negative bacteria, are considered to be the prototypical class of PAMPs. LPSs are specifically recognized by TLR4, a recognition receptor of the innate immune system. Other PAMPs include bacterial flagellin (recognized by TLR5), lipoteichoic acid from Gram-positive bacteria, peptidoglycan, and nucleic acid variants normally associated with viruses, such as double-stranded RNA, recognized by TLR3 or unmethylated CpG motifs, recognized by TLR9. DAMPs, also known as alarmins, are molecules released by stressed cells undergoing necrosis that act as endogenous danger signals to promote and exacerbate the immune and inflammatory response. DAMPs vary greatly depending on the type of cell (epithelial, mesenchymal, etc.) and injured tissue. Some endogenous danger signals include heat-shock proteins, high-mobility group box 1 (HMGB1), reactive oxygen intermediates, and extracellular matrix breakdown products such as hyaluronan fragments, neuromediators, and cytokines including the interferons. Non-protein DAMPs include ATP, uric acid, heparin sulfate, and DNA. Furthermore, accumulating evidence supports correlation between alarmins and changes in the microbiome. Increased serum or plasma levels of these DAMPs have been associated with many inflammatory diseases, including gastric and intestinal inflammatory diseases, graft-versus-host disease (GVHD), sepsis and multiple organ failure, allergies particularly in the lungs, atherosclerosis, age-associated insulin resistance, arthritis, lupus, neuroinflammation/degeneration, and more recently in tumors, which is particularly interesting with the emergence of immunotherapies. Therapeutic strategies are being developed to modulate the expression of these DAMPs for the treatment of these diseases.

A vast number of reviews have already been published in this area; thus, in an effort to not duplicate what has already been written, we will focus on recent discoveries particularly in disease models that are epidemic in Western society: intestinal chronic inflammatory diseases including GVHD and its relationship with the microbiome, chronic infectious diseases, allergies, autoimmune

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diseases, neuroinflammation, and cancers. We will also focus on the basic cellular roles of macrophages, T cells, and B cells.

This *research topic* brings together 16 articles that provide novel insights into the mechanisms of action of DAMPs/alarmins and their regulation and subsequent immunologically driven responses. The take-home messages from these 16 studies are summarized below.

Two articles focused on the basic mechanisms of activation of macrophages and B cells by TLRs. Chu et al. showed in an original research article that the Fab fragment of a human anti-Siglec-9 monoclonal antibody suppresses LPS-induced inflammatory responses in human macrophages, which has important therapeutic consequences for sepsis management. The mini-review by Suthers and Sarantopoulos explored the cross talk between TLR7/TLR9 and BCR signaling, which they suggest induces dangerous B cells. Although underexplored, it is now clear that a balance between TLR7 and TLR9 is pivotal in the development of B-cell autoreactivity, and one disease model to study this further is chronic GVHD, as the microenvironment after allogeneic hematopoietic stem cell transplantation contains large amounts of microbial-derived nucleic acids and B-cell-activating factor.

Five articles included in this *research topic* investigated the roles of PAMPs and DAMPs in the development of intestinal inflammation including acute GVHD. Pellegrini et al. reviewed how the canonical and non-canonical activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome regulates tolerance and inflammation in the intestine. Indeed, NLRP3 has a dual role in the pathogenesis of bowel inflammation. In some studies, NLRP3 has regulatory and reparative roles in the immune homeostasis and maintenance of the epithelial barrier integrity, whereas in others, overactivation of NLRP3 contributes to interruption of the intestinal immune balance. Lin et al. published an original article on how *Helicobacter pylori*, which infects more than half of the human population worldwide, activates HMGB1 expression, and recruits RAGE into lipids rafts to promote inflammation in gastric epithelial cells. In this translational work, the authors found that HMGB1 and RAGE expression levels are significantly greater in *H. pylori*-infected cells than in uninfected gastric cells. Blocking HMGB1 by a neutralizing antibody can abrogate *H. pylori*-elicited RAGE signaling by reducing nuclear factor (NF)-κB activation and interleukin (IL)-8 production. The current understanding and future perspectives of danger signals in GVHD were reviewed by Toubai et al. One interesting new finding in this field is the role of the HMGB1 receptor (Siglec-G)/CD24 axis in controlling the severity of GVHD (1). The mini-review by Apostolova and Zeiser summarizes the role of purine metabolites, particularly ATP/ectonucleotidases as novel DAMPs, in the development of acute GVHD. These interactions are influenced by the intestinal microbiome, which has been particularly well explored. Slingerland et al. reviewed the role of the microbiome in intestinal diseases and other diseases such as circulatory, integumentary, musculoskeletal, respiratory, neuromuscular, and systemic conditions, focusing on clinical evidence and one potential therapeutic intervention. Two original papers looked at danger signals in relation to autoimmunity development. Peng et al. showed increases in TLR activity and TLR ligands in patients with autoimmune thyroid diseases. Using peripheral blood mononuclear cells from 30 healthy controls, 36 patients with untreated Hashimoto's thyroiditis, and 30 patients with newly onset Graves' disease, they showed that TLR2, TLR3, and TLR9 expression and activation are increased in patients with autoimmune thyroid diseases, suggesting a role for TLRs in the pathogenesis. Yun et al. showed that the HMGB1–CXCL12 complex promotes T-cell infiltration in chronic experimental autoimmune uveitis. They demonstrated that at a very early stage of intraocular inflammation initiated by uveitogenic autoreactive T cells, synergism between HMGB1 and CXCL12 is crucial for the infiltration of inflammatory cells and that the induction of experimental autoimmune uveitis was significantly inhibited by a CXCR4 antagonist, AMD3100.

Three papers explored the role of danger signals in the brain. Terrando et al. showed that HMGB1 is rapidly released after tissue trauma and its neutralization prevents postoperative neurocognitive dysfunction in a model of aged rats. Indeed, postoperative neurocognitive disorders are common complications in elderly patients following surgery or critical illness, and these findings offer a better understanding of the neurocognitive dysfunction and therapeutic options. Lin et al. studied another neuroprotective effect through the activation of the cannabinoid receptor-2 with a selective agonist, JWH133, and they showed the protection to be due to suppressed neuroinflammation and upregulated expression of microglial macrophage M2-associated markers in an intracerebral hemorrhage model. Finally, Wilkins et al. reviewed the role of mitochondria-derived DAMPs in neurodegeneration. The roles of neuroinflammation in neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis are increasingly appreciated, but the field is still in its infancy. Initial interest in neuroinflammation as a causative factor in AD was triggered by the reduced risk for AD in long-term non-steroidal anti-inflammatory drug users, which was later linked to the apolipoprotein E ε4 allele (2). Furthermore, genome-wide association studies identified a triggering receptor variant expressed on myeloid cells 2 (TREM2) as a causative factor for AD (3). Since these initial studies were published, much progress has been made to clarify how mitochondrial dysfunction plays a fundamental role, as elegantly reviewed by the group of Russell Swerdlow, a pioneer in the field (Wilkins et al.).

Two studies investigated the importance of immune homeostasis disruption in lung diseases. Chen et al. demonstrated that a novel subset of IL-10-producing $CD1d^{hi}CD5^{+}$ regulatory B cells modulates immune homeostasis in patients with silicosis, a condition of chronic inflammation and fibrosis of the lung. The study reported by Lin et al. showed that LPS can attenuate proallergic cytokines such as thymic stromal lymphopoietin (TSLP) and IL-33 in respiratory epithelial cells stimulated with polyI:C and human parechovirus. This work supports the "hygiene hypothesis," which claims that childhood exposure to environmental microbial products is inversely related to the incidence of allergic diseases in later life. It also suggests that in addition to therapeutic targeting of TSLP and IL-33, local application of non-pathogenic LPS may be a rational strategy to prevent allergies.

Interestingly, one of the mediators recently identified in the pathogenesis of allergies is IL-33. Undeniably, the serum stimulation-2 (ST2)/IL-33 axis has been found to be rooted in

the pathogenesis of an increasing number of diseases, and this is reviewed in a manuscript published by Griesenauer and Paczesny. Briefly, ST2, the IL-33 receptor, exists in two forms as splice variants: a soluble form (sST2), which acts as a decoy receptor, sequesters free IL-33, and does not signal, and a membrane-bound form (ST2), which activates the MyD88/NF-κB signaling pathway to enhance mast cell, Th2, regulatory T cell, and innate lymphoid cell type 2 functions. Plasma/serum levels of sST2 are increased in patients with active inflammatory bowel disease (4), cardiac diseases (5), acute cardiac allograft rejection (6), and GVHD (7–14). The review also details the immune cells that express ST2 on their surface or secrete sST2 as well as the relevant signaling mechanisms. The ST2/IL-33 axis has recently been shown to be a potential novel checkpoint in the development of tumors, as reviewed by Wasmer and Krebs. Indeed, recent findings have shown a role of IL-33 in several cancers where it may exert multiple functions. The role of the ST2/IL-33 axis has been particularly well studied in colorectal cancer (15) and myeloproliferative neoplasms (16). Importantly, IL-33 could be used as potential tumor biomarker or therapeutic target.

In summary, the reviews and original articles collected for this research topic of *Frontiers in Immunology* convey to readers the multiplicity and implications of PAMPs/DAMPs/alarmins and their regulators in the development of inflammatory diseases. Importantly, learning from these mechanisms of action, each group of investigators has proposed novel targeted treatments.

AUTHOR CONTRIBUTIONS

All authors wrote and approved the manuscript.

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ORIGINAL RESEARCH
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The Fab Fragment of a Human Anti-Siglec-9 Monoclonal Antibody Suppresses LPS-Induced Inflammatory Responses in Human Macrophages

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Sepsis is a major cause of death for hospitalized patients and is characterized by massive overreaction of immune responses to invading pathogens which is mediated by cytokines. For decades, there has been no effective treatment for sepsis. Sialic acid-binding, Ig-like lectin-9 (Siglec-9), is an immunomodulatory receptor expressed primarily on hematopoietic cells which is involved in various aspects of inflammatory responses and is a potential target for treatment of sepsis. The aim of the present study was to develop a human anti-Siglec-9 Fab fragment, which was named hS9-Fab03 and investigate its immune activity in human macrophages. We began by constructing the hS9-Fab03 prokaryotic expression vector from human antibody library and phage display. Then, we utilized a multitude of assays, including SDS-PAGE, Western blotting, ELISA, affinity, and kinetics assay to evaluate the binding affinity and specificity of hS9-Fab03. Results demonstrated that hS9-Fab03 specifically bind to Siglec-9 antigen with high affinity, and pretreatment with hS9-Fab03 could attenuate lipopolysaccharide (LPS)-induced TNF-α, IL-6, IL-1β, IL-8, and IFN-β production in human PBMC-derived macrophages, but slightly increased IL-10 production in an early time point. We also observed similar results in human THP-1-differentiated macrophages. Collectively, we prepared the hS9-Fab03 with efficient activity for blocking LPS-induced pro-inflammatory cytokines production in human macrophages. These results indicated that ligation of Siglec-9 with hS9-Fab03 might be a novel anti-inflammatory therapeutic strategy for sepsis.

Keywords: Siglec-9, human anti-Siglec-9 antibody, Fab fragment, LPS, TLR4, sepsis, human macrophages

INTRODUCTION

Sepsis is a leading cause of mortality in intensive care units; recent statistics have indicated the occurrence about 19 million cases worldwide per year (1). Current treatments for sepsis are typically supportive and often ineffective, despite the fact that the number of deaths per year is around eight million (2). Moreover, more than 30% of survivors suffer from long-term functional disabilities

and persistent critical illness (3). Lethality caused by sepsis arises from a massive hyperinflammatory immune response to pathogens, such as Gram-negative organisms, Gram-positive organisms, and fungi (4). The uncontrolled pro-inflammatory responses that lead to organ dysfunction in sepsis are primarily initiated by the toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns (PAMPs), such as bacterial lipoproteins, lipopolysaccharide (LPS), and non-methylated CpG DNA (5). TLR4 activation results in the synthesis and release of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-8, and IFN-β, which act locally but are released systemically, initiating the cytokines storm that damage vital tissues (6, 7). Our recent studies indicated that anti-TLR4 Fab fragment could reduce the inflammatory responses by inhibiting LPS-induced TLR4 signaling pathway in mouse primary macrophages and human THP-1-differentiated macrophages (8, 9). Therefore, it is intriguing that macrophages and inflammatory cytokines could be potential therapeutic targets in patients with sepsis.

Sialic acid-binding immunoglobulin-type lectins (Siglecs) are a family of sialic acid-binding immunoglobulin-like lectins that are differentially presented on the surface of hematopoietic cells, which exert immunomodulatory functions *via* glycans or glycoproteins recognition during immune responses (10, 11). Siglecs can be categorized into two groups. CD169, CD22, MAG, and Siglec-15 are conserved across mammals. In comparison, the CD33-related Siglecs are variable across mammals (12). It has been suggested that CD33-related Siglecs may serve as a negative regulator for immunocytes behavior, such as inhibition of cellular activation, induction of apoptosis, and suppression of pro-inflammatory cytokines production (13). All of CD33-related Siglecs may transduct through their immunoreceptor tyrosine-based inhibitory motifs (ITIMs) located in the cytoplasmic region (except for Siglec-14), which are associated with SHP-1 and/or SHP-2 (14, 15). Siglec-9, as a member of the CD33-related Siglecs, is predominantly presented on neutrophils, monocytes, macrophages, and dendritic cells (DCs), whose mouse ortholog Siglec-E are expressed on neutrophils, monocytes, and conventional dendritic cells (16, 17). Siglec-9 has a characteristic N-terminal, Ig-like, V-type domain which could mediate its binding to sialic acid moiety of glycans and glycoproteins, a single transmembrane region, and a cytoplasmic tail that contain an ITIM and SLAM-like motif (18, 19). It is well established that ligation of the Siglec-9 induces phosphorylation of the tyrosine within the ITIM and recruit tyrosine phosphatase SHP-1 and SHP-2, then exerts its inhibition during innate and acquired immunity (20).

The cross talks between Siglecs family and TLRs are under intense investigation. Recently, Siglecs expressed on neutrophils, macrophages, and DCs could regulate TLRs-induced cytokines production through small RNA interference or *in vitro* ligation with Siglecs-specific antibodies. Results showed that Siglec-G could not regulate responses to microbial products directly, but instead it might interact with the receptor CD24 in *cis* to inhibit DC-initiated inflammatory reactions (21). Chen et al. showed that Siglec-G expression could be upregulated on macrophages after infection by vesicular stomatitis virus (VSV) or Sendai virus, which lead to the degradation of retinoic acid-inducible gene I and inhibition of the IFN-β production (22). Furthermore, recent results suggest that Siglec-9 inhibits the production of TNF-α while promotes the secretion of the IL-10 upon stimulation with LPS in macrophages, but the precise mechanism of Siglec-9-influenced LPS signaling pathway is still unknown (23).

Thus, we prepared the Fab fragments of human anti-Siglec-9 monoclonal antibody (hS9-Fab03) from human antibody library and phage display and examined whether treatment of hS9-Fab03 could regulate immune responses upon stimulation by LPS in human macrophages. In this study, we report that hS9-Fab03 not only attenuates LPS-induced TNF-α, IL-6, IL-1β, IL-8, and IFN-β production in human PBMC-derived macrophages but also slightly increases IL-10 production in an early time point.

MATERIALS AND METHODS

Cells and Reagents

The THP-1 cells were acquired from the cell bank of Shanghai Institute of Biochemistry and Biology (Chinese Academy of Sciences, Shanghai, China). RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA, USA). LPS (O111:B4), PMA, Ficoll-Paque Plus, and commercial anti-Siglec-9 antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA), Abs specific to GAPDH, total p38, phosphorylated JNK1/2, p38, p65, and IRF-3 were purchased from Cell Signaling Technology (Danvers, MA, USA). His-trap Lambda Fab Select column was obtained from GE Healthcare (Piscataway, NJ, USA). Anthrax chimeric Fab antibody was prepared in our lab.

Cell Culture and Differentiation

THP-1 cells were cultured in RPMI-1640 supplied with 10% FBS, 1% penicillin, and streptomycin in a 5%-CO_2 humidified incubator at 37°C. The THP-1 cells were stimulated with 10 nM PMA for 48 h, then THP-1-derived macrophages were differentiated. The PBMC-derived macrophages were cultured and differentiated as previously reported (24). Briefly, human PBMCs were separated by centrifugation on Ficoll-Paque Plus and purified by CD14-positive cells isolation kit (Miltenyi Biotec, CA, USA). The purified cells were differentiated in complete RPMI-1640 supplied with M-CSF (10 ng/ml) (BD Biosciences, CA, USA) for 6 days. Donor blood samples were randomly collected in the Jiangsu Province Blood Center. The study was approved by Ethical Committee of Anhui Medical University Affiliated with Bayi Clinical College and all participants signed an informed consent form when they filled the questionnaire.

Phage Library and Helper Phage

A human naive Fab phage library for Siglec-9 selection was generated as previously described (25). Before the first round panning, the phage library was titrated and 1×10^{13} phage clones were collected for panning.

Phage ELISA

Single phage clone from the *E. coli* XL1-Blue infected by the seventh round of eluted phage was randomly picked up and

grown in 1 ml super broth (SB) medium containing 100 μg/ml ampicillin and 1% glucose. VCSM13 helper phage (1×10^9) was added to each vial. Then, 50 μl of supernatant from each vial was added to each well of 96-well plate, which was pre-coated with 100 ng extracellular domain of Siglec-9 antigen. After incubation for 2 hours and washing for three times, 50 μl of

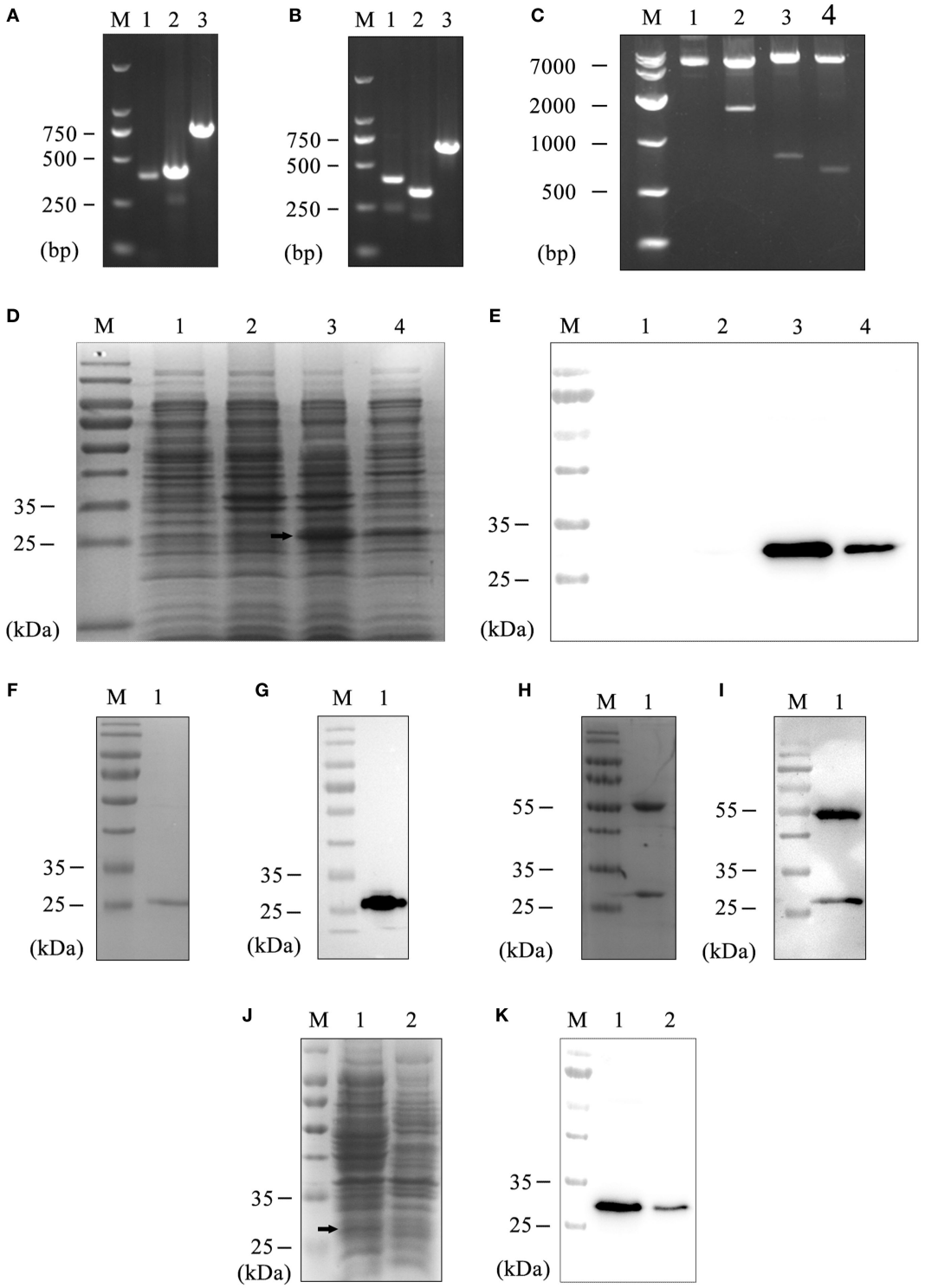


FIGURE 1 | **Preparation of the hS9-Fab03**.

(*Continued*)

FIGURE 1 | Continued
(A) The heavy chain PCR products of positive clone. Lane 1, V_H; Lane 2, C_H; Lane 3, V_H combined with C_H; Lane M, DNA maker. **(B)** The light chain PCR products of positive clone. Lane 1, V_L; Lane 2, C_L; Lane 3, V_L combined with C_L; Lane M, DNA maker. **(C)** The construction of pETDuet-hSiglec-9 Fab03. Lane 1, the plasmid without restriction endonuclease digestion; Lane 2, the plasmid was double digested with *Nco*I and *Xho*I; Lane 3, *Nco*I and *Hin*dIII were used for double digesting the plasmid; Lane 4, *Eco*RV and *Xho*I were used for double digesting the plasmid; Lane M, DNA marker. Coomassie blue staining **(D)** and Western blotting **(E)** detected the expression of the recombinant vector. Lane 1, whole lysate of untransfected *E. coli* BL21, as a negative control; Lane 2, whole lysate of pETDuet-hSiglec-9 Fab03-transfected *E. coli*; Lane 3, supernatant of sonicated lysate of pETDuet-hSiglec-9 Fab03-transfected *E. coli*; Lane 4, sediment of sonicated lysate of pETDuet-hSiglec-9 Fab03-transfected *E. coli*; Lane M, protein marker. All strains were induced by IPTG overnight. Coomassie blue staining **(F)** and Western blotting **(G)** detected the purified Fab fragments. Lane 1, supernatant of sonicated lysate of pETDuet-hSiglec-9 Fab03-transfected *E. coli* induced by IPTG overnight; lane M, protein marker. **(H)** Coomassie blue staining showed that the hS9-Fab03 was expressed, and the heavy chain Fd and light chain L were linked together. **(I)** horseradish-peroxidase-conjugated goat anti-human antibody (Fab specific) was used to detect the heavy chain Fd, and light chain L of the hS9-Fab03 was expressed and separated in Western blotting. Coomassie blue staining **(J)** and Western blotting **(K)** detected the efficiency of hS9-Fab03. Lane 1, supernatant of sonicated lysate of the pETDuet-hSiglec-9 Fab03-transfected *E. coli* was purified by the His-trap Lambda Fab Select column; lane 2, whole lysate of pETDuet-hSiglec-9 Fab03-transfected *E. coli* induced by IPTG overnight; lane M, protein marker.

horseradish-peroxidase (HRP)-conjugated anti-M13 antibody solution was added to each well. Finally, the highest absorbance of the positive clones was selected for further evaluation.

Construction of the hS9-Fab03 Expression Vector

Total RNA was extracted from positive cells by the TRIzol reagent (Invitrogen, CA, USA) and converted to complementary DNA (cDNA) by the Prime Script RT Master Mix kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. The variable regions of the heavy (V_H) chain and the light (V_L) chain were amplified with primers shown in Table S1 in Supplementary Material. The conserved regions of the heavy (C_H1) and light (C_L) chains were provided by the Barbas laboratory (Scripps Research Institute, USA). The V_H and V_L were amplified and cloned into pMD-18T vector, respectively. The heavy chain Fd and the light chain L were amplified with HF1, HR2, and LL1, LR2, respectively. The Fd and L were cloned into pETDuet-1 at *Nco*I/*Hin*dIII and *Eco*RV/*Xho*I, respectively.

Western Blotting

The expression of hS9-Fab03 in *E. coli* BL21 was detected by Western blotting as previously described (26). The concentrations of proteins were determined by bicinchoninic acid protein assay kit (Thermo, Waltham, MA, USA). The proteins from bacteria lysate were dissociated by 12% SDS-PAGE and then transferred onto nitrocellulose membrane (Bio-Rad, CA, USA). The membrane was blocked by 5% non-fat milk in TBST for 1 h at room temperature, which was then incubated with HRP-conjugated goat anti-human antibody (Fab specific) (Santa Cruz Biotechnology, CA, USA). The THP-1-derived macrophages (1×10^6 cells/well) were pretreated with the hS9-Fab03 (5 μg/ml) for 2 h and stimulated with LPS (1 μg/ml) for 0, 15, 30, and 60 min. The phosphorylation level of NF-κB/MAPKs/IRF-3 signaling pathway were detected and analyzed.

Fluorescence-Activated Cell Sorting (FACS)

The THP-1-derived macrophages were treated with the hS9-Fab03 for 2 h; the cells were then incubated with goat anti-human IgG (Fab specific)-FITC antibody (Sigma-Aldrich, MO, USA) at 37°C for 1 h. The cells were analyzed by LSRII flow cytometer (BD Biosciences, CA, USA).

ELISA

TNF-α, IL-1β, IL-6, IL-10, and IL-8 in supernatants were determined with ELISA kits (R&D Systems, MN, USA).

Surface Plasmon Resonance (SPR) Analysis of the hS9-Fab03

We optimized the coupling condition based on the isoelectric point of the Siglec-9 protein, and sodium acetate was chosen as the dilution buffer for Biacore X100 SPR system (GE, Sweden). The Siglec-9 protein was diluted to 30 μg/ml and coupled to the CM5 chip. The coupling level was set at 1,500 RU, the injection time was set to 180 s, the dissociation time was set to 15 min, and 50 mM Gly-HCl (pH = 1.7) was used as the regeneration buffer. Finally, the Siglec-9 protein was treated with serial concentrations of hS9-Fab03 to detected the antibodies' binding affinity.

Real Time Q-PCR

The total RNA was extracted using RNAfast200 (Feijie, Shanghai, China) following the manufacturer's instructions. A quantity of 0.5 μg total RNA was used in a 10-μl reverse-transcription reaction using the PrimeScript RT Master Mix kit, then the cDNA was diluted into 40 μl as template for real time Q-PCR. Q-PCR analysis was performed using LightCycler (Roche Diagnostic, IN, USA). The primers used for human GAPDH, TNF-α, IL-6, IL-1β, IL-8, IL-10, and IFN-β were as described previously (27). Data were normalized by the level of GAPDH expression in each sample.

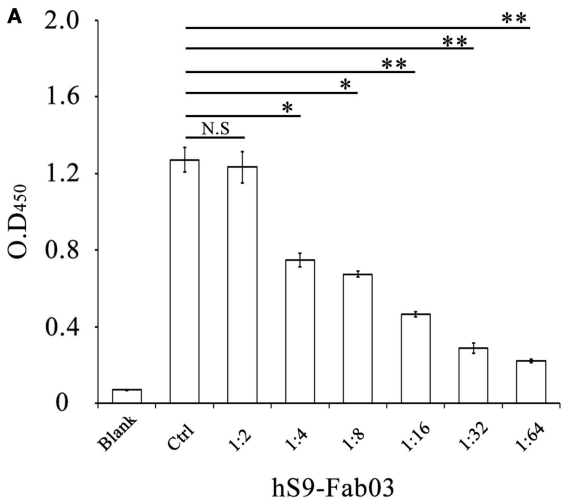
Statistical Analysis

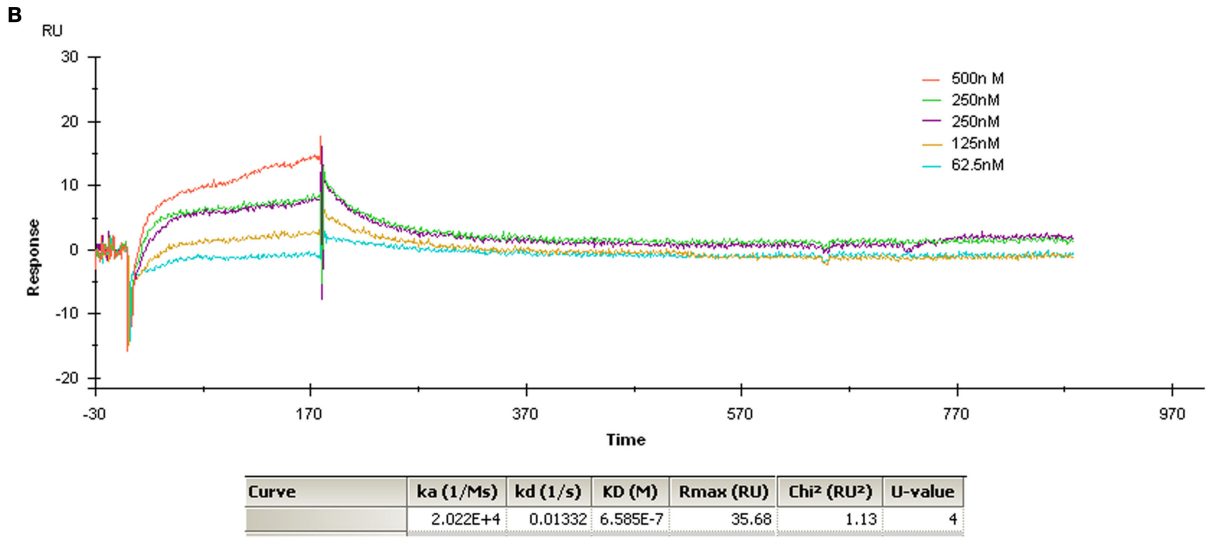
The significance of differences was analyzed by Student's *t*-test and Mean ± SD was calculated, with a value of $p < 0.05$ considered to be statistically significant.

RESULTS

Preparation of Human Anti-Siglec-9 Fab Fragments

Generally, with seven rounds of affinity panning, 50 single phages were selected and analyzed by phage ELISA. While





Curve	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Chi² (RU²)	U-value
	2.022E+4	0.01332	6.585E-7	35.68	1.13	4

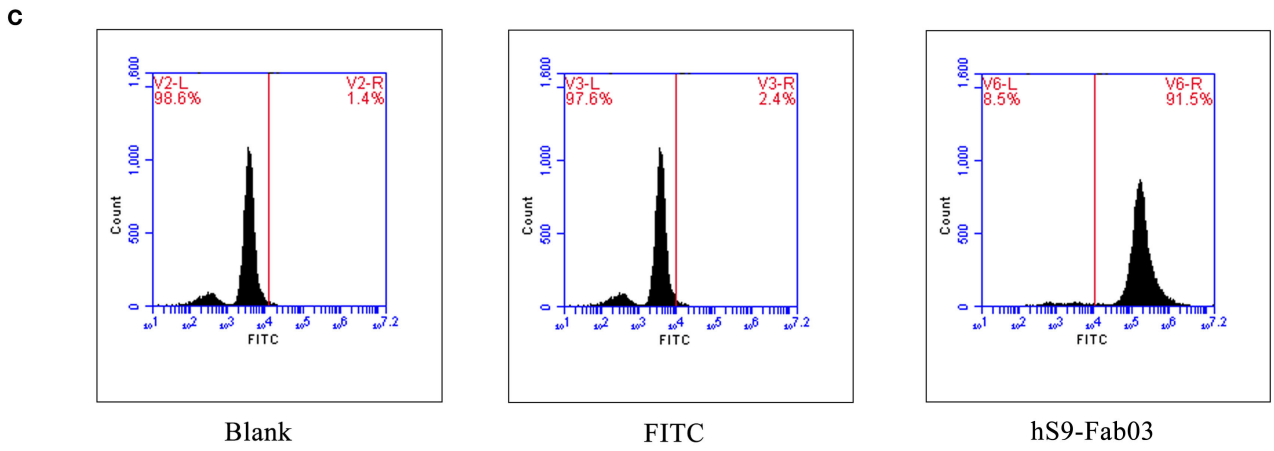


FIGURE 2 | **The hS9-Fab03 specifically binds the Siglec-9 antigen**. **(A)** 96-well plates were pre-coated with recombinant human Siglec-9, 10 μg/ml. Serial concentrations of the hS9-Fab03 were used as the primary antibody. Horseradish-peroxidase-conjugated goat anti-human antibody (Fab specific) was used as the secondary antibody. Commercial anti-Siglec-9 antibodies were used as positive control (Ctrl). The absorbance was read at 450 nm after color development. **(B)** The hS9-Fab03 was diluted with buffer solution to 30 μg/ml and then treated with a running buffer containing different concentrations of hS9-Fab03. Results were analyzed using the Biacore X100 software. **(C)** PMA-stimulated THP-1-differentiated macrophages were treated or untreated with hS9-Fab03 at 4°C for 1 h, Goat anti-human IgG (Fab specific)-FITC antibody was incubated with macrophages for 1 h in the dark at 37°C. The cells were determined by fluorescence-activated cell sorting. Experiments were repeated in triplicate. Data are shown as mean ± SD ($n = 3$, N.S., not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control).

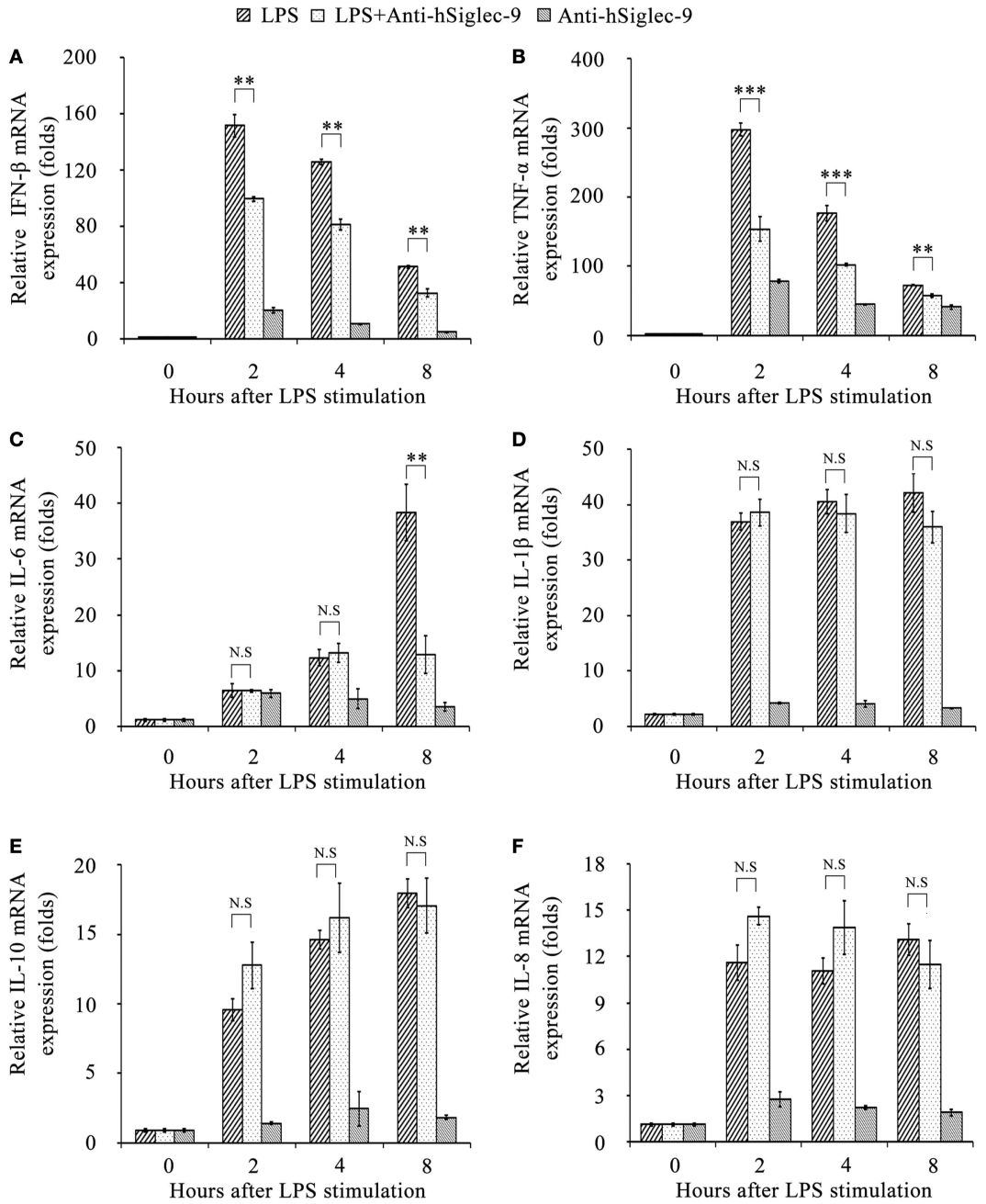


FIGURE 3 | The hS9-Fab03 inhibits lipopolysaccharide (LPS)-induced inflammatory cytokines production in THP-1-derived macrophages. PMA-stimulated THP-1-differentiated macrophages in 24 wells were pre-incubated with 5 μg/ml hS9-Fab03 for 2 h, then stimulated with 1 μg/ml LPS. Anthrax chimeric Fab antibody was used as the negative control. The mRNA expression levels of IFN-β **(A)**, TNF-α **(B)**, IL-6 **(C)**, IL-1β **(D)**, IL-10 **(E)**, and IL-8 **(F)** were detected by Q-PCR. Experiments were performed in triplicate. Data are shown as mean ± SD ($n = 3$, N.S., not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to negative control).

the positive and negative ratio was exceeded 4, the phage was considered positive. Ten positive phages were possessed, amplified, and confirmed by sequencing. After examining the sequence in VBASE2 database, the light chain was classified as lambda chain. Consequently, we amplified the V_L and C_L of selected positive clones with universe primers, which were designed by our lab as previously reported (28). The length of V_H and C_H1 were close to 400 bp (**Figure 1A**). Similarly, the length of V_L and C_L were approximately 400 and 350 bp, respectively (**Figure 1B**). Then, the heavy chain Fd and light chain L were spliced by overlap extension-polymerase chain reaction (29), which were approaching to 800 and 750 bp,

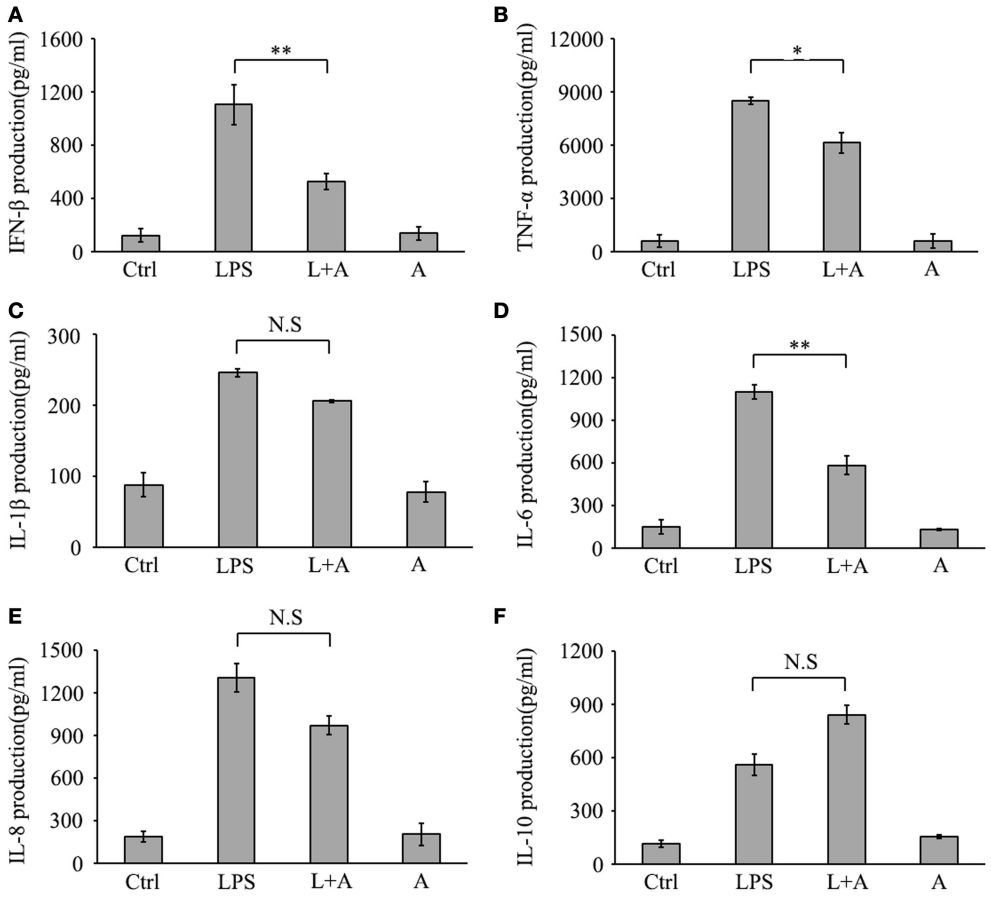


FIGURE 4 | Inhibitory effect of hS9-Fab03 on cytokine expression in cell culture supernatant of lipopolysaccharide (LPS)-stimulated THP-1-differentiated macrophages. THP-1-differentiated macrophages were pre-incubated with 5 μg/ml hS9-Fab03 for 2 h, then stimulated with 1 μg/ml LPS. Anthrax chimeric Fab antibody was used as the negative control. The production of IFN-β **(A)**, TNF-α **(B)**, IL-1β **(C)**, IL-6 **(D)**, IL-8 **(E)**, and IL-10 **(F)** in human THP-1-differentiated macrophage culture supernatant was determined by ELISA analysis. Data are shown as mean ± SD ($n = 3$, N.S., not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to negative control).

respectively. Finally, the Fd and L fragments were inserted into the prokaryotic expression plasmid pETDuet-1 sequentially affirmed by digestion and sequencing (**Figure 1C**). Therefore, the prokaryotic expression vector of human anti-Siglec-9 Fab fragments (pETDuet-hSiglec-9 Fab03) had been constructed successfully.

The vector of pETDuet-hSiglec-9 Fab03 was transformed into *E. coli* BL21 and induced with 1 mmol/l IPTG overnight at 16 or 37°C, the recombinant *E. coli* was preferred to express more Fab fragments (data not shown). Then the lysate of the recombinant *E. coli* was examined by SDS-PAGE gel with Coomassie brilliant blue staining, the bands of Fd and L were overlapped due to their similar DNA length (**Figure 1D**). To determine whether the Fab fragments were expressed in the inclusion body, western blotting was utilized. We observed that the recombinant proteins were primarily secreted in the component of ultrasonic supernatant but not that of sediment (**Figure 1E**). After purification, the Fab antibodies were presented at 27 kDa (**Figures 1F,G**). Compared with SDS-PAGE electrophoresis, Native-PAGE could reduce the possibility of protein denaturalization. As shown in **Figures 1H,I**, results showed that two obvious bands were at 27 and 55 kDa, respectively.

Considered to light chain of the recombinant protein was lambda chain; the purification of the human anti-Siglec-9 Fab fragments (hS9-Fab03) was achieved by His-trap Lambda Fab Select column. The purification efficiency of target proteins was above 95% with the concentration of hS9-Fab03 up to 1.0 mg/ml (**Figures 1J,K**). Collectively, the hS9-Fab03 was successfully expressed at high concentration to explore its affinity and immune activity.

The hS9-Fab03 Specifically Binds Siglec-9

To examine whether the hS9-Fab03 could specifically bind to Siglec-9 antigen, the ELISA assay was carried out with different concentration of hS9-Fab03, and the commercial anti-Siglec-9 antibody was used as positive control. Next, the concentration of hS9-Fab03 was diluted from 0.5 to 0.07 mg/ml. The absorbance

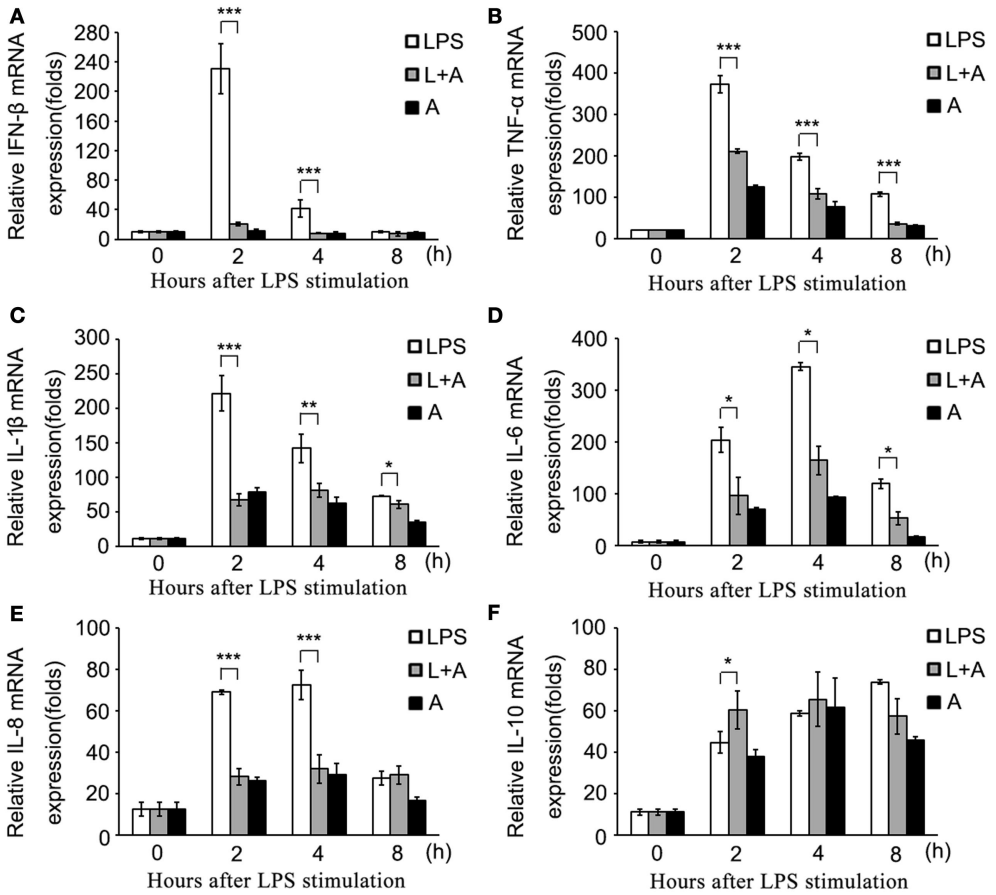


FIGURE 5 | The hS9-Fab03 inhibits lipopolysaccharide (LPS)-induced inflammatory cytokines production in human PBMC-derived macrophages. Human PBMC-differentiated macrophages in 24 wells were pre-incubated with 5 μg/ml hS9-Fab03 for 2 h, then stimulated with 1 μg/ml LPS. Anthrax chimeric Fab antibody was used as the negative control. The mRNA expression levels of IFN-β **(A)**, TNF-α **(B)**, IL-1β **(C)**, IL-6 **(D)**, IL-8 **(E)**, and IL-10 **(F)** were detected by Q-PCR. Experiments were performed in triplicate. Data are shown as mean ± SD ($n = 3$, N.S., not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to negative control).

value at 450 nm was from 1.174 to 0.216 (**Figure 2A**). Finally, the ELISA results showed that the hS9-Fab03 could particularly bind to Siglec-9 antigen in a dose-dependent manner, which was as good as the commercial antibody.

Aiming to characterize the binding capacity of hS9-Fab03, we further calculated the affinity constant using the following formula: affinity constant (KD) = dissociation constant (Kd) ÷ binding constant (Ka) (30). Results from the Biacore X100 SPR analysis demonstrated that the affinity constant of hS9-Fab03 was 6.58×10^{-7} (**Figure 2B**). Taken together, these results indicated that hS9-Fab03 could effectively bind to Siglec-9 protein.

The binding ability of hS9-Fab03 was further analyzed with Flow cytometry in Siglec-9-positive THP-1-derived macrophages. Our results revealed that the population of hS9-Fab03-treated THP-1 cells was divided from unbinding cells, whereas no apparent difference was detected in blank and FITC antibody-treated cells (**Figure 2C**). This implied that hS9-Fab03 could effectively bind to Siglec-9 expressed on the cells' surface.

The hS9-Fab03 Inhibits LPS-Induced Inflammatory Cytokines Production *In Vitro*

Human macrophages have been widely used for studying TLRs signaling (31). To investigate the role of Siglec-9 during LPS-initiated inflammatory responses, we determined the efficient pre-incubated time and optimized concentrations of hS9-Fab03 and LPS. When the concentration of hS9-Fab03 range from 0.1 to 5 μg/ml, the inhibition efficiency of IFN-β and TNF-α mRNA levels were increased in a concentration-dependent manner (Figures S1A,B in Supplementary Material). Then, when the concentration of hS9-Fab03 was at 5 μg/ml, the pre-incubated time of hS9-Fab03 was ranging from 30 min to 4 h, the inhibition efficiency of IFN-β and TNF-α mRNA levels was better at 2 h (Figures S1C,D in Supplementary Material). Furthermore, treatment with 5 μg/ml of hS9-Fab03 reduced LPS-initiated inflammatory responses by approximately 85% of IFN-β expression and by approximately 70% of TNF-α expression than without antibody

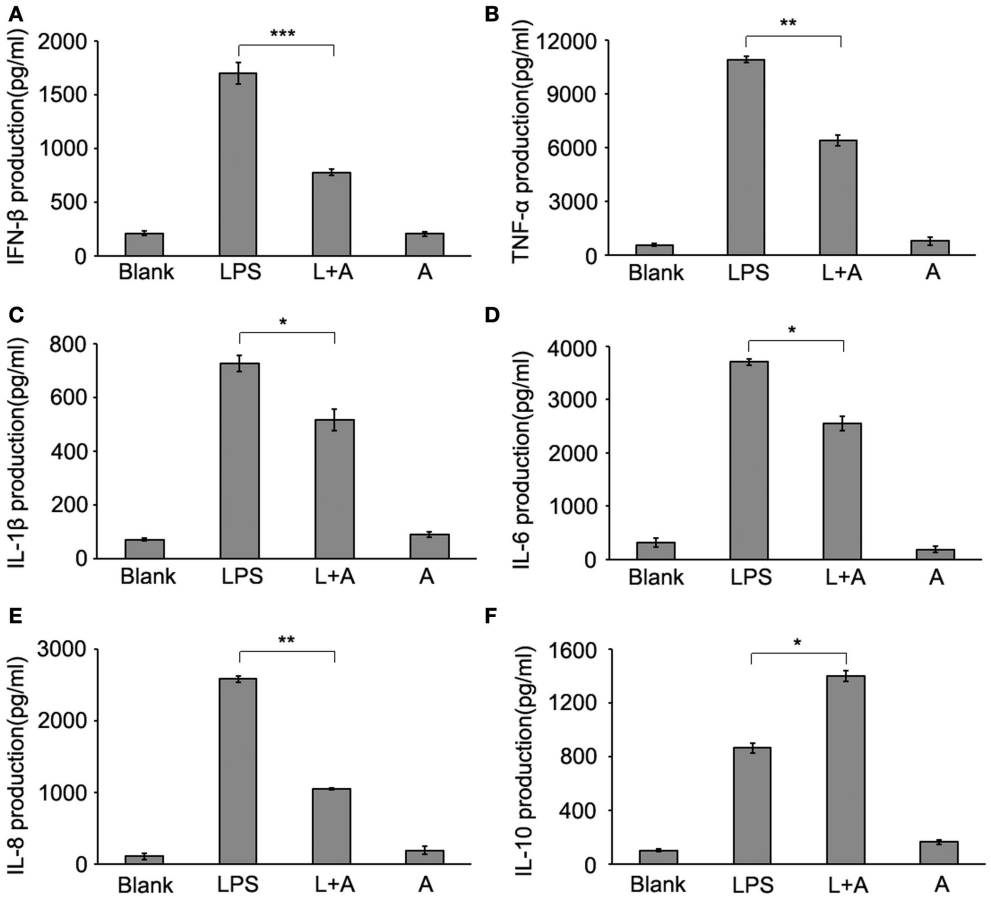


FIGURE 6 | Inhibitory effect of hS9-Fab03 on cytokine expression in cell culture supernatant of lipopolysaccharide (LPS)-stimulated human PBMC-differentiated macrophages. Human PBMC-differentiated macrophages were pre-incubated with 5 μg/ml hS9-Fab03 for 2 h, then stimulated with 1 μg/ml LPS. Anthrax chimeric Fab antibody was used as the negative control. The production of IFN-β **(A)**, TNF-α **(B)**, IL-1β **(C)**, IL-6 **(D)**, IL-8 **(E)**, and IL-10 **(F)** in human PBMC-differentiated macrophage culture supernatant were determined by ELISA analysis. Data are shown as mean ± SD ($n = 3$, N.S., not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to negative control).

treatment (Figures S1E,F in Supplementary Material). Finally, we conducted the following experiments with pre-incubation time at 2 h and the concentration of hS9-Fab03 and LPS at 5 and 1 μg/ml, respectively.

Next, we investigated whether hS9-Fab03 ligation could regulate the expression of pro-inflammatory cytokines in human macrophages. The THP-1-differentiated macrophages were pre-treated with the hS9-Fab03 and stimulated with LPS for different time. The mRNAs were collected to evaluate the expression of pro-inflammatory cytokines. We found that hS9-Fab03 could obviously inhibit LPS-initiated production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IFN-β, no effect on the expression of IL-1β, IL-8, and IL-10 in THP-1-differentiated macrophages (**Figures 3** and **4**). Similarly, we observed that pre-incubation of hS9-Fab03 could also suppress the TNF-α, IL-6, IL-1β, IL-8, and IFN-β expression of pro-inflammatory in human PBMC-derived macrophages stimulated with LPS, but slightly increased IL-10 production in an early time point compared to group with LPS only (**Figures 5** and **6**). We also incubated different concentrations of hS9-Fab03 macrophages and found that hS9-Fab03 could effectively block LPS-induced TNF-α and IFN-β expression in a dose-dependent manner (Figures S1A,B in Supplementary Material). Thus, the results implied that the hS9-Fab03 could inhibit LPS-induced pro-inflammatory cytokines production in human macrophages.

Suppression of LPS-Initiated TLR4 Signaling by the hS9-Fab03

To investigate the inhibitory effects of the hS9-Fab03 on TLR4 signaling transduction induced by LPS ligation, we detected the phosphorylation levels of NF-κB, MAPKs, and IRF-3 signaling pathways. As shown in **Figure 7**, LPS ligation significantly increased the phosphorylation level of these proteins in THP-1-differentiated macrophages, but the phosphorylation level of p65, p38, JNK1/2, and IRF-3 was suppressed when pretreated with 5 μg/ml of the hS9-Fab03. Thus, these results demonstrated that the hS9-Fab03 could suppress LPS-triggered TLR4 signaling transduction.

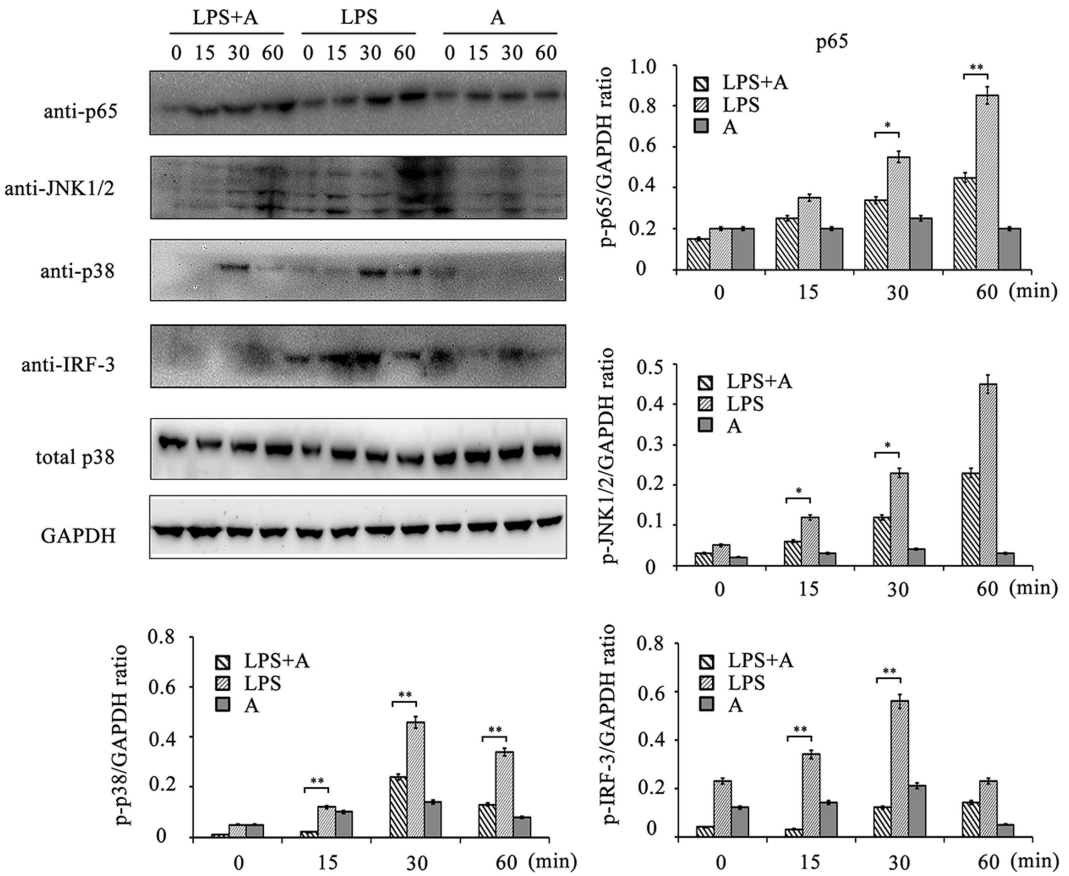


FIGURE 7 | Western blot analysis for inhibition of lipopolysaccharide (LPS)-induced NF-κB, MAPKs, and IRF-3 activation by hS9-Fab03. Cells were *pretreated* with 5 μg/ml of the hS9-Fab03 for 2 h and further incubated in presence or absence of LPS (1 μg/ml). After immunoblotting, the phosphorylation levels of p65, JNK1/2, p38, and IRF-3 were identified using phosphor-specific antibodies. Total p38 and GAPDH were used to ensure equal loading. L, LPS; A, hS9-Fab03. Data are shown as mean ± SD ($n = 3$, N.S., not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to negative control).

DISCUSSION

In this study, we utilized the human Fab phage library to construct an active anti-Siglec-9 antibody Fab fragments (hS9-Fab03) that could recognize recombinant Siglec-9 protein. The screening strategy was applied by the repeated panning with coated recombinant Siglec-9 protein in microtiter plates to assure the efficiency of specific Siglec-9 binding phage. After seven rounds of screening, 10 of 50 selected candidate phage clones displayed strong positive signal, and ELISA results indicated that these clones possess the specifically binding ability. After this, the prokaryotic expression vector pETDuet-hSiglec-9 Fab03 was successfully constructed. Our reliable approach ensured that the two different chains are comparatively expressed and efficiently dimerized into active hS9-Fab03, which ensured the production of stable antibody fragments (32). Series of western blotting and SDS-PAGE with Coomassie brilliant blue staining confirmed the efficient expression and purification of hS9-Fab03. Moreover, FACS confirmed the specifically binding ability of hS9-Fab03 to Siglec-9 protein expressed on the surface of THP-1-differentiated macrophages. Thus, these results demonstrated that the antibody engineering process couldn't change the specificity of the human anti-Siglec-9 Fab fragments.

Sepsis is a systemic infection characterized by the release of pro-inflammatory cytokines inducing systemic immune activation and coagulation activation and causing severe sepsis and septic shock (33). TLRs are fundamental for the initiation of the protective pro-inflammatory responses, which served as the first line of host defense against infection (34, 35). The uncontrolled pro-inflammatory cytokine production is critically involved in a variety of pathologies, such as sepsis and autoimmune diseases (36). TLR4 were mainly responded to PAMPs (such as LPS) and DAMPs (such as HMGB1), whose excessive activation could stimulate macrophages, DCs, and neutrophils which play a vital role in adaptive immunity by contributing to sepsis (37, 38). Accumulating data showed that inhibition of TLR4 signaling by means of antibodies or soluble decoy receptors have proven to obtain a clinical benefit to sepsis (39). In this study, we found that the production of TNF-α, IL-1β, IL-6, IL-8, and IFN-β were downregulated in hS9-Fab03-treated LPS-stimulated human macrophages compared with control group, but slightly increased IL-10 production in an early time point. The downregulation of

pro-inflammatory cytokines and the enhanced expression of IL-10 could be beneficial in preventing macrophage activation. It is well established that C/EBP and Sp1 are involved in activating the expression of IL-10 after stimulation by LPS (40). As shown in **Figure 5F**, pretreatment of hS9-Fab03 could enhance the IL-10 expression after the LPS stimulation, which might contribute to the late tolerance in human macrophages. Siglecs could negatively regulate innate immunity by recruiting tyrosine phosphatase SHP-1 and -2 through their ITIMs (41). However, we did not detect the phosphorylation level of SHP-1 or -2 in the present study. Thus, the exact roles of hS9-Fab03 in enhanced production of IL-10 remain to be further investigated.

Human antibodies are broadly used in clinical diagnosis and treatment of diseases, which are produced by various DNA recombination technologies. Meanwhile, the phage display utilizes the advantage of being inexpensive and efficient. Previously, we have constructed a fully human Fab phage library for Fab investigation, then smaller antibody fragments (Fabs or scFvs) could be comprised with phage display (9, 28). Considerable results have showed that these fragments preserve high efficiency in penetrating into the targeted tissue with high concentration and validity (42).

The exact mechanism of how hS9-Fab03 block TLR4 signaling transduction is still unknown. One possibility is that hS9-Fab03 could directly bind to the Siglec-9 and induce the activation of SHP-1 or SHP-2, whose tyrosine phosphatase activity might inhibit the phosphorylation of downstream molecules of TLR4 signaling pathway. In this case, the inhibition of LPS-induced IFN-β and TNF-α levels were increased in an antibody concentration-dependent manner (Figures S1A,B in Supplementary Material), while Western blotting data showed that hS9-Fab03 could suppress the phosphorylation of key molecules downstream of TLR4 pathway as shown in **Figure 7**. Additional studies should be performed to clarify the mechanism and exploit its biological effects *in vivo*.

In conclusion, we introduce the preparation and characteristics of a human anti-Siglec-9 antibody Fab fragment (hS9-Fab03), in this study, which could specifically bind to Siglec-9 with high affinity. Pretreatment with hS9-Fab03 could suppress LPS-stimulated TLR4 signaling transduction and inhibit the production of pro-inflammatory cytokines in human macrophages. Our data suggest ligation of Siglec-9 with hS9-Fab03 might be a novel anti-inflammatory therapeutic strategy for sepsis.

AUTHOR CONTRIBUTIONS

MW, JZ, and XZ designed the experiments. SC, XZ, NY, WZ, BC, YW, and QS performed the experiments. SC, XZ, NY, WZ, FZ, and SN analyzed the data. ZY, XZ, and CW contributed reagents/materials/analysis tools. SC and XZ wrote the paper.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00649/full#supplementary-material.

FIGURE S1 | The optimization of pre-incubation time and concentration of hS9-Fab03 and lipopolysaccharide (LPS). **(A,B)** PMA-stimulated THP-1-differentiated macrophages in 24 wells were pre-incubated with different concentrations of hS9-Fab03 for 2 h and then were stimulated with 1 μg/ml LPS. **(C,D)** The macrophages in 24 wells were pre-incubated with 5 μg/ml hS9-Fab03 with different times then stimulated with 1 μg/ml LPS. **(E,F)** The macrophages in 24 wells were pre-incubated with 5 μg/ml hS9-Fab03 for 2 h and then stimulated with different concentrations of LPS. The mRNA expression levels of IFN-β and TNF-α were detected by Q-PCR. Anthrax chimeric Fab antibody was used as the negative control. Data are shown as mean ± SD ($n = 3$, N.S., not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to negative control).

TABLE S1 | Primers used for the construction of the hS9-Fab03 gene.

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TLR7/TLR9- and B Cell Receptor-Signaling Crosstalk: Promotion of Potentially Dangerous B Cells

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B cells are capable of receptor-mediated responses to foreign antigens. Recognition of microbial-derived nucleic acid (NA) by toll-like receptors (TLRs) 7 and 9 in B cells has been substantiated. Endogenous NA released from damaged or dying cells can also be immunogenic in certain contexts and can incite aberrant activation of B cells. When TLR-driven B cell receptor (BCR)-activated B cells are not properly constrained, pathologic autoantibodies are produced. It is also clear that endosomal TLR7/TLR9 can operate in conjunction with BCR. In addition to BCR signaling, a balance between TLR7 and TLR9 is pivotal in the development of B cell autoreactivity. While TLR9 is important in normal memory B cell responses through BCR, TLR9 activation has been implicated in autoantibody production. Paradoxically, TLR9 also plays known protective roles against autoimmunity by directly and indirectly inhibiting TLR7-mediated autoantibody production. Herein, we summarize literature supporting mechanisms underpinning the promotion of pathological BCR-activated B cells by TLR7 and TLR9. We focus on the literature regarding known points of TLR7/TLR9 and BCR crosstalk. Data also suggest that the degree of TLR responsiveness relies on alterations of certain intrinsic B-cell signaling molecules and is also context specific. Because allogeneic hematopoietic stem cell transplantation is a high NA and B cell-activating factor environment, we conclude that B cell studies of synergistic TLR–BCR signaling in human diseases like chronic graft-versus-host disease are warranted. Further understanding of the distinct molecular pathways mediating TLR–BCR synergy will lead to the development of therapeutic strategies in autoimmune disease states.

Keywords: B cell signaling, TLR9, TLR7, chronic graft-versus-host disease, allogeneic hematopoietic stem cell transplantation, B cell receptor, autoantibody production, B cell biology

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INTRODUCTION

Toll-like receptor (TLR) responses to nucleic acids (NAs) have been extensively studied in monocytes and macrophages (1). In B cells, TLRs, such as TLR7 and TLR9, have been shown to mediate cell responses to both immunogenic NAs and NA-containing immune complexes (ICs) (2–4). Under normal conditions, B cells can respond immediately to initial microbial insults through NA recognition. B cells can also mount recall responses to previously encountered infectious agents and perpetuate life-long serological memory (5). However, when excessive cellular or tissue damage occurs and B cell responses to endogenous cellular NA are not restrained, autoantibodies and autoimmunity

are promoted (6). A body of evidence has elucidated cooperative TLR7 or TLR9 (TLR7/TLR9) and B cell receptor (BCR) activation in aberrantly activated B cells. Further understanding of potential molecular synergy between BCR and TLR7/TLR9 pathways in B cells will enable development of agents that can potentially prevent autoimmune states in patients.

TLR7 AND TLR9 ACTIVATION VERSUS ATTENUATION OF AUTOREACTIVE B CELLS

A number of murine models have been employed to substantiate the roles of TLR9 and/or TLR7 in the production of DNA-associated and RNA-associated autoantibody production, respectively (**Table 1**). TLR7-deficient autoimmune-prone mice display reduced or absent RNA-associated antibodies, whereas Tlr9-deficient mice have lower amounts of anti-nucleosome and anti-chromatin antibodies (7–9). A pathogenic role for TLR7 was revealed *via* characterization of the Y chromosome-linked autoimmune accelerating (Yaa) mouse that has known TLR7 overexpression due to gene duplication (10, 11). When Yaa are combined with systemic lupus erythematosus (SLE) mice and the *Tlr9* gene knocked out, mice have increased RNA-associated antibodies, exacerbated clinical symptoms, and accelerated mortality (12). Unexpectedly, in all autoimmune-prone mouse models, including MRL/lpr, B6/lpr, Balb/c-Pristane, B6.Nba2.Yaa, B6 Yaa, and Ali5 deficient in TLR9, RNA-associated antibodies are increased, suggesting a more complex role for TLR9 in SLE (8, 9, 12–17). In fact, on an autoimmune-prone background, *Tlr9* deficiency alone leads to overall increased immune activation, exacerbation of pathogenesis, and in some cases increased mortality (8, 9, 12–15). By contrast, *Tlr7*-deficiency in autoimmune-prone mice leads to a significant decrease in overall immune activation and disease severity (9, 14). Thus, TLR7 and TLR9 have opposing pathogenic and protective roles, respectively, in autoimmune disease.

Nundel et al. found that TLR9 directly constrains BCR–TLR7-dependent responses, suggesting a B-cell intrinsic protective role for TRL9 (18). By contrast, *Tlr7*-deficient B cells are not responsive to DNA-containing ICs and have increased death rates. Interestingly, BCR–TLR9-mediated post-proliferative cell death of B cells when TLR7 is absent can be blocked by the TNF family survival cytokine B cell-activating factor (BAFF). Nickerson et al. observed that TLR9 was associated with anti-dsDNA B cell sequestration and deletion, corroborating a protective role for TLR9 (19). The relative contributions of B cell-intrinsic TLR7 and TLR9 on autoimmunity were addressed by Jackson et al. This group generated mixed bone marrow (BM) chimeras by adoptively transferring BM from wild type, Wiskott–Aldrich syndrome (WAS) protein-deficient, *Was*-deficient-*Tlr7*-deficient, or *Was*-deficient-*Tlr9*-deficient mice with μMT BM (20:80) into lethally irradiated μMT recipient mice (20). In this chimeric WAS model, B cells were the predominant cells rendered WAS-deficient and hyperactive. Since immune dysregulation and autoimmunity was largely confined to the B cell compartment, results suggest that the TLR9 and TLR7 effects were B-cell intrinsic (20). Further studies in B cell-specific knockout models are needed to clarify any impact from the 20% myeloid cells also found in this TLR7/TLR9-deficient chimeric model (20). Together, data highlight a need to better understand the molecular mechanisms that underpin pathological or protective responses of TLR7/TLR9 responses in B cells.

TABLE 1 | TLR7/TLR9 responses have substantiated roles in both autoantibody production and autoimmunity, especially in B cell receptor (BCR)-activated B cells.

	TLR7 and TLR9 functions in B cell autoimmunity	Reference
TLR7	RNA-associated antigen recognition	(11)
	RNA-associated autoantibody production	(9)
	Pathogenic role in development of autoimmunity (murine models)	(7, 14, 18, 20)
	1. Increased IgG production 2. Increased immune (B and T) cell activation 3. Promoted survival of plasmablast/antibody forming cells 4. Increased systemic lupus erythematosus (SLE)-related mortality and pathogenesis	
TLR9	Endogenous double-stranded deoxyribonucleic acid (dsDNA) and chromatin antigen recognition	(8, 9, 14)
	Anti-dsDNA and chromatin-associated autoantibody production	
	Protective role in autoimmunity (by limiting potentially pathogenic role of TLR7 in murine models)	(12, 13, 15–18, 20)
	1. Attenuated IgG production (both total and pathologic) 2. Decreased immune activation 3. Induced of B cell tolerance and cell death 4. Decreased SLE-related mortality and pathogenesis	
	Functional synergy of BCR–TLR7/TLR9 pathways	
BCR–TLR7/TLR9	BCR activation results in increased TLR9	(29)
	BCR dictates subcellular location of TLR9	(28)
	BCR and TLR7/TLR9 increases proliferation, cytokine, and autoantibody production	(25–27, 29, 32)
	BCR and TLR7 operate together to confer autoimmunity, by attenuating TLR7 tolerance	(35)
	BCR and TLR9 synergize to confer central tolerance	(36)
	Proximal BCR-signaling components and TLR7/TLR9 autoimmune responses	
	Syk inhibition of B cells blocked the CpG response	(40)
	Btk and Syk mediate TLR crosstalk	(38, 41, 42)
	Btk is dispensible for TLR7 and 9 (ligands and immune complex) proliferation	(39)
	Lyn negatively regulates:	(44–46)
	1. Both anti-RNA and anti-dsDNA antibody production (both global deletion and B-cell specific) 2. IgG class-switching 3. B cell activation 4. Cytokine production (pro-inflammatory) 5. Autoimmune pathology	

TLR7– AND TLR9–BCR RESPONSES ARE LIMITED BY AVAILABILITY AND TRAFFICKING OF NA LIGAND

TLR7 and TLR9 are located in endosomal compartments and as a consequence, are usually sequestered away from

NA-associated ligands. Immunogenic NA is derived from microbes or from damaged or dying cells located in the extracellular matrix (21). In both the physiological and autoimmune settings, endogenous NAs are more likely to form complexes with proteins or antibodies. As depicted in **Figure 1A**, TLR7/TLR9 ligands like NA-bound proteins can be brought into the B cell *via* several potential mechanisms. Endocytosis of NA-bound protein and diffusion of a synthetic agent (e.g., imiquimod/R848 or CpG) are known examples. Alternatively, NA or NA-ICs can be recognized and internalized by BCRs or Fc receptors and then presented to endosomal TLR7 or TLR9 for subsequent activation (6, 21). Trafficking of TLR7 and TLR9 from the endoplasmic reticulum to endosomal compartments is tightly regulated by the chaperone protein, UNC93B1 (22). The balance of TLR7:TLR9 determines downstream effector function in part because of outcompetition of TLR9 binding to UNC93B1 (23, 24).

Dual engagement of BCR and activation of TLR7/TLR9 were first shown in seminal papers by Marshak-Rothsteins' group (25, 26). These investigators employed transgenic (Tg) mice that express rheumatoid factor (RF) AM14 BCR. AM14 BCR specifically binds with low affinity to IgG2a that is bound to endogenous or synthetic, highly purified NA. These IgG-NA ICs are "dual specific" and bind to BCR and various forms of NA (chromatin, dsNDA, RNA, SnRNP). A series of studies using this unique set of tools has now substantiated a requirement for BCR-IC internalization in TLR7/TLR9-mediated autoantibody production (25–27).

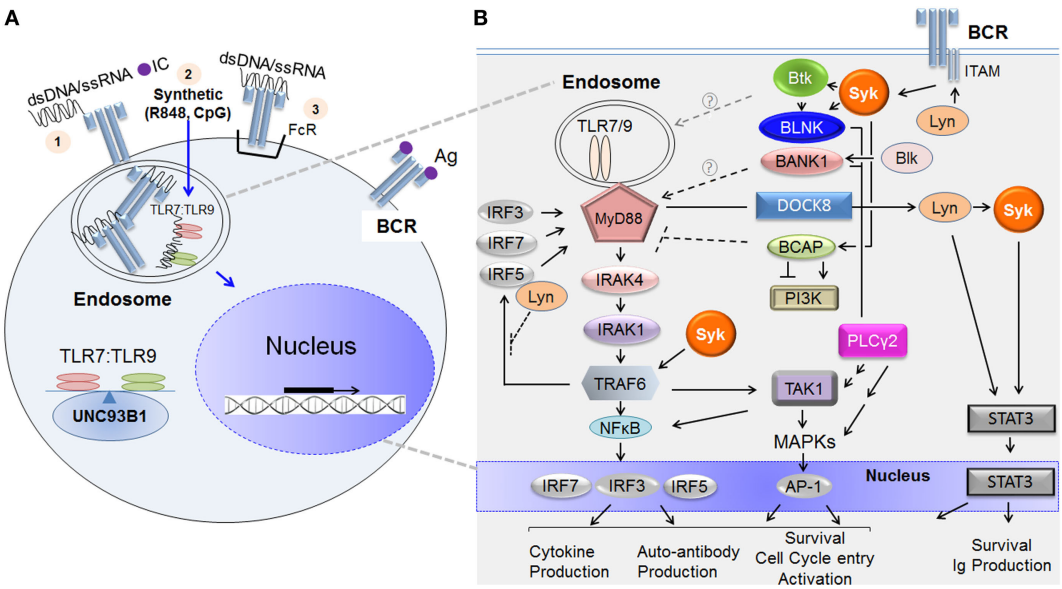

FIGURE 1 | TLR7/TLR9 and B cell receptor (BCR) ligands incite B cell signaling cascades. **(A)** Depiction of how immunogenic extracellular nucleic acid (NA) antigens are internalized to activate endosomal TLR7 and TLR9. NA antigens comprising double-stranded deoxyribonucleic acid (dsDNA) or single-stranded ribonucleic acid (ssRNA) or synthetic analog reach the endosomal compartment *via* any of the following potential mechanisms: (1) membrane uptake of immune complex (IC) (NA protein or antibody); (2) diffusion (synthetic compounds or oligonucleotides), or (3) BCR- or Fc receptor (FcR)-mediated internalization when either is recognized directly. Once internalized, the NA component of the IC binds the appropriate toll-like receptor (TLR) molecule, initiating dimerization and signal transduction. TLR7/TLR9 and BCR pathway stimulation leads to the activation of nuclear factors and to transcription of additional activation genes, such as mediators of proliferation and effector cytokines. Trafficking of TLR7 and TLR9 from the endoplasmic reticulum to endosomal compartments is tightly regulated by the chaperone protein, UNC93B1. **(B)** The major molecular activators immediately downstream of the BCR and TLR7/TLR9 and molecular points of crosstalk between the two signaling pathways. On the left-hand side, initial activation of TLR7/TLR9 by NA in the endosome leads to the recruitment and binding of MyD88 to their intracellular domains. This TLR7/TLR9 activation leads to Myddosome complex composed of MyD88, IRAK1, IRAK4, and subsequent recruitment of TNF receptor-associated factor 6 (TRAF6), each activated sequentially. Ubiquitinated TRAF6 associates with and polyubiquitinates the TAK1 complex (including proteins TAB 1 and TAB 2, not depicted). TAK1 then undergoes autophosphorylation, initiating the MAPK or NFκB pathways. These pathways can each result in the activation of important transcription factors including NFκB, AP-1, and IRFs (3, 5, and 7) that govern B cell fate. On the right-hand side, BCR ligation activates proximal kinase proteins including Lyn, Blk, Syk, and Btk. These kinases phosphorylate adaptor molecules including, BLNK, BCAP, and BANK1, which function as scaffolding proteins and allow for the many divergent pathways activated downstream of BCR including PLCγ2, MAPK, PI3K, and NFκB pathways. Molecules known to convey crosstalk between the BCR–TLR pathways upon ligation by NA-ICs include Lyn, Syk, Btk, BANK1, BCAP, TAK1, and DOCK8. The proposed mechanism of positive or negative regulation of TLR signaling is shown. Key: arrows = activation; multiple arrows = indirect activation; perpendicular lines = inhibition; broken lines = unknown in B cells because published work done on non-B cells; solid lines = protein association; gray broken arrow = contradictory regulation of TLR signaling; circled question-mark = mechanism of TLR regulation unknown. Abbreviations: Lyn, Lck/Yes-related novel protein tyrosine kinase; Blk, B-lymphoid tyrosine kinase; Syk, spleen tyrosine kinase; Btk, Bruton tyrosine kinase; BLNK, B cell linker protein; BCAP, B cell adaptor for phosphoinositide 3-kinase; BANK1, B cell scaffold protein with ankyrin repeats 1; PLCγ2, phosphoinositide-specific C phospholipase gamma 2; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; TAK1, TGFβ-activated kinase 1; MAPK, mitogen-activated kinase; MyD88, myeloid differentiation primary response gene 88; IRAK, interleukin-1 receptor-associated kinase; TRAF, TNF receptor-associated factor; IRF, interferon regulator factor.

TLR7/TLR9 ACTIVATION OF B CELLS RELIES ON BCR ACTIVATION IN CERTAIN CONTEXTS

The role of the BCR is not simply to internalize and present NA antigen. After BCR activation, both total and endosomal TLR9 levels increase, suggesting that BCR directly regulates TLR9 (28, 29) (**Table 1**). Several signaling molecules downstream of BCR operate in concert with TLR pathways to modulate TLR responses (30, 31). In the healthy state, dual BCR and TLR7/TLR9 engagement confer synergistic responses, including cytokine production, antibody production, and class-switch recombination (32, 33). In autoimmune disease, synergistic BCR–TLR7/TLR9 activation by NA-IC results in increased B cell proliferation and autoantibody production (25–27). For full activation of autoreactive RF-B cells, combined signals from the BCR and either TLR7/TLR9 are required (30, 34). Dual engagement of BCR and TLR9 by chromatin-IC leads to distinct functional outcomes (29). BCR activation can operate with TLR7 to attenuate peripheral B cell tolerance (35). Conversely, BCR–TLR9 synergy induces central tolerance through AID expression in autoreactive immature/T1 B cells (36). Together, data reveal a pivotal role for BCR in regulating TLR7 and TLR9 responses for maintaining the balance between normal and pathological B cell activation. A unique pattern of gene expression induced after co-stimulation with BCR and chromatin-ICs versus stimulation with TLR9 or BCR alone further suggests synergistic BCR–TLR signaling (29) Specific molecular mechanisms underpinning BCR–TLR signaling crosstalk are emerging as summarized below.

PROXIMAL BCR-SIGNALING COMPONENTS IMPORTANT FOR TLR7/TLR9 AUTOIMMUNE RESPONSES

Data reveal that BCR-proximal kinases Syk, Btk, and Lyn are involved in BCR–TLR7/TLR9 crosstalk (**Table 1**). The proximal BCR-signalosome molecules, Syk and Btk, have been associated with TLR7/TLR9 activation (37–39). In human B cells, Syk appears to be a positive regulator of TLR and TLR–BCR synergism, since inhibition of Syk blocks TLR responses (40, 41). The role of Btk in BCR–TLR signaling is less clear. Btk has been implicated in downstream signaling of TLRs, including TLR9 in B cells (42). Studies in the autoimmune setting revealed that Btk was dispensable for TLR7/TLR9 or BCR–TLR IC responses. In the absence of *Btk*, AM14-Tg B cells had diminished responses to BCR–TLR IC that was related to increased overall cell death, rather than to the level of activation of individual B cells (39). Lyn, a src kinase molecule associated with the positive and negative regulation of the BCR pathway (43) has been shown to negatively regulate TLR7/TLR9 activation (44–46). *Lyn*-deficient or B cell-specific *Lyn*-deficient mouse models had increased NA-associated autoantibodies, cytokine production (including IL-6 and IL-10), and autoimmune pathology (44–46). The exact mechanism of this negative regulatory role in TLR signaling has not been defined in B cells, although it is well established that Lyn is required to phosphorylate and activate CD22, an important negative regulator of BCR signaling (43). While the mechanistic role for Btk remains somewhat contradictory, roles for Lyn and Syk are more defined.

BCR AND TLR7/TLR9 SIGNALING CASCADES: CROSSTALK AND POTENTIAL ABERRANT B CELL ACTIVATION

Figure 1B is a simplified depiction of the major molecular components TLR7/TLR9 and BCR signaling. As depicted on the left in **Figure 1B**, TLR7 and TLR9, unlike BCR, signal in a MyD88-dependent fashion. After ligand associates with TLR7 or TLR9 in the endosomal compartment, TLR monomers dimerize and recruit the adaptor protein MyD88 to the intracellular domains. MyD88 then binds the kinase interleukin-1 receptor-associated kinase (IRAK)-4, promoting its autophosphorylation. IRAK4 subsequently associates with and phosphorylates IRAK1 (47). The resultant multimeric MyD88–IRAK4–IRAK1 complex (often referred to as the "Myddosome") is critical for downstream effector signaling. Phosphorylation of IRAKs is required for recruitment of the E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) to the complex (48). TRAF6, together with two other ubiquitin-conjugating enzymes (not depicted), itself becomes ubiquitinated before translocation into the cytosol where it activates transforming growth factor-beta-activated kinase 1 (TAK1) (48). TAK1 activation results in the phosphorylation and subsequent activation of MAPKs and/or NFκB. NFκB activation leads to phosphorylation of downstream transcription factors, including interferon regulatory factors (IRFs)—IRF3, IRF5, or IRF7. After phosphorylation by TRAF6, these IRFs are translocated to the nucleus where they ultimately determine cell fate (47, 49, 50). As shown on the right in **Figure 1B**, BCR activation incites signaling through activation of proximal BCR molecules Syk, Lyn, and Btk. When soluble antigen ligates the BCR or when BCR is cross-linked by anti-IgM surrogate antigen, several src kinases including Lyn are rapidly activated. In turn, immuno-receptor tyrosine-based activation motifs (ITAM) within the cytoplasmic domains of the CD79a/CD79b heterodimer complex of the BCR are phosphorylated by Lyn (43). Dual phosphorylation of ITAM tyrosine residues allows the association and subsequent activation of Syk tyrosine kinase (51). Syk can then associate with and activate a number of other kinases, including Btk and adaptor proteins such as B cell linker protein (BLNK) and B cell adaptor for phosphoinositide 3-kinase (BCAP) (51). Another adaptor protein, B cell scaffold protein with ankyrin repeats 1 (BANK1) is primarily activated by B-lymphoid tyrosine kinase (Blk) (52). Adaptor proteins, BANK1 and BCAP, lack kinase activity but function as scaffold proteins in the formation of macromolecular complexes that enable efficient effector signal transduction. Subsequent downstream signals include PLCγ2 activation, calcium mobilization, MAPK, NFκB pathways, and BCAP–PI3K-mediated pathways (52–54).

Improved understanding of distinct molecular mediators of BCR–TLR crosstalk in normal versus aberrant B-cell signaling is emerging. As shown in **Figure 1B**, BCR-proximal kinases Lyn and Syk have been specifically implicated in molecular BCR–TLR7/

TLR9 crosstalk. Lyn has been shown to negatively regulate TLR activation *in vivo* (44–46). While not yet studied directly in B cells, the molecular mechanism may be similar to that found in dendritic cells, where Lyn directly associates with IRF5 and in doing so, inhibits the ability of TRAF6 to associate with and activate the transcription factor (55). Syk is a positive regulator of TLR signaling (40, 41). Syk activation has been associated with TRAF6 expression in B cells from patients with SLE (38). The association between Syk and TRAF6 suggests an important point of crosstalk in the context of autoimmune disease, suggesting a potential mechanism for how Syk blockade attenuates the TLR9 responses (40).

B cell adaptor proteins, BCAP and BANK1, are also potential components of BCR-endosomal TLR signaling crosstalk (**Figure 1B**). BCAP negatively regulates inflammatory responses mediated by TLRs 4, 7, and 9 by linking TLR–PI3K pathways (56–58). This has been shown to occur through a hidden TIR domain in the full-length BCAP protein, which allows its direct association with TLR adaptor proteins (58, 59). Recently, Halabi et al. published that BCAP binds directly with TLR adaptor proteins to facilitate PLCγ2- and PI3K-mediated depletion of the cell membrane phospholipid component of macrophages (59). Without these phospholipid substrates, the TLR adaptor proteins could not associate with the cell membrane. This potentially results in inhibition of subsequent signal transduction (59). Similar mechanisms downstream of endosomal TLR7/TLR9 may be utilized by B cells, but this requires further investigation. B cell adaptor proteins may also positively regulate TLR7/TLR9. BANK1 in B cells has been shown to augment TLR7/TLR9 signaling; however, the exact mechanism remains unknown (60, 61). Splenic B cells from *Bank1*-deficient mice had significantly decreased TLR9 responses mediated *via* a p38MAPK-dependent mechanism (61). This positive regulatory role of BANK1 was further supported in the autoimmune setting. *Bank1*-deficient mice crossed with B6Sle1.Yaa mice (the TLR7 overexpressing autoimmune model) resulted in reduced IgG production (importantly the pathogenic-associated IgG2a isotype) and reduced IL-6 and BAFF serum levels (60). This was confirmed to be B-cell intrinsic, as *ex vivo* TLR7 responses were impaired (60). Thus, early evidence has identified important roles for BCR adaptor proteins in aberrant B cell responses to TLR in autoimmune disease.

Two other proteins have been implicated in BCR–TLR crosstalk. TAK1 has been suggested to be at the exact point of synergism between the BCR–TLR9 pathways (62). Synergistic BCR–TLR9 activation was abrogated when TAK1 was inhibited and silenced (62). Jabara et al. identified the adaptor dedicator of cytokinesis-8 (DOCK8) as the link between TLR9 and STAT3 activation in B cells (63). This link was dependent upon the BCR-signaling components Lyn and Syk (63). CpG activation of TLR9 induced migration of the preexisting complex consisting of MyD88, DOCK8, and Pyk2 to the cytoplasmic tails of TLR9. This association induced phosphorylation of DOCK8 by Pyk2 and allowed Lyn to bind to DOCK8. Binding of DOCK8 to Lyn leads to subsequent activation of Syk, and activation of STAT3, resulting in gene expression important for long-lived memory B cell survival and antibody production (63). Importantly, DOCK8 was not required for initial BCR signaling. This suggests a pivotal role for the integration of DOCK8 and TLR–BCR signaling cascades in BCR activation by low affinity antigen, although studies examining simultaneous BCR and TLR9 activation are required to address this. Thus, in the physiological context NA-ICs when low affinity BCR is activated before NA is presented to TLR9, DOCK8, Syk, and Lyn may all play even more significant roles in integration of BCR–TLR9 signaling.

FUTURE STUDIES: TLR–BCR-MEDIATED AUTOIMMUNITY IN HUMANS

We now know there are a number of molecular links between BCR and TLR7/TLR9 signaling. Further studies are required to determine distinct pathologic signaling pathways so that agents can be used to block aberrant TLR/BCR signaling in patients. Future studies should address mechanisms that restrain potentially dangerous responses to antigen in autoimmune-provoking environments. To do this, more physiological research tools are required to further define mechanisms of TLR–BCR signaling, particularly for studies of human B cells. Many studies use anti-IgM surrogate antigen for the BCR-activation component, which does not precisely recapitulate the more physiological setting of BCR–TLR activation by ICs that requires internalization. Additional technical challenges need to be addressed. Potentially pathogenic B cells are already in an activated state, hampering the ability to stimulate and delineate meaningful mechanistic studies *ex vivo* without inducing cell death. Gene knockdown is also challenging in primary disease-state B cells. Until these technical and logistical barriers are overcome, studies of synergistic BCR–TLR signal transduction in the physiological setting in human B cells remain challenging.

Understanding B-cell intrinsic BCR–TLR signaling and activation in the context of human disease will also require investigation of extrinsic factors involved in the promotion of autoreactive B cells. BAFF plays a pivotal role in B cell development and the maintenance of B cell homeostasis (64). Elevated BAFF levels have been implicated in breaking B cell tolerance in systemic autoimmune diseases including SLE and Sjorgren's syndrome (SS) (65). Elevated serum BAFF levels have been correlated with circulating autoantibodies, disease progression, and anti-dsDNA antibodies in SLE patients (66, 67) and with autoantibody levels in SS patients (68). One mechanism by which BAFF breaks this tolerance in a lupus-like disease model has been shown to be TLR-dependent (69). Data suggest a model whereby excess BAFF expands autoreactive B cells, and BAFF signals directly promote TLR activation and internalization of dsDNA or NA-IC autoreactive BCRs. BAFF also increases TLR7/TLR9 expression, and TLR7/TLR9 signaling promotes BAFF receptor expression, thus providing a positive feedback loop (69). Further study will elucidate how the extrinsic factor BAFF dysregulates intrinsic BCR–TLR B cell signals and promotes aberrant B cell activation and pathogenesis.

IMPLICATIONS FOR A DISEASE THAT DEVELOPS IN AN AUTOIMMUNE-PROVOKING ENVIRONMENT: ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (HCT)

Current evidence as summarized above compels examination of aberrant B cells in patients with autoimmune pathology. This includes patients who develop chronic graft-versus-host disease (cGVHD) that develops after HCT. cGVHD is a B-cell mediated autoimmune disease-like state that is unacceptably debilitating and difficult to treat. Persistently altered B cell homeostasis in patients with cGVHD is potentially perpetuated by global B cell depletion strategies (70–72). In cGVHD, intrinsic abnormalities in the proximal BCR machinery of B cells are being defined. Thus, we and others are interested in developing ways to target only B cells from patients with cGVHD that are hyperactivated and primed for survival *in vivo via* BAFF- and BCR-associated pathways (73). Based on murine and human studies that demonstrated a role for BCR-activated B cells in the pathophysiology of cGVHD (74, 75), the novel application of signaling pathway inhibitors is being tested in clinical trials.

After HCT, B cells are recovering in an NA and alloantigen (76) rich environment that may promote pathological B cells. Circulating monocytes in cGVHD patients upregulate gene pathways involved in innate cellular damage responses (77). Some of these genes include TLR7, BAFF and Type 1 interferons. No definitive examination of TLR7/TLR9 in cGVHD has yet been performed, but studies suggest that there is a muted signaling response to TLR9 agonists by plasmablast-like cells that normally regulate immune responses *via* the production of cytokines including IL-10 (78). We conclude that studies of cGVHD addressing TLR9 and TLR7 signaling of BCR-activated B cells after HCT are warranted. Such studies will inevitably lead to further understanding of human B cell tolerance and will likely compel the expanded use of targeted therapeutic agents in patients.

AUTHOR CONTRIBUTIONS

SS and AS both researched the topic, wrote and edited the manuscript, and made the table and figure for this manuscript.

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REVIEW
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Canonical and Non-Canonical Activation of NLRP3 Inflammasome at the Crossroad between Immune Tolerance and Intestinal Inflammation

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Several lines of evidence point out the relevance of nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain-containing protein 3 (NLRP3) inflammasome as a pivotal player in regulating the integrity of intestinal homeostasis and shaping innate immune responses during bowel inflammation. Intensive research efforts are being made to achieve an integrated view about the protective/detrimental role of canonical and non-canonical NLRP3 inflammasome activation in the maintenance of intestinal microenvironment integrity. Evidence is also emerging that the pharmacological modulation of NLRP3 inflammasome could represent a promising molecular target for the therapeutic management of inflammatory immune-mediated gut diseases. The present review has been intended to provide a critical appraisal of the available knowledge about the role of canonical and non-canonical NLRP3 inflammasome activation in the dynamic interplay between microbiota, intestinal epithelium, and innate immune system, taken together as a whole integrated network regulating the maintenance/breakdown of intestinal homeostasis. Moreover, special attention has been paid to the pharmacological modulation of NLRP3 inflammasome, emphasizing the concept that this multiprotein complex could represent a suitable target for the management of inflammatory bowel diseases.

Keywords: canonical, non-canonical, NLRP3, bowel inflammation, intestinal homeostasis, immune system, enteric microbiota

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INTRODUCTION

A growing body of evidence highlights the relevance of the nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain-containing protein 3 (NLRP3) inflammasome in the pathophysiology of several autoinflammatory syndromes [i.e., cryopyrin-associated autoinflammatory syndromes (CAPS), Schnitzler's syndrome], as well as metabolic and/or inflammatory disorders (i.e., obesity, atherosclerosis, type 2 diabetes, gout, and intestinal inflammation) (1–3). In the setting of intestinal microenvironment, NLRP3 inflammasome plays a pivotal role both in regulating the integrity of intestinal homeostasis and in shaping innate immune responses during bowel inflammation (3). In particular, the NLRP3 inflammasome, through the adaptor protein apoptosis-associated

speck-like protein (ASC), recruits and activates caspase-1 leading to processing and release of IL-18 and IL-1β. These are two key cytokines involved both in the control of immune tolerance and support to immune and tissue events occurring in the presence of inflammation (4). This pathway is currently designated as "canonical NLRP3 inflammasome activation." In addition, a "non-canonical NLRP3 inflammasome activation," which depends on caspase-11 in mice (human orthologs are caspase 4 and caspase 5), has been described to be pivotal in the maintenance of intestinal immune homeostasis (5).

Several lines of preclinical evidence have unraveled a dual role of NLRP3 inflammasome in the pathogenesis of bowel inflammation (6). In particular, some studies showed a regulatory and reparative role of NLRP3 in the maintenance of immune tolerance and epithelial barrier integrity (7, 8). Conversely, others reported that the overactivation of NLRP3 inflammasome during intestinal inflammation is associated with a breakdown of intestinal immune balance, with consequent detrimental effects to the host (9). In this context, research efforts are currently being focused on a better understanding of the role of canonical and non-canonical NLRP3 inflammasome in the pathophysiology of intestinal inflammation.

Based on the above background, the present review has been intended to provide an integrated and critical appraisal of the available knowledge about the protective or detrimental role of canonical and non-canonical NLRP3 inflammasome activation in the maintenance of intestinal homeostasis as well as in sustaining the pathophysiological events underlying bowel inflammation. Special attention has been paid to point out how NLRP3 inflammasome influences the dynamic interplay between microbiota, intestinal epithelium, and innate immune system, as well as how the pharmacological modulation of this enzymatic complex could represent a suitable strategy in the management of inflammatory bowel diseases (IBDs).

MECHANISMS OF CANONICAL AND NON-CANONICAL NLRP3 INFLAMMASOME ACTIVATION

Canonical NLRP3 inflammasome activation requires two parallel and independent steps: transcription and oligomerization (**Figure 1**) (10). The first step is regulated by innate immune signaling, mediated primarily by toll-like receptor (TLR)-adaptor molecules myeloid differentiation primary response 88 (MyD88) and/or cytokine receptors, such as the tumor necrosis factor receptor, which, in turn, activate pro-IL-1β and NLRP3 transcription *via* nuclear factor-κB (NF-κB) activation (11). The second step results in NLRP3 inflammasome oligomerization, leading to caspase-1 activation and, in turn, IL-1β and IL-18 processing and release (12). Various stimuli associated with infections, including an increase in extracellular adenosine triphosphate (ATP), extracellular osmolarity or pH alterations, β-amyloid fibers and degradation of extracellular matrix components, increase in potassium efflux, reactive oxygen species (ROS), cathepsin activation, and deubiquitination, can promote NLRP3 inflammasome oligomerization and activation by initiating assembly of a multiprotein complex consisting of NLRP3, the adaptor protein ASC, and pro-caspase-1. The recruitment of ASC is pivotal for the activation of pro-caspase-1 into its cleaved form (13–18). Caspase-1 activation promotes also, independently from IL-1β maturation, pyroptosis, a key defense mechanism against microbial infections, which blocks the replication of intracellular pathogens *via* cytoplasmic swelling and promotes phagocytosis of surviving bacteria (19–21). In particular, recent evidence has shown that caspase-1 cleaves the linker between the amino-terminal gasdermin-N and carboxy-terminal gasdermin-C domains in gasdermin D, an acid cytoplasmic protein, which plays a critical role in the process of pyroptosis (22, 23). Pyroptosis then promotes the release of additional cytosolic proteins, such as high mobility group box 1 (HMGB1) alarmin, a pro-inflammatory mediator significantly involved in the pathogenesis of several inflammatory chronic diseases (**Figure 1**) (24–26).

Besides canonical NLRP3 inflammasome activation, a non-canonical caspase-11-dependent NLRP3 activation has been characterized (**Figure 1**) (5). In particular, Gram-negative bacteria (i.e., *Citrobacter rodentium*, *Escherichia coli*, *Legionella pneumophila*, *Salmonella typhimurium*, and *Vibrio cholerae*) activate the TLR4–MyD88 and toll/IL-1 receptor homology-domain-containing adapter-inducing interferon-β (TRIF) pathways, with a consequent nuclear translocation of NF-κB, which in turn promotes the transcription of IL-1β, IL-18, and NLRP3 as well as interferon regulatory factor (IRF)-3 and IRF7 genes (27, 28). Subsequently, the IRF3–IRF7 complex elicits the expression of interferon (IFN)-α/β, which binds the IFN-α/β receptor 1 (IFNAR)/IFNAR2 receptor leading to activation of the JAK/STAT pathway and consequent transcription of caspase-11 gene (19, 29–31). In addition, binding of lipopolysaccharide (LPS) to caspase-11 and/or as-yet-unidentified scaffold proteins or receptors induced by Gram-negative bacteria, escaping phagosomes, have been shown to activate the effector functions of caspase-11 (32, 33). In particular, once activated, caspase-11 induces pyroptosis through cleavage of gasdermin, as well as HMGB1 and IL-1α release, and promotes IL-1β processing and release through activation of the NLRP3-ASC-caspase-1 pathway (**Figure 1**) (22, 23, 32).

These different NLRP3 activation processes occur independently. However, caspase-11 enhances the canonical caspase-1 processing and IL-1β/IL-18 production in the presence of specific stimuli (e.g., *cholerae toxin* or *E. coli*) (5, 22). In this setting, further *in vitro* experiments on cultured cells should be implemented to clarify the molecular mechanisms underlying the interplay between caspase-1 and -11 in promoting the canonical and/or non-canonical NLRP3 inflammasome activation.

NLRP3 INFLAMMASOME IN THE PATHOPHYSIOLOGY OF BOWEL INFLAMMATION

A dynamic interplay between enteric microbiota, intestinal epithelium, and mucosal immune system contributes to the maintenance of intestinal homeostasis (34). Indeed, dysbiosis, alterations of intestinal epithelial barrier and uncontrolled

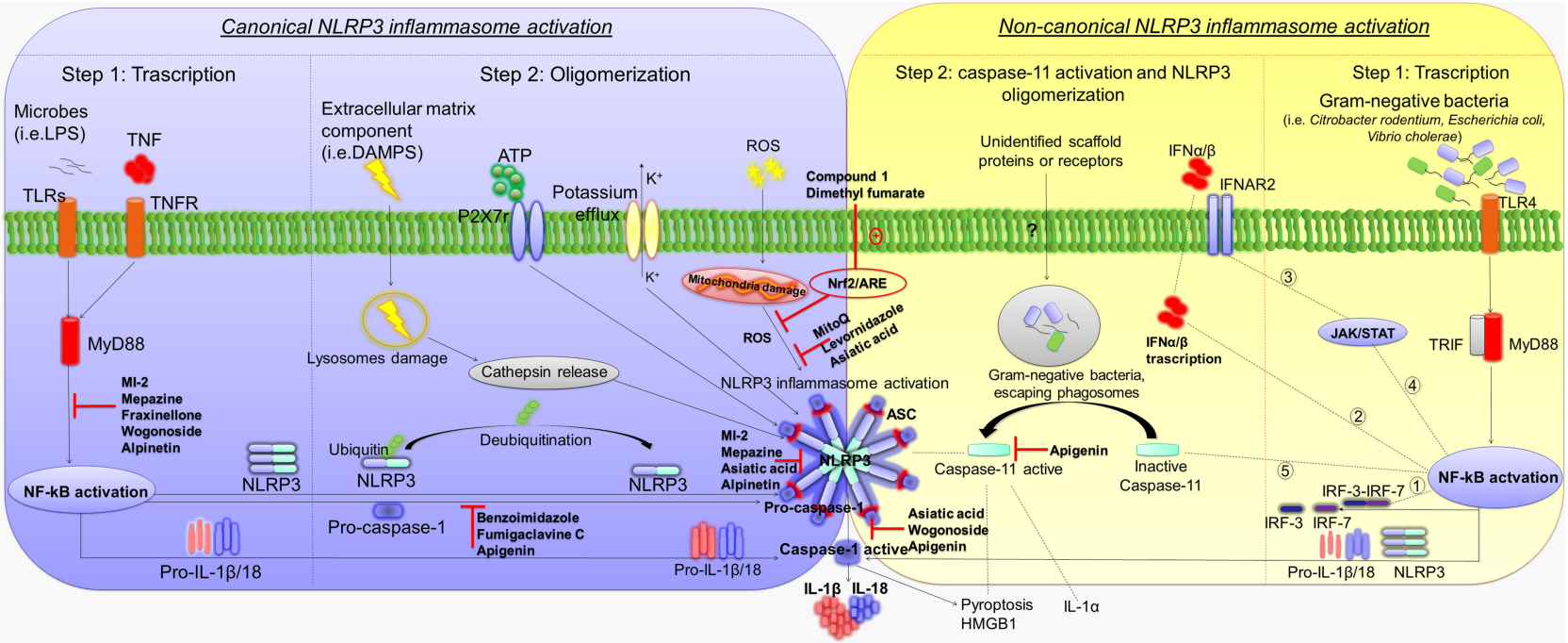

FIGURE 1 | **Mechanisms of canonical and non-canonical NLRP3 inflammasome activation**. Diagram showing the canonical and non-canonical NLRP3 inflammasome activation, and representation of the molecular mechanisms through which several compounds inhibit NLRP3 activation and counteract intestinal inflammation. Left panel: first step of canonical NLRP3 inflammasome activation by TLRs–MyD88 and/or TNFR, which activate pro-IL-1β and NLRP3 transcription *via* NF-κB activation. The second step results in NLRP3 inflammasome oligomerization, leading to caspase-1 activation as well as IL-1β and IL-18 release. Extracellular ATP, degradation of extracellular matrix components, increase in potassium efflux, ROS, cathepsin activation, and deubiquitination promote NLRP3 inflammasome oligomerization and activation. Caspase-1 activation promotes also pyroptosis and HMGB1 release. Right panel: first step of non-canonical NLRP3 inflammasome activation. Gram-negative bacteria (i.e., *Citrobacter rodentium*, *Escherichia coli*, and *Vibrio cholerae*) activate the TLR4–MyD88 and TRIF pathways, with consequent nuclear translocation of NF-κB, which promotes the transcription of IL-1β, IL-18, and NLRP3 as well as IRF-3 and IRF7 genes. The IRF3–IRF7 complex (1) elicits the expression of IFN-α/β (2) that binds the IFNAR1/IFNAR2 receptor (3), leading to activation of the JAK/STAT pathway (4) and transcription of caspase-11 gene (5). In the second step, unidentified scaffold proteins or receptors induced by Gram-negative bacteria cleave and activate caspase-11, which induces pyroptosis as well as HMGB1 and IL-1α release, and promotes the activation of NLRP3-ASC-caspase-1 pathway. Abbreviations: NLRP3, nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain-containing protein 3; TLRs, toll-like receptors; MyD88, adaptor molecules myeloid differentiation primary response 88; TNFR, tumor necrosis factor receptor; NF-κB, nuclear factor-κB; ATP, adenosine triphosphate, ROS, reactive oxygen species; HMGB1, high mobility group box 1; TRIF, toll/IL-1 receptor homology (TIR)-domain-containing adapter-inducing interferon-β; IRF, interferon regulatory factor; IFN, interferon; IFNAR, interferon-α/β receptor; IL, interleukin; P2X, purinergic receptor 7; JAK/STAT, janus kinase/signal transducers and activators of transcription; NRF2/ARE, nuclear factor (erythroid-derived 2)-like 2/antioxidant response element.

immune responses to pathogenic stimuli represent the main factors implicated in the pathogenesis of bowel inflammation. IBDs, including Crohn's disease and ulcerative colitis, comprise chronic and relapsing inflammatory disorders that affect the gastrointestinal tract (35). In this context, NLRP3 inflammasome has been found to act as a key player both in the maintenance and breakdown of intestinal immune tolerance. Indeed, through the regulation of intestinal epithelial and immune innate cells (monocytes, macrophages and dendritic cells), it contributes to maintaining intestinal homeostasis, while sustaining also the pathophysiological events underlying bowel inflammation (36). However, despite more than a decade has passed since the discovery of inflammasomes, the role of NLRP3 inflammasome in the intestinal homeostasis as well as in the pathophysiology of bowel inflammation remains multifaceted and controversial (6). A number of preclinical investigations have attempted to unravel the role played by NLRP3 inflammasome in this setting. Accordingly, current data on the involvement of canonical and non-canonical NLRP3 pathways in the pathophysiology of bowel inflammation are addressed in the following section.

Canonical NLRP3 Inflammasome Activation

In an attempt of understanding the role of canonical NLRP3 inflammasome in the pathophysiology of bowel inflammation, several efforts have been made to implement research on the effects of NLRP3 gene deletion and its components on immune and non-immune cell activity, as well as on pathophysiological events downstream its activation in preclinical models of colitis (see **Table 1**). Two initial reports showed that NLRP3 plays a key role in the regulation of intestinal homeostasis, maintaining the epithelial barrier integrity and reducing mortality during experimental colitis (7, 37). In particular, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *caspase-1*$^{-/-}$ mice were found to be more susceptible to colitis induced by dextran sodium sulfate (DSS) and 2,4,6-trinitrobenzenesulfonic acid (TNBS), both characterized by body weight loss, diarrhea, rectal bleeding, and mortality, suggesting a protective role of NLRP3 inflammasome in the digestive tract. Such a favorable action was ascribed to the ability of NLRP3 of inducing IL-18 release, a crucial mediator in the repair of colonic mucosal barrier that, through binding IL-18 receptors on intestinal epithelial cells, exerts a restorative effect on the enteric epithelium (**Figure 2**). In addition, *Nlrp3*$^{-/-}$ mice showed an elevation of nitric oxide (NO) levels, likely resulting from an increase in inducible NO synthase (iNOS) activity, and a decrease in the anti-inflammatory IL-10 cytokine and protective growth factor TGF-β expression, thus suggesting the ability of NLRP3 to regulate the production of pro- and anti-inflammatory mediators in the presence of bowel inflammation (7). However, the molecular mechanisms underlying NLRP3 inflammasome-dependent regulation of these inflammatory factors remain to be determined. In the same paper, the authors showed also that NLRP3 activation modulated the activity and trafficking of neutrophils as well as leukocyte recruitment. In particular, NLRP3-deficient neutrophils showed a pattern of altered migration, attenuated chemotactic responses, and enhanced spontaneous apoptosis (7). These findings support the view that NLRP3 inflammasome acts in a reparative key, regulating neutrophil and leukocyte phagocytic activity.

Consistent with the above results, Zaki et al. observed that the induction of colitis in *Nlrp3*$^{-/-}$ mice was associated with a disruption of intestinal epithelial barrier and an increase in mucosal permeability as compared to DSS wild-type (WT) mice, with consequent bacterial translocation into the mucosa and systemic dissemination (37). Such detrimental effect on the intestinal epithelial barrier, besides a decrease in the inflammasome-dependent IL-18 cytokine release, resulted from the ability of NLRP3 to regulate crypt bactericidal capacity and the expression of colonic β-defensin, an antimicrobial peptide released by macrophages implicated in the resistance of epithelial surfaces to microbial colonization (**Figure 2**) (7). These findings highlight the relevance of NLRP3 in modulating the interplay between intestinal epithelium and innate immune cells, suggesting a

TABLE 1 | Summary of current pre-clinical evidence supporting the differential role of NLRP3 inflammasome in intestinal inflammation.

Animal model	Genetic phenotype	Treatment/timing	Outcome	Role of NLRP3	Reference
DSS	*Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *caspase-1*$^{-/-}$	2.5% (w/v)/6 days	✓ Body weight loss ✓ Diarrhea ✓ Rectal bleeding mortality	Protective	(7)
TNBS	*Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *caspase-1*$^{-/-}$	(30 mg/mL)/3 days	✓ Body weight loss ✓ Diarrhea ✓ Rectal bleeding mortality	Protective	(7)
DSS	*Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *caspase-1*$^{-/-}$	✓ 3% (w/v)/5 days and sacrifice at day 7 ✓ 3% (w/v)/7 days and sacrifice at day 9	✓ Disruption of the intestinal epithelial barrier ✓ Increase in mucosal permeability ✓ Bacterial translocation ✓ Systemic dissemination	Protective	(37)
DSS	*Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *caspase-1*$^{-/-}$	2% (w/v)/9 days	✓ Less severity of colitis ✓ Reduced pro-inflammatory cytokines levels	Detrimental	(9)
DSS	*Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *caspase-1*$^{-/-}$	2% (w/v)/9 days	✓ Less severity of colitis ✓ Reduced pro-inflammatory cytokines levels	Detrimental	(41)
IL-10$^{-/-}$	*IL-10*$^{-/-}$	n.a.	✓ Increase in colonic IL-1β and IL-17 levels	Detrimental	(42)

DSS, dextran sodium sulfate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; w/v, weight/volume; IL-1β, interleukin-1beta; IL-17, interleukin-17.

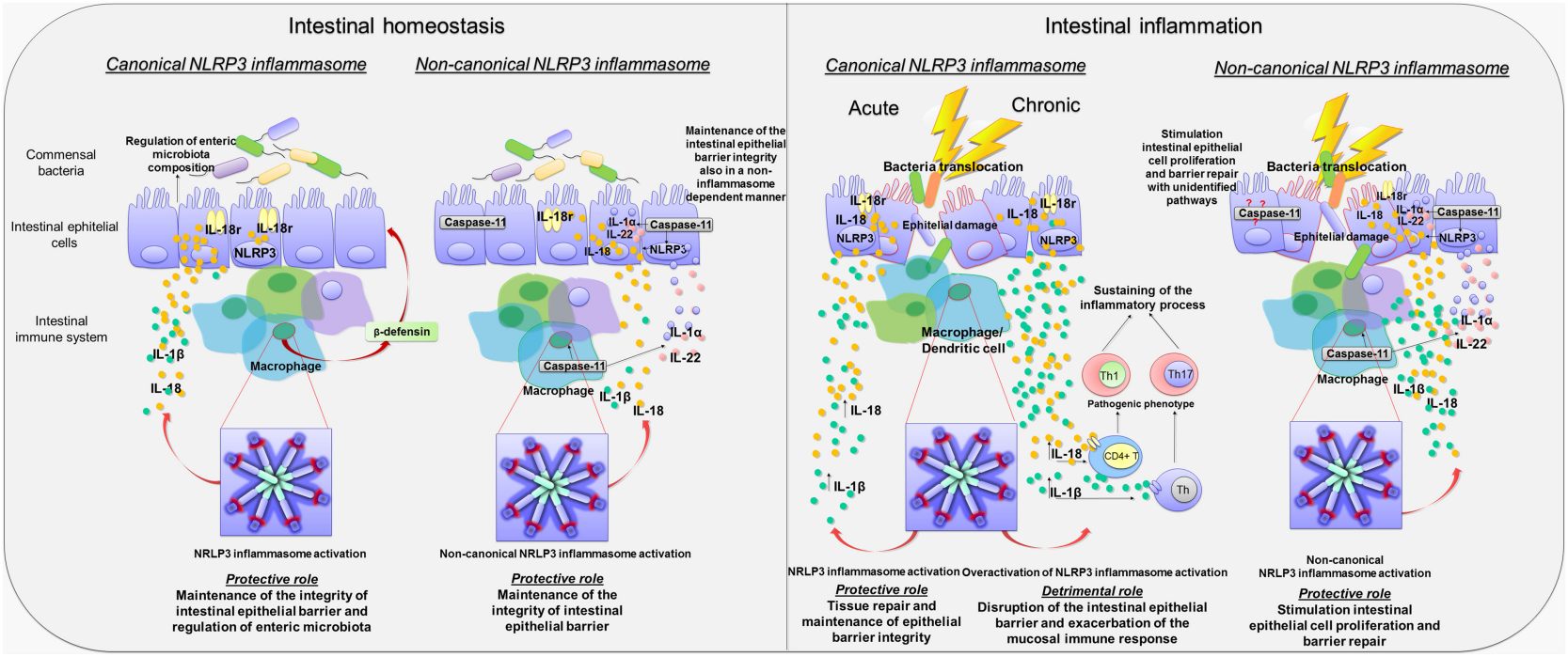

FIGURE 2 | Canonical and non-canonical activation of nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain-containing protein 3 (NLRP3) inflammasome pathways in intestinal homeostasis and inflammation. Diagram showing the role of canonical and non-canonical activation of NLRP3 inflammasome pathways in intestinal homeostasis and inflammation. Left panel: Canonical and non-canonical NLRP3 inflammasome activation in intestinal homeostasis. Canonical NLRP3 inflammasome activation plays a key role in the maintenance of the integrity of intestinal epithelial barrier as well as in the enteric microbiota composition through the release of IL-18 by macrophages and intestinal epithelial cells, the regulation of crypt bactericidal capacity, and the release of colonic β-defensin by macrophages. Likewise, non-canonical NLRP3 inflammasome activation contributes to maintain the integrity of intestinal epithelial barrier through IL-18 release by macrophages and intestinal epithelial cells. In addition, caspase-11 contributes, in a NLRP3-independent manner, to the maintenance of intestinal homeostasis promoting the release of Il-1α and IL-22. Right panel: Canonical and non-canonical NLRP3 inflammasome activation in intestinal inflammation. In the acute phase of inflammation, canonical NLRP3 inflammasome activation promotes the release of IL-1β and IL-18, contributing to tissue repair and maintenance of epithelial barrier integrity. Conversely, in the chronic phase of inflammation, canonical NLRP3 inflammasome overactivation is associated with an increase in IL-1β and IL-18 release that is harmful to the host. In addition, IL-1β and IL-18 release induce the differentiation of T cells into pathogenic Th1 and Th17 phenotypes, which contribute to sustain the inflammatory response. Non-canonical NLRP3 inflammasome activation plays a protective role during bowel inflammation likely *via* IL-18 release that stimulates intestinal epithelial cell proliferation and barrier repair. In addition, the release of IL-22 and IL-1α contributes to the repair of intestinal epithelial barrier. However, caspase-11 contributes also to promote intestinal epithelial cell proliferation and barrier repair by recruitment of yet unidentified inflammasome-independent pathways.

key role of NLRP3 inflammasome in the maintenance of the integrity of intestinal epithelial barrier as well as in orchestrating the mucosal innate immune response during inflammation. However, further investigations should be implemented to identify the exact mechanism through which NLRP3 modulates the intestinal epithelium–innate immune system interplay both under physiological conditions and in the presence of bowel inflammation.

Besides the regulation of non-immune and immune cells activity, NLRP3 inflammasome influences also the composition of enteric microbiota (7, 38). In particular, fecal microbiota in NLRP3$^{-/-}$ mice did dramatically differ, in terms of load and species, from WT mice, and such microbial shifts occurred in *Nlrp3*$^{-/-}$ mice prior to the induction of colitis, suggesting that a reduced inflammasome functionality is associated with enteric bacterial dysbiosis (37). Taken together, these findings expand further available knowledge about the regulatory role of inflammasome in homeostasis, since it appears to coordinate a dynamic interplay between gut microbiota and epithelium–innate immune system, contributing to the maintenance of intestinal microenvironment integrity (**Figure 2**).

Several lines of evidence point out the concept that deficiencies in NLRP3 inflammasome components can protect mice from DSS-induced colitis (9, 39). In particular, in a study by Bauer et al., DSS *Nlrp3*$^{-/-}$ mice developed a less severe colitis and produced lower levels of pro-inflammatory cytokines as compared with DSS WT mice. In addition, the pharmacological inhibition of caspase-1 with pralnacasan protected from colonic mucosal damage as with NLRP3 deficiency, suggesting that NLRP3 inflammasome contributes to the pathophysiology of intestinal inflammation and that NLRP3 blockade could represent a viable pharmacological strategy for the management of bowel inflammation (39, 40). Different findings about the different roles of NLRP3 inflammasome in bowel inflammation might be ascribed to different experimental conditions. For instance, in reports showing a protective and regulatory action of NLRP3, experiments were performed at the seventh day after 5 days of 3% DSS treatment and 2 days without DSS exposure, or 7 days of continued 2.5% DSS exposure. By contrast, in the paper by Bauer et al., describing a detrimental role of NLRP3 in colitis, mice received 2% DSS for 9 days. It is therefore conceivable that, extending DSS exposure, the overactivation of NLRP3 becomes detrimental for the intestinal microenvironment. Indeed, 6 days after colitis induction, the histopathological score was significantly reduced in DSS NLRP3$^{-/-}$ mice, as compared with DSS WT (39).

Interestingly, in a subsequent paper, Bauer et al. hypothesized a putative role of NLRP3 inflammasome both in the innate and adaptive immune response. In particular, unlike intestinal epithelial cells, where inflammasome-induced IL-18 release promoted a mucosal repair after DSS-induced damage, NLRP3-induced IL-18 release from lamina propria macrophages and dendritic cells elicited the activation and differentiation of CD4$^+$ T cells into the Th1 pro-inflammatory phenotype (**Figure 2**) (41). The relevance of NLRP3 in the modulation of immune cell differentiation was further confirmed by observing that lamina propria dendritic cells in NLRP3$^{-/-}$ mice expressed a tolerogenic phenotype (CD103$^+$ DC) both under physiological and inflammatory conditions, which may, at least in part, explain the reduced susceptibility of *Nlrp3*$^{-/-}$ mice to colitis (41). However, the molecular mechanisms through which NLRP3 triggers the differentiation of dendritic cells into a pro-inflammatory phenotype are still unclear and deserve further investigations.

In support of the above results, showing a detrimental role of NLRP3 in bowel inflammation, Zhang et al. (9) observed a causative link between NLRP3 inflammasome activation and development of chronic intestinal inflammation, showing that the increase in colonic IL-1β levels in *IL-10*$^{-/-}$ mice promoted IL-17 release, known to contribute to the pathogenesis of chronic colitis both in animal models and IBD patients (42). Treatment with IL-1 receptor antagonist or caspase-1 inhibitors suppressed IL-1β and IL-17 production, thus ameliorating spontaneous colitis in *IL-10*$^{-/-}$ mice. In this setting, it appears that the lack of anti-inflammatory IL-10 cytokine triggers unknown molecular mechanisms that could influence IL-1β release through gene transcription and/or direct regulation of canonical caspase-1-dependent inflammasome activation (9). Based on these findings, the authors hypothesized that, in the absence of anti-inflammatory IL-10 cytokine, intestinal inflammasomes undergo a condition of prolonged activation, leading to an uncontrolled and aberrant inflammasome-mediated immune response that contributes to the development of chronic colitis (9). Therefore, since IL-10 appears to modulate inflammasome activation, an *in vivo* pharmacological modulation of IL-10 in animal models of hapten-induced colitis could help to unravel the mechanisms underlying the negative regulation of inflammasome by IL-10.

Another considerable issue pertains to the relationship between NLRP3 and gut microbiota. Indeed, Bauer et al. (41) observed that cohousing of *Nlrp3*$^{-/-}$ mice with WT animals abrogated the protective effect of NLRP3 deficiency during colitis, and increased DSS susceptibility. Based on these results, it is conceivable that changes in enteric bacterial composition and a condition of NLRP3 hypo-functionality could contribute to the pathophysiology of bowel inflammation (41). Therefore, although these findings are in contrast with previous observations, showing a protective role of NLRP3 during colitis (37), both highlight the relevance of NLRP3-enteric microbiota interplay in the maintenance of intestinal homeostasis.

Non-Canonical NLRP3 Inflammasome Activation

Besides canonical NLRP3 inflammasome activation, over the last years, a pivotal role in the pathophysiology of intestinal inflammation has been proposed also for non-canonical caspase-11-dependent NLRP3 inflammasome activation. In particular, caspase-11, widely expressed in both hematopoietic- and non-hematopoietic cells, including macrophages and epithelial cells, once activated by Gram-negative bacteria, promotes NLRP3 inflammasome assembly and subsequent release of IL-1β, IL-18 and regulates IL-1α and HMGB1 release and pyroptosis (43, 44).

The contribution of caspase-11 to NLRP3 inflammasome activation has been initially investigated in animal models of acute exposure to enteric bacteria, sepsis, and endotoxic shock

(45, 46). Recently, several lines of evidence have shown that caspase-11-induced inflammasome activation plays a protective role during intestinal inflammation (8, 47). In particular, Williams et al. (8) observed that *Casp11*$^{-/-}$ mice displayed a significantly increased morbidity, colonic tissue damage, and leukocyte infiltration following DSS exposure, thus suggesting an increased susceptibility to DSS-induced colitis that was ascribed to a decrease in colonic inflammasome-induced IL-1β and IL-18 release (8). DSS *Casp11*$^{-/-}$ mice showed also a significant increase in caspase-1 expression, which, however, was not associated with an increased inflammasome activity (8). In support of the protective role of caspase-11, it has been observed that both chimeric *Casp11*$^{-/-}$ mice, receiving WT bone marrow (WT → *Casp11*$^{-/-}$), and chimeric WT mice, receiving *Casp11*$^{-/-}$ bone marrow (*Casp11*$^{-/-}$ → WT), were more sensitive to DSS-induced colitis as compared to WT mice receiving WT bone marrow. In particular, chimeric WT → *Casp11*$^{-/-}$ or *Casp11*$^{-/-}$ → WT DSS mice displayed a significantly increased histopathological damage, epithelial tethering, large areas of erosion, extensive areas of ulceration, enhanced inflammatory cell infiltration, and increased crypt atrophy as compared with chimeric DSS WT → WT animals (8). A proposed mechanism, underlying the protective role of caspase-11-induced inflammasome activation, calls into play its ability of regulating the release of IL-18, IL-22, and IL-1α cytokines, known to promote intestinal epithelial cell proliferation and barrier repair (47). Indeed, *Casp11*$^{-/-}$ DSS mice showed reduced colonic IL-18, IL-22, and IL-1α levels in comparison with WT DSS animals, thus suggesting that decreased levels of these cytokines, in particular IL-18, could contribute to the increase in epithelial barrier permeability, with consequent bacterial translocation into the lamina propria and exacerbation of the inflammatory response (48–50). Taken together, these findings suggest that caspase-11, *via* non-canonical inflammasome activation, regulates mucosal and epithelial barrier integrity during intestinal inflammation by increasing epithelial cell proliferation and inhibiting cell death (**Figure 2**).

The protective role of caspase-11-induced non-canonical NLRP3 inflammasome activation has been shown in a model of Gram-negative *C. rodentium* infection-induced colitis (51). In particular, in autophagy defective mice, made knock out for nucleotide-binding oligomerization domain-like receptors NLRs (NOD2) and recruit receptor interacting protein 2, infected with *C. rodentium,* the increase in oxidative stress activated the c-Jun N-terminal kinase (JNK) signaling that, in turn, increased caspase-11 expression and non-canonical NLRP3 inflammasome activation, with consequent protection of colonic epithelial barrier (51). These results corroborate previous findings, supporting the regulatory role of non-canonical NLRP3 inflammasome activation in the maintenance of intestinal homeostasis, and, most importantly, they show that, besides the TLR4–TRIF–IFN-β pathway, JNK signaling promotes non-canonical inflammasome activation. In this context, despite the observation that JNK signaling can be activated also by TLR stimulation (52), it is not clear whether the stimulation of TLR–MyD88–JNK pathway promotes caspase-11-dependent non-canonical NLRP3 inflammasome activation during intestinal inflammation. Therefore, there is still need to evaluate the role of this pathway in animal models of colitis.

At odds with the above data, Demon et al. (53) suggested that sensitivity to DSS colitis in *Casp11*$^{-/-}$ mice is independent from caspase-1-induced canonical inflammasome activation, since the colonic levels of IL-1β and IL-18, as well as circulating HMGB1 in DSS *Casp11*$^{-/-}$ mice, did not differ from DSS WT mice, suggesting that caspase-11 protects against colitis independently from inflammasome activation and, therefore, hypothesizing an unidentified pathway for caspase-11 in bowel inflammation (**Figure 2**) (53). These conflicting findings about the relevance of caspase-11-induced non-canonical inflammasome activation could result from different experimental designs, as well as from environmental variability. For instance, since caspase-11 is activated by Gram-negative bacteria, changes in gut microbiota composition could (i) influence hypo- or hyper-activation of caspase-11-induced non-canonical NLRP3 inflammasome, (ii) alter basal IFN production (54, 55), with subsequent changes in caspase-11 expression and/or function, or (iii) trigger yet unidentified molecular NLRP3-independent pathways involved in the pathophysiology of gut inflammation.

Discussion

Current data allow to hypothesize that NLRP3 inflammasome can play both protective and detrimental roles in bowel inflammation, depending on the choice of colitis models and variations of commensal enteric microflora. In particular, in the model of DSS-induced acute colitis, which causes a direct damage to the epithelial barrier, with consequent stimulation of innate immune cells by commensal bacteria, infiltration of myeloid cells and massive inflammation, IL-1β and IL-18 appear to be essential for tissue repair and the maintenance of epithelial barrier integrity (56), thus suggesting a protective role of NLRP3. By contrast, in animal models of chronic colitis, the inflammasome-induced IL-1β release induces differentiation of T cells into pathogenic Th17 phenotypes, thus contributing to sustain the inflammatory process (9). Despite these conflicting and heterogeneous findings, it appears that, in the first acute phase of inflammation, the NLRP3 inflammasome acts as a key player to restore intestinal homeostasis. Conversely, in chronic colitis, where a disruption of the intestinal epithelial barrier and an exacerbation of the mucosal immune response occur, the overactivation of NLRP3 inflammasome results to be harmful to the host (**Figure 2**). However, the results from knockout mouse models, where there is a complete removal of NLRP3 protein complex, cannot be easily and fully translated into the clinical setting, since NLRP3 gene deletion might trigger unknown compensatory immune mechanisms that influence the disease outcome. Furthermore, it is also noteworthy that in animals with complete deletion of NLRP3 gene no distinction between canonical and/or non-canonical NLRP3 inflammasome activation can be made. Therefore, the *in vivo* pharmacological modulation of canonical NLRP3 inflammasome in more predictive animal models of colitis should be investigated, in order to clarify the role of this enzymatic complex in the pathophysiology of bowel inflammation. Data on the effects stemming from the pharmacological modulation of NLRP3 inflammasome are discussed in the following sections.

With regard to non-canonical NLRP3 inflammasome activation, the majority of current data suggest that caspase-11-dependent NLRP3 activation, although dispensable for caspase-1-inflammasome assembly, contributes to protection against DSS-induced colitis regulating the epithelial barrier integrity. However, owing to scarce, conflicting and heterogeneous findings, it remains unclear whether caspase-11, expressed in the colonic mucosa, plays a protective role also in intestinal inflammation independently from canonical NLRP3 inflammasome activation. Accordingly, there is a strong need for further experiments, aimed at evaluating how the simultaneous caspase-1 and caspase-11 gene deletion, as well as the pharmacological modulation of caspase-11, could interfere with NLRP3 assembly and consequently with the pathophysiology of bowel inflammation. In addition, given the relevance of intestinal microbiota in caspase-11 activation, extensive investigations are needed to evaluate whether changes in enteric bacteria composition could influence caspase-11 activity and consequent non-canonical inflammasome assembly.

PHARMACOLOGICAL MODULATION OF NLRP3 INFLAMMASOME IN BOWEL INFLAMMATION

The involvement of inflammasome pathways in the pathophysiology of intestinal inflammation is fostering research on the potential therapeutic benefits, in terms of anti-inflammatory activity, resulting from the pharmacological targeting of NLRP3 inflammasome. At present, the majority of available studies have investigated the role of NLRP3 in several experimental models of colitis, displaying remarkable beneficial effects by the pharmacological modulation of this enzymatic complex (57–59). In particular, DSS-induced colitis has been largely employed, since in this model lysosomal damage and increased ROS levels can lead to an overactivation of NLRP3 inflammasome (39). Following DSS administration, surface molecules produced by microorganisms or other inflammatory factors (i.e., LPS) can promote also the first step of NLRP3 assembly, through the activation of NF-κB transcription, with subsequent increase in NLRP3 as well as pro-IL-1β and pro-IL-18 protein levels. Furthermore, DSS treatment is associated with an increase in extracellular ATP or bacterial toxins, which are able to stimulate caspase-1 activation directly, thereby releasing IL-1β and IL-18 (39, 58). Accordingly, several targets have been identified for inhibiting the assembly of NLRP3 (**Figure 1**).

A pioneering study by Dashdorj et al. (59) showed that MitoQ, a mitochondria-targeted derivative of the antioxidant ubiquinone, endowed with antioxidant and anti-apoptotic properties, exerted beneficial effects on experimental colitis through a decrease in colonic NLRP3 and caspase-1 expression, with consequent decrease in IL-1β and IL-18 release (59). The molecular mechanism underlying NLRP3 inflammasome blockade was proposed to depend on the ability of MitoQ to suppress ROS-induced dissociation of thioredoxin-interacting protein (TXNIP), from thioredoxin, thus inhibiting the interaction of TXNIP with NLRP3 (59). Indeed, although Masters et al. (60) showed that TXNIP is not essential to NLRP3 activation in bone marrow-derived macrophages primed with LPS and then stimulated with *S. aureus*, silica, or ATP, in the setting of colitis, where an increase in oxidative stress and activation of different inflammatory pathways occur, the dissociation of TXNIP could represent one of the mechanisms underlying NLRP3 activation (59).

The inhibition of mitochondrial ROS generation, as a suitable pharmacological target for inhibiting NLRP3 inflammasome assembly, has been confirmed by a subsequent study, showing that *in vivo* administration of levornidazole, the levo isomer of ornidazole generally used for protozoan infections, to DSS mice exerted enteric anti-inflammatory effects through the blockade of NLRP3 inflammasome assembly by suppression of ROS generation. These findings suggest that the blockade of NLRP3 upstream signaling could represent a suitable pharmacological target for the management of intestinal inflammation (61).

Consistent with the above data, two recent papers by Wang et al. (58) and Liu et al. (62) have reported that the inhibition of ROS formation exerted beneficial effects in colitis through the blockade of NLRP3 assembly (58, 62). In particular, these authors observed that two small molecules, 3-(2-oxo-2-phenylethylidene)-2,3,6,7-tetrahydro-1H-pyrazino-[2,1-a] isoquinolin-4(11bH)-one (compound 1) and dimethyl fumarate, promote the transcription of genes coding for various detoxification and antioxidant enzymes, through the activation of NFE-related factor 2 (Nrf2). The subsequent inhibition of ROS formation has been shown to exert inhibitory effects on NLRP3 assembly (58, 62). Indeed, after exposure to environmental or intracellular stresses, such as ROS, Nrf2 translocates into the nucleus and binds to antioxidant response elements (AREs), which in turn induce the production of cytoprotective enzymes, such as heme oxygenase 1, NAD(P)H quinine oxidoreductases, and glutathione *S*-transferases, that are pivotal to maintain optimal cellular functions (63). In this respect, the stimulation of Nrf2/ARE pathway could represent an indirect molecular target to inhibit NLRP3 inflammasome activation. However, further extensive investigations are needed to characterize the actual molecular mechanisms underlying the Nrf2–ROS–NLRP3 interplay and, most importantly, the correlation between Nrf2 stimulation and NLRP3 inhibition in the setting of bowel inflammation.

Besides targeting the inflammasome upstream signaling, NLRP3 blockade *via* caspase-1 inhibition has been shown also to exert anti-inflammatory effects in DSS mice (57, 64). In particular, a synthetic benzimidazole derivative and fumigaclavine C, a fungal metabolite, through the inhibition of caspase-1 activation, exerted beneficial effects on colonic inflammation reducing protein and mRNA levels of colonic TNF, IL-1β, and IL-17 pro-inflammatory cytokines (57, 64). However, even if both compounds have caspase-1 inhibition as ultimate goal, they influence intracellular signaling in different ways. Indeed, the benzimidazole derivative has been shown to inhibit MAPK and STAT1 signaling without interaction with NF-κB-mediated transcription, while fumigaclavine C was found to significantly interfere with NF-κB activation, STAT3 and STAT1 signaling.

These findings suggest that the inhibition of the primary TRL–MyD88–NF-κB step involved in NLRP3 inflammasome activation, though at different steps of the intracellular cascade, represents a suitable pharmacological target for inhibiting the NLRP3 assembly, and therefore a promising strategy for treatment of bowel inflammation.

In further support of the above data, a recent paper by Liu et al. (65) showed that the inhibition of mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1 (MALT1), a scaffold protein, which recruits the IκB kinase complex leading to release and activation of NF-κB, ameliorated clinical symptoms and histopathologic features of DSS-induced colitis through NF-κB and NLRP3 inhibition, thus interfering with both inflammasome activation steps (65, 66). In particular, treatment with two specific MALT1 inhibitors, MI-2 and mepazine, dose-dependently attenuated the symptoms of colitis in mice through a decrease in protein and mRNA levels of colonic TNF, IL-1β, IL-6, IL-18, IL-17A, and IFN-γ pro-inflammatory cytokines (66). The mechanisms underlying the inhibitory effects of MALT1 in DSS-induced colitis have been ascribed to the inhibition on NF-κB and NLRP3 inflammasome activation in macrophages, thus implying that MALT1-NF-κB signaling regulates NLRP3 inflammasome activation. However, since the NF-κB pathway is involved in the transcription of both pro- and anti-inflammatory mediators (i.e., IL-10 and TGF-β), extensive investigations are required to identify more selective targets to inhibit NLRP3 inflammasome oligomerization, thereby counteracting the pathophysiological events underlying NLRP3 activation, and attenuating bowel inflammation.

Of interest, several lines of evidence have shown that various NLRP3-targeting natural compounds are able to exert anti-inflammatory effects on DSS-induced colitis in mice. Guo et al. (66) observed that oral administration of asiatic acid, a natural triterpenoid compound, dose-dependently attenuated body weight loss, shortening of colon length, histopathologic scores, myeloperoxidase activity, and colonic TNF, IL-1β, IL-6, and IFN-γ levels in mice with DSS-induced colitis through the inhibition of NLRP3 inflammasome activation (67). In particular, the authors found that asiatic acid inhibited the upstream signaling of inflammasome oligomerization by suppressing mitochondrial ROS generation, as well as caspase-1 activation and inflammasome assembly. Likewise, treatment with fraxinellone, a natural lactone endowed with immunosuppressive activity, significantly reduced weight loss, diarrhea and colonic macroscopic damage, as well as myeloperoxidase, alkaline phosphatase, and colonic TNF, IL-1β, IL-6, and IL-18 levels in DSS-induced colitis mice (68). Such anti-inflammatory effects were ascribed to the inhibition of $CD11b^+$ macrophage infiltration, as well as the decrease in mRNA levels for colonic macrophage-related proteins, including intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), iNOS, and cyclooxygenase-2 (COX-2), through NF-κB signaling and NLRP3 inhibition. These findings represent a point of novelty, since they support the view that the blockade of NLRP3 assembly can influence also the activation of infiltrating macrophages by inhibiting the release of intercellular adhesion molecules and pro-inflammatory mediators contributing to the inflammatory process.

Recent evidence has shown that several flavonoid derivatives exerted anti-inflammatory effects on colitis *via* NF-κB/NLRP3 inhibition (69–71). In particular, treatment with wogonoside, a glucuronide metabolite of the bioactive flavonoid wogonin, reduced significantly colonic NF-κB and NLRP3 expression, as well as caspase-1 expression and activity in mice with colitis, exerting beneficial effects on colonic inflammation (69). Likewise, the administration of alpinetin, a novel plant flavonoid isolated from *Alpinia katsumadai Hayata*, significantly attenuated diarrhea, colonic shortening, histological damage, and myeloperoxidase activity as well as colonic TNF and IL-1β expression in mice with DSS-induced colitis, likely by suppressing TRL4-NF-κB and NLRP3-ASC-caspase-1 signaling (70). However, the authors documented the ability of alpinetin of inhibiting NLRP3 activation in *in vitro* THP-1 cells, omitting the evaluation of alpinetin effects on NLRP3 activation in DSS mice.

The protective effects of flavonoids *via* NLRP3 inhibition have been shown also in a mouse model of DSS-induced colitis (71). In particular, a dietary apigenin (API) enrichment decreased the macroscopic and microscopic signs of colitis and reduced colonic PGE, COX-2, and iNOS expressions as well as serum matrix metalloproteinase (MMP-3) levels. In addition, API diet reduced IL-1β and TNF pro-inflammatory cytokine release in primary LPS-stimulated splenocytes. The beneficial effects of API on colonic inflammation result from the inhibition of both canonical and non-canonical NLRP3 inflammasome pathways, through the regulation of caspase-1 and caspase-11 enzyme expression and activity (71). Indeed, although caspase-11 has been proposed to mediate a protective role in the host during the acute phases of colitis, it appears to be detrimental in chronic inflammation, where it is significantly upregulated and promotes IL-1β and IL-18 release (8, 47). These findings demonstrate, for the first time, that the pharmacological blockade of both canonical and non-canonical NLRP3 activation could represent a suitable and promising pharmacological target for treatment of bowel inflammation. Nevertheless, the molecular mechanisms through which API can block canonical and non-canonical NLRP3 assembly remain to be clarified.

OVERALL CONCLUSION AND FUTURE DIRECTIONS

Studies aimed at characterizing the molecular mechanisms and downstream signaling underlying the canonical and non-canonical NLRP3 inflammasome activation have unraveled the pivotal and dual role of this enzymatic complex in the intestinal homeostasis. According to current information, NLRP3 regulates the integrity of intestinal mucosal barrier under physiological conditions, but it can shape also the immune response against commensal microbiota during bowel inflammation.

One considerable deficiency in our knowledge concerns how canonical and non-canonical NLRP3 inflammasome coordinate differently the dynamic interplays among gut microbiota–epithelium–innate immune system. For instance, it remains unclear whether alterations of enteric bacteria composition promote an abnormal caspase-1- and/or caspase-11-dependent NLRP3 inflammasome activation, or whether, *vice versa*, the

overactivation of NLRP3 alters epithelial barrier integrity and gut microbiota with consequent alteration of intestinal homeostasis. In this context, given the high complexity of the NLRP3 inflammasome system, strong efforts are needed to better understand how canonical and non-canonical NLRP3 manage their downstream signaling to maintain or break down intestinal homeostasis. Nevertheless, despite current heterogeneous and conflicting evidence, beneficial effects resulting from the pharmacological modulation of NLRP3 in animal models of colitis have led to postulate that the overactivation of NLRP3 during bowel inflammation is detrimental for the host. Therefore, the blockade of NLPR3 activation could represent a suitable pharmacological approach for the management of inflammatory intestinal disorders.

A recent study by Coll et al. (72) showed that the pharmacological blockade of canonical and non-canonical NLRP3 activation with MCC950, a recognized selective, small molecule inhibitor of NLRP3, reduced IL-1β tissue levels and attenuated the severity of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (72). Furthermore, MCC950 rescued neonatal lethality in a mouse model of CAPS, and it was effective in reducing IL-1β levels in human peripheral blood mononuclear cells from patients with Muckle–Wells syndrome, thus suggesting its putative therapeutic activity in NLRP3-associated syndromes, including autoinflammatory and autoimmune diseases (72). Based on these observations, testing MCC950 in animals with colitis (i.e., DSS, TNBS, or *IL-10*$^{-/-}$) would allow a better characterization of the anti-inflammatory effects resulting from selective inhibition of canonical and non-canonical NLRP3 inflammasome activation.

Another relevant issue concerns the inhibition of NLRP3 downstream signaling through the blockade of IL-β receptor. In this respect, several lines of evidence have shown that treatment with IL-β receptor antagonists (i.e., anakinra) exerted beneficial effects in patients with immune-mediated inflammatory diseases (i.e., rheumatoid arthritis, ankylosing spondylitis, and gout) (73). In addition, anakinra reduced postoperative inflammation and ameliorated postoperative ileus in mice (74), thus suggesting that IL-1β receptor blockade exerts beneficial effects during intestinal inflammation. Based on these findings, it is conceivable that both upstream and downstream inhibition of NLRP3 inflammasome could represent suitable pharmacological approaches for treatment of bowel inflammation. Therefore, the possible effects of the pharmacological blockade of IL-1β receptor in different animal models of colitis should be investigated, in order to find out the better strategy to inhibit the NLRP3 inflammasome pathway and counteract bowel inflammation.

In support of preclinical findings, clinical evidence has documented an increased IL-1β secretion from colonic tissues and macrophages of IBD patients, these patterns being correlated with the severity of disease, thus suggesting IL-1β as a key pro-inflammatory cytokine for the pathogenesis of IBDs (75). In addition, the activation of NLRP3 inflammasome in monocytes infiltrating the lamina propria and M1 pro-inflammatory macrophages isolated from intestinal specimens of IBD patients, seems to contribute to the disruption of epithelial barrier through a deregulation of tight junction proteins (i.e., claudin-1, claudin-2, and junctional adhesion molecule-A), as well as to induce epithelial cell apoptosis (76). In particular, NLRP3 inflammasome-induced IL-1β and IL-18 release from monocytes infiltrating the lamina propria alters tight junctions and promotes apoptosis in intestinal epithelial cells, and, subsequently M1 macrophages, recruited into the lamina propria, contribute to sustain the immune innate response, thus suggesting a detrimental role of NLRP3 inflammasome in IBD patients (76). Accordingly, a translation of preclinical evidence into clinical practice could allow a better understanding of protective/detrimental shift of NLRP3 in IBD patients.

In conclusion, given the heterogeneity of preclinical studies and the paucity of human studies, extensive investigations are awaited for better understanding the NLRP3 inflammasome functionality in non-immune and immune cells since early stages of intestinal inflammation, in order to clarify the relevance of NLRP3 inflammasome in the pathophysiology of IBDs. In addition, considering the close inflammasome-gut microbiota interplay, and that genetic NLRP3 mutations and environmental factors, altering the gut microbiota, are involved in the pathogenesis of IBDs, future investigations should be addressed to characterize the correlation between changes in enteric bacteria composition and hypo- or hyperfunctionality of NLRP3 inflammasome.

AUTHOR CONTRIBUTIONS

CP, GL-C, and LA wrote the first draft of the manuscript. CP prepared the figures. CB and MF revised the manuscript.

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Helicobacter pylori Activates HMGB1 Expression and Recruits RAGE into Lipid Rafts to Promote Inflammation in Gastric Epithelial Cells

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Helicobacter pylori infection is associated with several gastrointestinal disorders in the human population worldwide. High-mobility group box 1 (HMGB1), a ubiquitous nuclear protein, mediates various inflammation functions. The interaction between HMGB1 and receptor for advanced glycation end-products (RAGE) triggers nuclear factor (NF)-κB expression, which in turn stimulates the release of proinflammatory cytokines, such as interleukin (IL)-8, and enhances the inflammatory response. However, how *H. pylori* activates HMGB1 expression and mobilizes RAGE into cholesterol-rich microdomains in gastric epithelial cells to promote inflammation has not been explored. In this study, we found that HMGB1 and RAGE expression increased significantly in *H. pylori*-infected cells compared with -uninfected cells. Blocking HMGB1 by neutralizing antibody abrogated *H. pylori*-elicited RAGE, suggesting that RAGE expression follows HMGB1 production, and silenced RAGE-attenuated *H. pylori*-mediated NF-κB activation and IL-8 production. Furthermore, significantly more RAGE was present in detergent-resistant membranes extracted from *H. pylori*-infected cells than in those from -uninfected cells, indicating that *H. pylori* exploited cholesterol to induce the HMGB1 signaling pathway. These results indicate that HMGB1 plays a crucial role in *H. pylori*-induced inflammation in gastric epithelial cells, which may be valuable in developing treatments for *H. pylori*-associated diseases.

Keywords: *Helicobacter pylori*, HMGB1, RAGE, cholesterol, interleukin-8

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INTRODUCTION

Helicobacter pylori, a Gram-negative bacterium, colonizes the human stomach and infects more than half of the human population worldwide (1, 2). Persistent infection by *H. pylori* in the stomach induces the production of proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α (3), which are closely associated with several gastroenterological diseases, including gastritis, peptic ulcer, and gastric adenocarcinoma (4, 5). Moreover, *H. pylori* possesses a set of virulence factors that allow the bacterium to persistently colonize the hostile environment of gastric mucus. These factors include urease, flagella, adhesins, and two major virulence factors, vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA) (6).

The major components of lipid rafts (also called cholesterol-rich microdomains) are phospholipids, sphingolipids, and cholesterol, which together form tight interactions and create rigid microdomains in the cytoplasm membrane (7). VacA was the first *H. pylori* toxin shown to hijack membrane cholesterol for its own oligomerization and delivery into target cells (8). Translocation, as well as phosphorylation, of CagA into gastric epithelial cells was previously shown to be cholesterol dependent (9). Accordingly, disruption of cholesterol-rich microdomains abolishes the actions of VacA and CagA, mitigating *H. pylori*-associated pathogenesis (9–11). These findings indicate that *H. pylori* orchestrates the exploitation of cholesterol for its intricate infection strategy.

High-mobility group box 1 (HMGB1) is a ubiquitous nuclear protein that stabilizes nucleosomes, enables nicking of DNA, and facilitates transcription (12). HMGB1 has been shown to function as a proinflammatory protein that mediates endotoxin-induced lethality, tissue damage, and systemic inflammation (13, 14). Receptor for advanced glycation end-products (RAGE), a single transmembrane-spanning domain belonging to the immunoglobulin superfamily, serves as a receptor for HMGB1 in the amplification of proinflammatory signaling (15). Interaction of RAGE with HMGB1 triggers mitogen-activated protein kinases (MAPKs) and subsequently activates nuclear factor (NF)-κB (16, 17), thereby stimulating the release of multiple proinflammatory cytokines (18). Moreover, HMGB1 has been implicated in several bacterial diseases that are mediated by inflammatory responses (19–21).

Recently, a study of *H. pylori* revealed that VacA induces programed necrosis of cells, releasing HMGB1, and resulting in a proinflammatory response (22). However, the mechanisms by which *H. pylori* activates HMGB1 expression and mobilizes RAGE into cholesterol-rich microdomains to promote inflammation in gastric epithelial cells have yet to be studied. Therefore, we explored the role of HMGB1 during *H. pylori* infection of gastric epithelial cells. In addition, we investigated whether cholesterol-rich microdomains are involved in the induction of HMGB1 and RAGE expression and the subsequent inflammatory response.

MATERIALS AND METHODS

Reagents and Antibodies

Alexa Fluor 647-conjugated cholera toxin subunit B (CTX-B), Alexa Fluor 488-conjugated goat anti-rabbit IgG, 4′,6-diamidino-2-phenylindole (DAPI), and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Anti-HMGB1 (ab18256), anti-RAGE (ab37647), and anti-actin antibodies were purchased from Abcam (Cambridge, MA, USA). Methyl-β-cyclodextrin (MβCD) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Luciferase substrate and β-galactosidase expression vector were purchased from Promega (Madison, WI, USA).

Bacterial Culture

Helicobacter pylori 26695 (ATCC 700392) was recovered from frozen stocks on *Brucella* agar plates (Becton Dickinson, Franklin Lakes, NJ, USA), containing 10% sheep blood (23). Boiled *H. pylori* and bacterial lysates were prepared, as described previously (24).

Cell Culture

Human AGS cells (ATCC CRL 1739) were cultured in F12 medium (Invitrogen). SCM-1 and TSGH9201 cells were cultured in RPMI 1640 medium (Invitrogen) (24). All culture media were supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA). For transient transfection, AGS cells were incubated in OPTI-MEM (Invitrogen), 1 μg NF-κB reporter genes, and 1 μl Lipofectamine 2000 for 6 h at 37°C. Transfected cells were then cultured in complete medium for 24 h before further analysis.

Western Blot Analysis

Helicobacter pylori-infected AGS cells were harvested and then boiled in SDS-PAGE sample buffer for 10 min. The protein lysate was then resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with antibodies against HMGB1 or RAGE at room temperature for 1 h. The blots were washed and then incubated with horseradish peroxidase-conjugated secondary antibody (Millipore). The proteins of interests were detected using the ECL Western Blotting Detection kit (GE Healthcare, Piscataway, NJ, USA).

Transfection of Small Interfering RNAs

Small interfering RNAs (siRNAs) for RAGE [On-Target*plus* Human AGER (177) siRNA] and scrambled control (sc-37007) were purchased from Thermo Fisher Scientific (Lafayette, CO, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. AGS cells were transfected with siRNAs (50 nM) by use of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Quantitative Real-time Reverse Transcription-PCR

Receptor for advanced glycation end-products mRNA levels were analyzed by quantitative real-time PCR using SYBR Green I Master Mix and a model 7900 Sequence Detector System, as described previously (25). The oligonucleotide primers used were corresponded to human RAGE (forward, 5′-CTACCGAG TCCGTGTCTACCA-3′ and reverse, 5′-CATCCAAGTGCCA GCTAAGAG-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5′-CCCCCAATGTATCCGTTGTG-3′ and reverse, 5′-TAGCCCAGGATGCCCTTTAGT-3′). The program was pre-incubated at 50°C for 2 min and 95°C for 10 min;

PCR was performed with 40 cycles of 95°C for 10 s and 60°C for 1 min.

Reporter Activity Assay

AGS cells were transfected with the NF-κB reporter constructs by using Lipofectamine 2000 prior to infection with *H. pylori* (MOI = 100) (26). Reporter lysis buffer (Promega) was added to the wells, and cells were scraped from the dishes. Equal volumes of luciferase substrate were added to the samples, and luminescence was detected using a microplate luminometer (Biotek, Winooski, VT, USA). Luciferase activity was normalized to transfection efficiency by determining the β-galactosidase activity generated from a co-transfected β-galactosidase expression vector (Promega) (10).

Determination of IL-8 Production

The concentration of IL-8 was determined by enzyme-linked immunosorbent assay (ELISA), as described previously (27). Briefly, AGS cells were transfected with RAGE siRNA followed by infection with *H. pylori* (MOI = 100) for 6 h. The IL-8 concentration was determined using a sandwich ELISA kit (R&D Systems).

Immunofluorescence Labeling

AGS cells (2×10^5) were seeded on coverslips in six-well plates and infected with *H. pylori* at an MOI of 100 for 6 h. The cells were fixed with 3.7% paraformaldehyde at room temperature for 1 h and then permeabilized with 0.1% TritonX-100 for 5 min. To label HMGB1 and RAGE, cells were incubated for 30 min with antibodies against HMGB1 and RAGE, followed by probed with Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG, respectively. The stained cells were analyzed using confocal microscopy (LSM 780; CarlZeiss, Göttingen, Germany) with a 100× objective (oil immersion; numerical aperture, 1.3).

Analysis of Proteins in Detergent-Resistant Membrane

To isolate detergent-soluble and -resistant fractions, *H. pylori*-infected AGS cells were lysed with ice-cold TNE buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA), containing 1% (vol/vol) Triton X-100, as described previously (28). Cell lysates were centrifuged at 18,000 × *g* at 4°C for 30 min to separate detergent-soluble and -resistant fractions, as described previously (27). The proteins of interests in each fraction were assessed by Western blot.

Statistical Analysis

Experimental results are expressed as means ± SEM. The Student's *t*-test was used to calculate the statistical significance of differences between two groups. The difference was considered significant when $P < 0.05$. Statistical analyses were carried out using SPSS program (version 11.0, SPSS Inc., Chicago, IL, USA).

RESULTS

H. pylori Infection Induces HMGB1 and RAGE Expression in Gastric Epithelial Cells

We first investigated whether *H. pylori* infection induces HMGB1 and RAGE expression in gastric epithelial cells. AGS cells were infected with *H. pylori* at various MOIs (0–500) for 6 h, and the expression levels of HMGB1 and RAGE were determined by Western blot assay. As shown in **Figures 1A–C**, HMGB1 and RAGE expression levels were markedly increased in cells infected with *H. pylori* at an MOI of 100, whereas they were decreased at higher MOIs of 200 and 500. In addition, AGS cells were infected with *H. pylori* (MOI = 100) for different durations (0–24 h) in parallel. *H. pylori*-induced HMGB1 and RAGE expression peaked with 6 h of infection and decreased after incubation for 16–24 h (**Figures 1D–F**). These results suggest that *H. pylori* induces HMGB1 and RAGE expression in AGS cells, and that the optimal conditions for infection are an MOI of 100 and incubation for 6 h.

Live *H. pylori* Is Essential for Enhancing HMGB1 and RAGE Expression in Gastric Epithelial Cells

We then explored whether increased HMGB1 expression could be seen in AGS and two other gastric epithelial cell lines (SC-M1 and TSGH9201). As shown in **Figure 2A**, the expression levels of HMGB1 were significantly elevated in the three *H. pylori*-infected gastric epithelium-derived cell lines. AGS cells were found to be the most susceptible; therefore, this line was chosen for the following investigations. We next analyzed the effects of live or killed *H. pylori* with the ability to elicit HMGB1 and RAGE expression in AGS cells. Live bacteria, boiled bacteria (heat-killed), and bacterial lysates (crude extracts) were examined for their capacity to induce HMGB1 and RAGE. As shown in **Figure 2B**, HMGB1 and RAGE expression in AGS cells in response to live *H. pylori* increased significantly, whereas boiled bacteria and bacterial lysates only slightly increased the expression of HMGB1 and RAGE in these cells. Our data showed that the expression levels of HMGB1 and RAGE were elevated in *H. pylori*-infected AGS cells and that live bacteria were required.

H. pylori-Induced RAGE Expression Is Elicited by HMGB1

Confocal microscopy was used to observe HMGB1 expression in AGS cells. As shown in **Figure 3**, without *H. pylori*, the image showed faint HMGB1 staining in cell nuclei. In contrast, the distribution of fluorescence clearly showed that HMGB1 localized in both the nucleus and the cytoplasm of cells upon *H. pylori* infection. We then analyzed RAGE expression in response to *H. pylori*-induced HMGB1. AGS cells were mock-treated or -pretreated with isotype IgG or neutralizing antibody against HMGB1 (α-HMGB1) for 30 min and then incubated with *H. pylori* for 6 h. As shown in **Figure 4**, blocking of HMGB1 by α-HMGB1 significantly reduced *H. pylori*-induced RAGE mRNA and protein levels, whereas this mock-treated cells or cells treated with isotype IgG showed no such effect. These results indicate that *H. pylori* infection induces HMGB1 expression, which in turn elicits the production of RAGE in gastric epithelial cells.

Silencing RAGE mRNA Ameliorates *H. pylori*-Induced Inflammation

AGS cells were then transfected scrambled control siRNA (SiCon) or RAGE siRNA (SiRAGE) for 24 h following incubation with

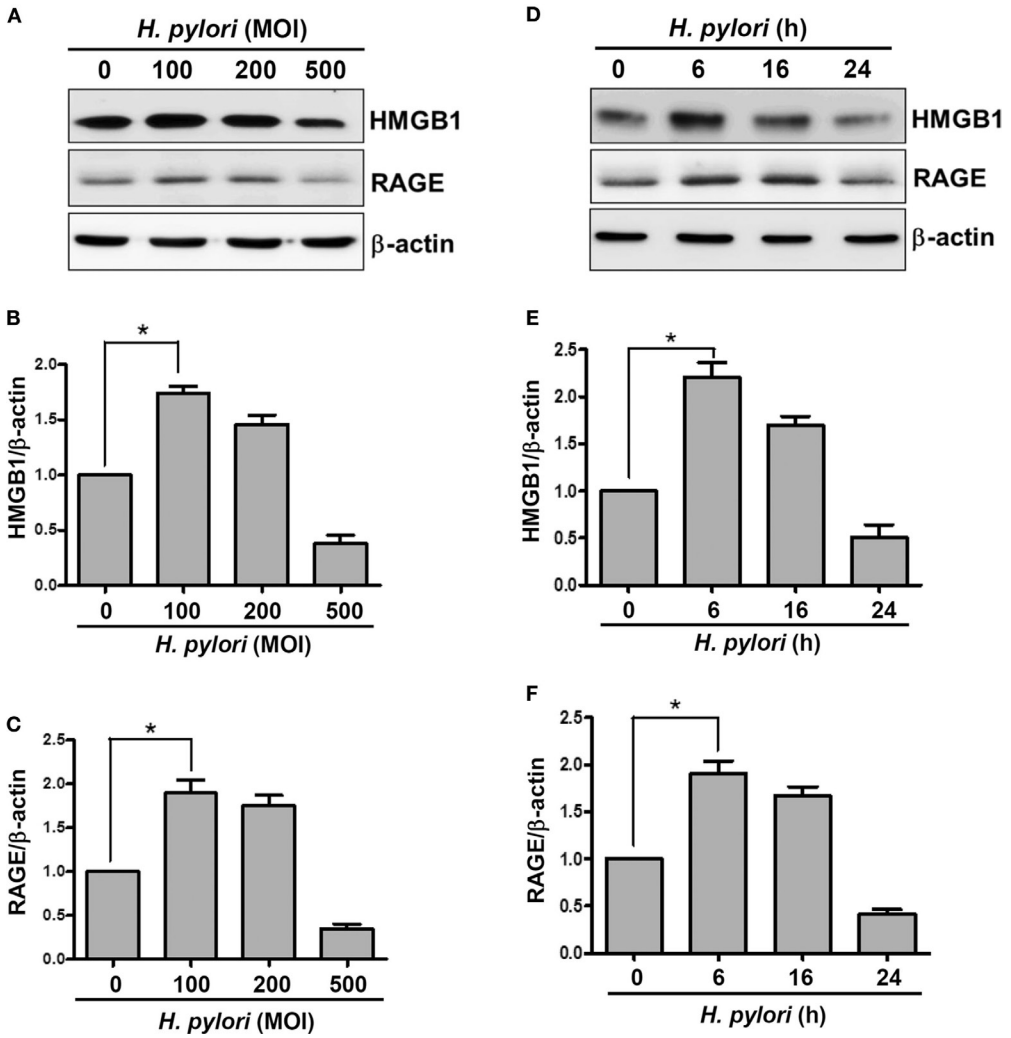

FIGURE 1 | ***H. pylori*** **induces HMGB1 and RAGE expression in gastric epithelial cells**. AGS cells were infected with *H. pylori* for 6 h with various MOIs **(A–C)**, including an MOI of 100 at different time points **(D–F)**. Total cell lysates were prepared to evaluate HMGB1 and RAGE expression by Western blot analysis. Protein expression levels were quantified by densitometric analysis and normalized to β-actin **(B,C,E,F)**. Statistical significance was evaluated by Student's *t*-test (*$P < 0.05$).

H. pylori for 6 h. A quantitative real-time PCR analysis showed that SiRAGE transfection significantly reduced the level of RAGE mRNA when compared to SiCon transfection (**Figure 5A**). Additionally, *H. pylori*-induced RAGE mRNA expression was markedly suppressed by transfection with siRAGE. We therefore analyzed whether silencing RAGE decreased NF-κB promoter activity and IL-8 production in *H. pylori*-infected cells. Cells were co-transfected with SiRAGE and an NF-κB/wt luciferase reporter prior to incubation with *H. pylori* for 6 h and then subjected to luciferase activity assay. Culture supernatants were harvested to evaluate IL-8 production by ELISA. Our data showed that both NF-κB promoter activity and IL-8 production were significantly reduced by knocking down RAGE in cells infected with *H. pylori* (**Figures 5B,C**). These results confirm *H. pylori*-induced inflammation in response to reciprocally elicited HMGB1 and RAGE expression.

Mobilization of RAGE into Cholesterol-Rich Microdomains by *H. pylori* Induces IL-8 Production

The involvement of cholesterol-rich microdomains in the induction of RAGE by *H. pylori* infection was explored next. The colocalization of RAGE with CTX-B, a raft-associated molecule that binds to the ganglioside GM1, was clearly observed around the cytoplasmic membrane in *H. pylori*-infected cells (**Figures 6E–H**); this effect was minimal in uninfected cells (**Figures 6A–D**). The merged images were then analyzed by confocal microscopy *z*-section. As shown in **Figures 6I–L**, the adhered bacteria (arrows) clearly appeared to colocalize with RAGE and CTX-B in the cytoplasmic membrane. These results indicate that the recruitment of RAGE into membrane rafts occurs in response to *H. pylori* infection.

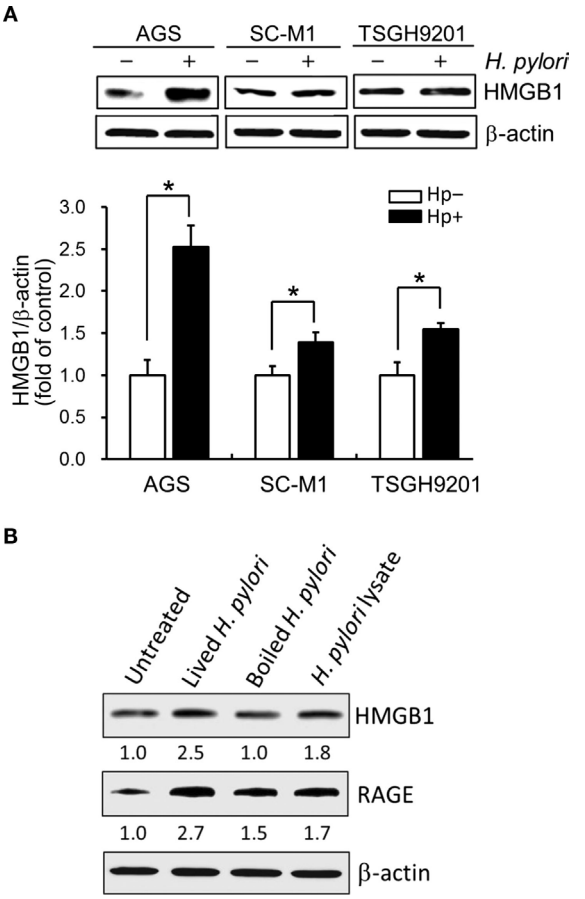

FIGURE 2 | Live *H. pylori* is essential for enhancing HMGB1 and RAGE expression. Gastric epithelial cell lines, AGS, SC-M1, and TSGH9201 cells, were infected with *H. pylori* at an MOI of 100 for 6 h. **(A)** Cells from these cells lines were uninfected or infected with *H. pylori* (MOI = 100) for 6 h. Cell lysates were prepared to analyze HMGB1 expression by Western blot. Protein expression levels were quantified by densitometric analysis and normalized to β-actin. Statistical significance was evaluated by Student's *t*-test (*$P < 0.05$). **(B)** AGS cells were untreated or treated with live *H. pylori* or heat-killed *H. pylori* (boiled *H. pylori*) at an MOI of 100, or crude extracts prepared from *H. pylori* (*H. pylori* lysate). Cell lysates were prepared to measure HMGB1 and RAGE protein expression by Western blot, with β-actin was used as the protein loading control. The expression level of each protein was quantified by signal intensity, and the respective value is indicated at the bottom of each lane.

We further investigated whether *H. pylori*-induced HMGB1 and RAGE expression required lipid raft integrity. Western blot analysis showed that CTX-B was enriched in the detergent-resistant membrane (DRM) fraction (**Figure 7A**), whereas disrupting lipid rafts with MβCD reduced the presence of CTX-B in the DRM. During *H. pylori* infection, HMGB1 and RAGE were abundant in the DRM fraction. Moreover, treatment of cells with MβCD led to a significant reduction in *H. pylori*-induced HMGB1 and RAGE expression in the DRM (**Figures 7B,C**), suggesting that cholesterol-rich microdomains play an important role in *H. pylori*-triggered HMGB1 and RAGE expression.

We next examined whether cholesterol-rich microdomains were essential for *H. pylori*-induced IL-8 production. AGS cells were untreated or pretreated with MβCD and then incubated with *H. pylori* for 6 h. Results showed that MβCD treatment significantly suppressed *IL-8* promoter activity in *H. pylori*-infected cells (**Figure 8A**). Similarly, *H. pylori*-induced IL-8 production in cells was markedly reduced when cholesterol-rich microdomains were disrupted by MβCD (**Figure 8B**). Taken together, results from this study demonstrate that depletion of cholesterol inhibits the mobilization of RAGE into cholesterol-rich microdomains, thereby mitigating *H. pylori*-induced inflammation.

DISCUSSION

Infection with *H. pylori* is associated with sustained inflammation, which may lead to severe gastric diseases (5). Previous studies have indicated that HMGB1 can be secreted by *H. pylori* VacA-treated cells, which then underwent necrosis, inducing a proinflammatory response (22). Moreover, *H. pylori* infection increases the expression of RAGE, which subsequently interacts with its ligand HMGB1, and is believed to amplify the inflammation cascade (29). Despite the fact that the interaction of HMGB1 and RAGE can be linked to necrosis and a proinflammatory response in cells (30), the detailed mechanism by which *H. pylori* induces HMGB1 and RAGE expression and triggers IL-8 secretion to promote inflammation of gastric epithelial cells remains unclear. To elucidate the direct mechanical effects of bacterial infection, we employed antibody neutralization of HMGB1 and siRNA for RAGE and demonstrated that *H. pylori*-induced RAGE following the elevation in HMGB1 levels. Furthermore, RAGE was mobilized into lipid rafts, which contributed to the induction of NF-κB activation and IL-8 production during *H. pylori* infection. Notably, depletion of cholesterol diminishes *H. pylori*-induced signaling, confirming the recruitment of RAGE into lipid rafts by *H. pylori* to promote inflammation in gastric epithelial cells.

High-mobility group box 1 has been recognized as a damage-associated molecular pattern (DAMP), and it has been implicated in several bacterial diseases, including inflammatory lung injury (20), pneumonia (19), sepsis (31), and keratitis (32). Accumulating evidence indicates that HMGB1 functions as an alarmin, forming immune stimulatory complexes with chemotactic factors that promote the migration of leukocytes, activation of lymphoid cells, and augment the inflammatory response (30, 33, 34), which correlate with severity of infection (21). RAGE, a ligand for HMGB1, is involved in activating NF-κB and stimulating proinflammatory factors (35). Treatment of mice with neutralizing α-HMGB1 reduced the bacterial burden and ameliorated tissue injury (20, 32). Similarly, blocking HMGB1 reduced *H. pylori*-elicited RAGE expression, resulting in the attenuation of NF-κB activation and thereby mitigating inflammation in gastric epithelial cells. Our findings are in accordance with previous studies with other bacteria, indicating a potential pathogenic role for HMGB1 and RAGE.

In this study, we showed that *H. pylori* infection elicits HMGB1 and RAGE expression, which enhances IL-8 production. In contrast, silencing RAGE appears to reduce *H. pylori*-mediated NF-κB and IL-8 activities. However, NF-κB and IL-8

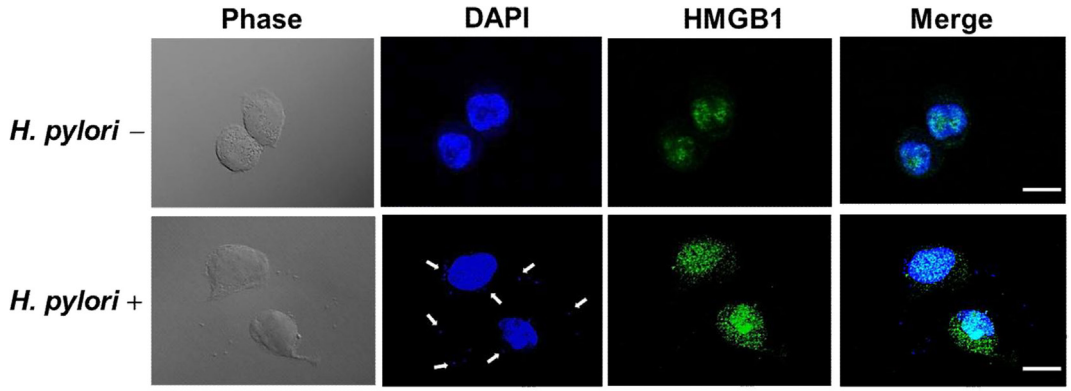

FIGURE 3 | HMGB1 expression in response to *H. pylori* infection. AGS cells were uninfected or infected with *H. pylori* (MOI = 100) at 37°C for 6 h. Cells were fixed and probed with antibody against HMGB1 (green) or stained with DAPI (blue) to visualize cell nuclei and *H. pylori* (arrows). The stained samples were analyzed by confocal microscopy. Scale bars, 10 μm.

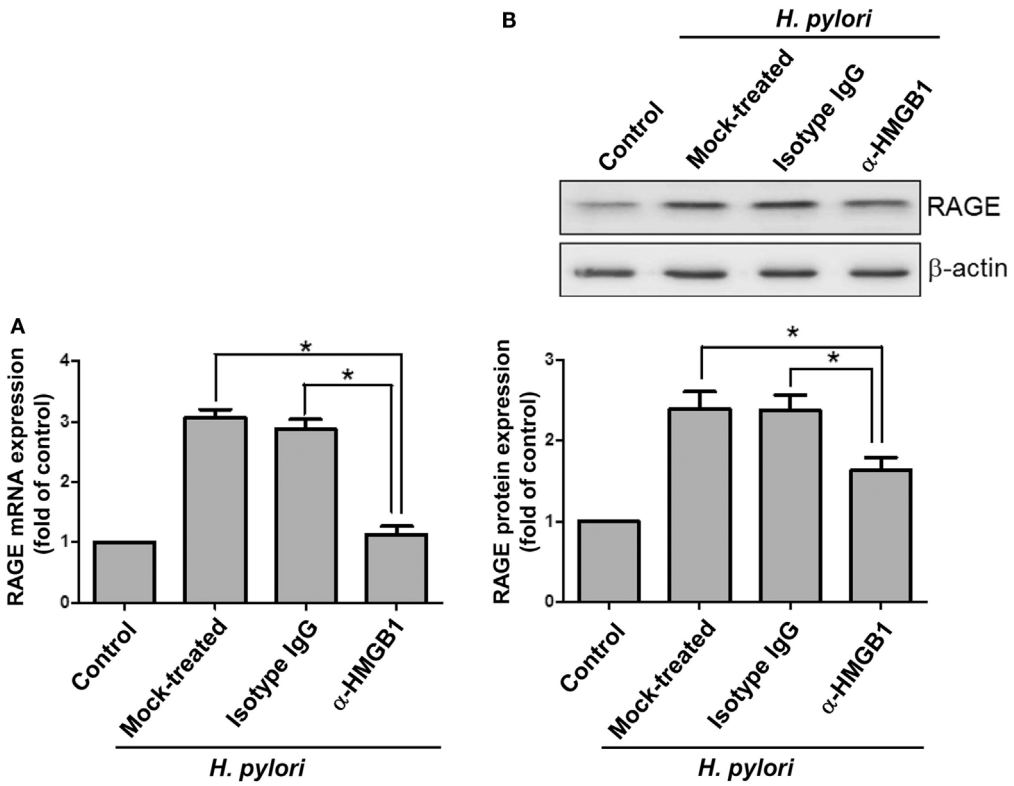

FIGURE 4 | HMGB1 is crucial for RAGE expression in *H. pylori*-infected cells. AGS cells were untreated or pretreated with 1 μg/ml of isotype IgG or anti-HMGB1 at 37°C for 30 min and then infected with *H. pylori* at an MOI of 100 for 6 h. RAGE mRNA and protein expression levels were measured by **(A)** quantitative real-time PCR and **(B)** Western blot analysis, respectively. Results are expressed as means ± SDs. *$P < 0.05$.

activities were still greater in siRAGE-transfected cells infected with *H. pylori* than in transfected cells that were uninfected. These results suggest that there are diverse receptors and ligands for HMGB1 and RAGE that interact and contribute to *H. pylori*-induced inflammation. For instance, HMGB1 is able to trigger a proinflammatory response by interacting with either IL-1β, CXCL12 to form immune stimulatory complexes, or several cell surface receptors, including RAGE, toll-like receptor 2 (TLR2), and TLR4 (15, 36). Our recent findings support the explanations that infection of gastric epithelial cells with *H. pylori* induces

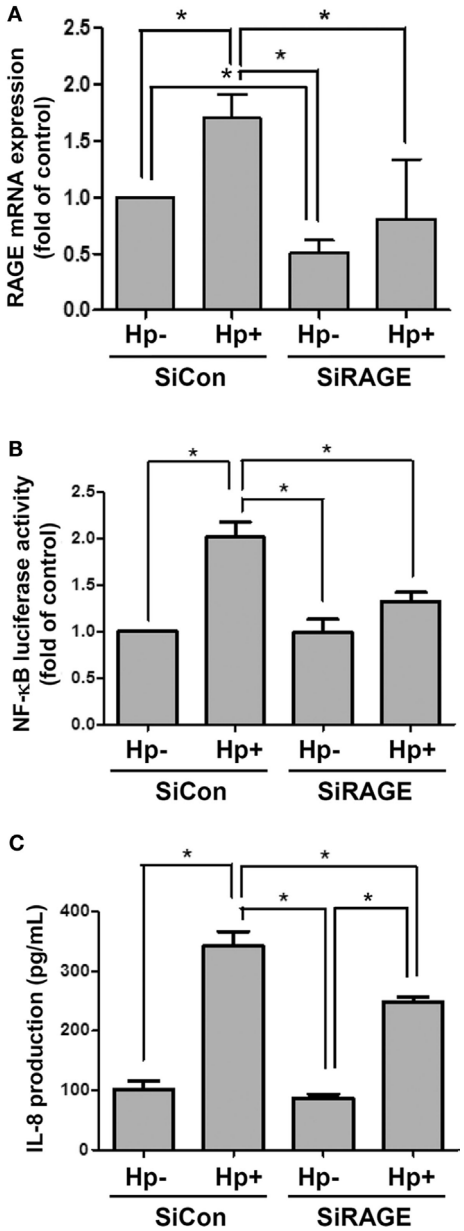

FIGURE 5 | Knocking down RAGE reduces NF-κB promoter activity and IL-8 production in *H. pylori*-infected AGS cells. Cells were transfected with control siRNA (SiCon) or RAGE siRNA (SiRAGE) for 24 h prior to infection with *H. pylori* (MOI = 100) for 6 h. **(A)** The RAGE mRNA level was determined by quantitative real-time PCR. **(B)** Cells were co-transfected with SiRAGE and NF-κB/wt luciferase reporter for 24 h and cultured with *H. pylori* (MOI = 100) for an additional 6 h. NF-κB promoter activity was analyzed by luciferase reporter assay. **(C)** The level of IL-8 in the culture supernatant was determined using a standard ELISA. Results were expressed as means ± SDs. *$P < 0.05$.

TLR4/MD-2 expression, which contributes to the inflammatory response (27). On the other hand, RAGE can bind ligands other than HMGB1, including amyloids and members of the S100 protein family (37, 38). Understanding the interactions other than those of HMGB1 and RAGE is required to further investigation the molecular patterns involved in immune sensing following infection with *H. pylori*.

Damage-associated molecular patterns are endogenous danger signals that have been identified, including HMGB1, S100A8/9, IL-1α, and IL-33/ST2 (39–41). Activation of HMGB1 signal is mediated by several pattern-recognition receptors (PRPs), such as RAGE and toll-like receptors (TLRs), that are important for *H. pylori*-induced inflammation has been revealed in our and other studies (24, 29, 42). Similar to HMGB1, IL-1α and IL-33/ST2 also are types of alarmins, which are abundantly expressed in epithelial and endothelial cells (43, 44). Expression of IL-1α and IL-33/ST2 has been reported in several bacterial infectious diseases. For example, IL-1α production was essential for the early recruitment of neutrophils to the lungs infected with *Legionella pneumophila* (45). In patients with *Staphylococcus aureus* infection on the skin, IL-33 is markedly increased as compared to the healthy controls and suggested that IL-33 possesses antimicrobial and wound-healing effects (46). However, limited reports indicated that IL-1α and IL-33/ST2 can be upregulated in cells treated with the virulence factors from *H. pylori* (47, 48), but their role in *H. pylori*-induced pathogenesis is ill defined. Although these DAMPs have been found to be associated with necroptosis, which is an important process for induction of inflammatory diseases (41), the exact role in *H. pylori*-induced inflammation remains to be investigated.

This study presents a model of the early *H. pylori*-induced gastric epithelial cell inflammatory response. The expression of HMGB1 and RAGE was only tended to increase with infections for 6 h. After incubation for a longer time, the expression levels of HMGB1 and RAGE were diminished. This trend can also be seen in infections with *S. aureus* and other Gram-negative bacteria in mouse models (19, 20, 49). One possible explanation for this observation is that cytokine production was substantially reduced at a time point later than 6 h, which may result in a reciprocal reduction in HMGB1 release and amelioration of bacteria-induced pathogenesis.

Although an inflammatory response with the recruitment of leukocytes is crucial for eradicating intracellular pathogens, prolonged activation of neutrophils may result in serious tissue damage in the stomach (50). IL-8 is recognized as one of the most important chemokines that cause neutrophils to infiltrate into sites of bacterial infections (51). Moreover, HMGB1 is reported to be a chemoattractant for neutrophils during inflammation (52). In this study, we showed that *H. pylori* exploits cholesterol to induce inflammation through activation of the HMGB1–RAGE–IL-8 axis. Silencing RAGE significantly attenuated *H. pylori*-induced NF-κB activation and IL-8 production. Our results, combined with the findings of others, indicate that HMGB1 might be a key target for the development of therapeutic agents against *H. pylori*-induced inflammation.

Although our study has demonstrated that *H. pylori* exploits cholesterol to induce inflammation through activation of the HMGB1–RAGE–IL-8 axis, the limitation of this work is that it lacks *in vivo* data. It has been reported that the human serum HMGB1 levels are significantly and sequentially increased during gastric cancer progression (53). Similarly, in a previous study, the HMGB1 expression in gastric cancer

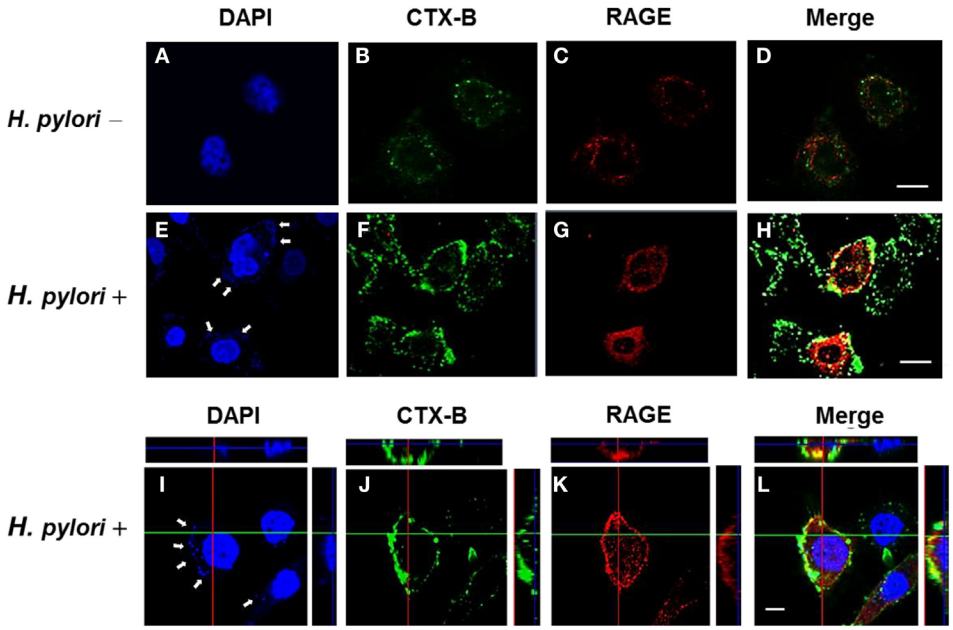

FIGURE 6 | Mobilization of RAGE into lipid rafts at sites of *H. pylori* infection. AGS cells were uninfected or infected with *H. pylori* (MOI = 100) for 6 h. Cells were fixed and stained with DAPI (blue) **(A,E,I)** to visualize *H. pylori* (arrows) and cell nuclei, with Alexa Fluor 488-conjugated cholera toxin subunit B (CTX-B) to visualize GM1 (green) **(B,F,J)**, or with antibody against RAGE (red) **(C,G,K)**, and then the merged images were observed by confocal microscopy **(D,H,L)**. Merged confocal *z*-section images **(I–L)** show bacteria colocalized with RAGE and CTX-B (cyan). Bars, 10 μm.

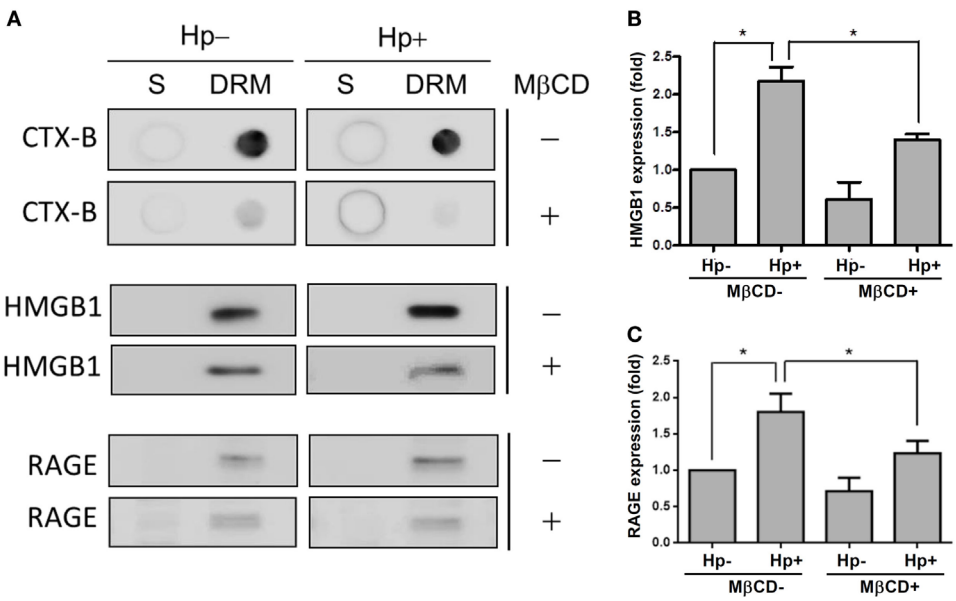

FIGURE 7 | Role of cholesterol-rich microdomains in *H. pylori*-induced HMGB1 and RAGE expression. AGS cells were untreated or pretreated with 5 mM MβCD at 37°C for 1 h. Cells were then washed and infected with *H. pylori* at an MOI of 100 for 6 h. **(A)** Detergent-resistant membrane (DRM) and detergent-soluble (S) fractions were prepared and subjected to cold detergent extraction using 1% Triton X-100 at 4°C followed by centrifugation. Each fraction was analyzed by dot blot or Western blot using cholera toxin subunit B (CTX-B) conjugated to horseradish peroxidase or antibodies against HMGB1 and RAGE, respectively. Protein expression levels of **(B)** HMGB1 and **(C)** RAGE were quantified by densitometric analysis (*$P < 0.05$).

tissues was increased as compared to that in non-cancerous tissues (54). Moreover, a markedly higher percentage of RAGE expression was found in *H. pylori*-infected biopsies with dysplasia or *in situ* carcinoma as compared to that in the control groups (55). Most importantly, it has been proven that overexpressed HMGB1 enhances IL-8 secretion in tumor cells

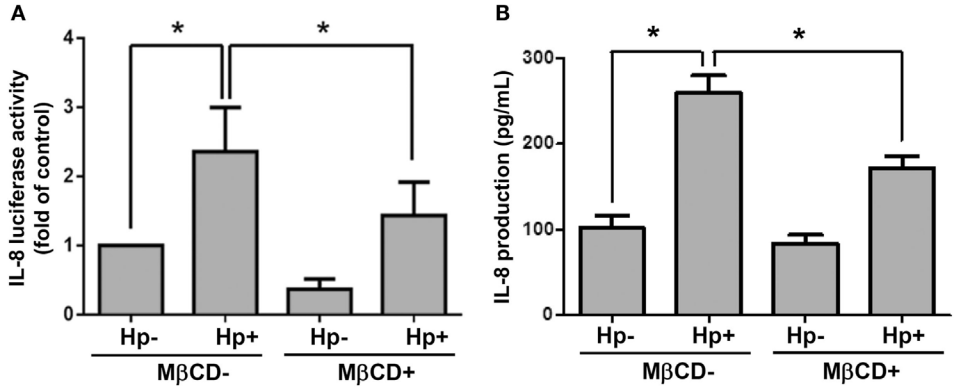

FIGURE 8 | Disruption of lipid rafts reduces *H. pylori*-induced IL-8 production. AGS cells were transfected with an *IL-8* luciferase reporter in the absence or presence of 5 mM MβCD prior to infection with *H. pylori* (MOI = 100) for 6 h. **(A)** Cell lysates were subjected to luciferase activity assay to assess *IL-8* promoter activity. **(B)** IL-8 secretions in the cell culture supernatants were assessed by ELISA. Statistical significance was evaluated by Student's *t*-test (*$P < 0.05$).

and over-secreted IL-8 promotes EMT activation in gastric cancer cells (56). Accordingly, blocking HMGB1 suppresses gastric cancer cell proliferation, whereas inducing IL-8 reverses this anti-tumor effect. These findings demonstrated the role of HMGB1 and RAGE as inducers of inflammation in the context of gastric cancer and suggested that they could be attractive targets for diagnosis and therapy of patients with incipient gastric cancer. Thus, our results supported by evidence in the existing literature, and further revealed that HMGB1–RAGE–IL-8 axis may play an important role in clinical features of *H. pylori*-induced inflammation. Although the present work did not include human studies, we believe that it deserves to be explored *in vivo* and that will definitely fill a gap in the translational research.

Here, we report that *H. pylori*-induced RAGE expression follows HMGB1 production. Our study shows that *H. pylori* infection mobilizes RAGE into cholesterol-rich microdomains, which contributes to NF-κB activation and IL-8 secretion. Furthermore, we elucidate the role for reciprocal, cholesterol-dependent interactions of HMGB1, and RAGE in IL-8 production during the early phase of *H. pylori*-induced inflammation in gastric epithelial cells.

AUTHOR CONTRIBUTIONS

Conception or design of this work: H-JL, H-CL, and C-HL. Experimental study: F-YH, W-WC, C-HL, and C-JC. Data analysis and interpretation: Y-JL, Y-YC, M-ZH, M-CK, and Y-AC. Writing the manuscript: H-JL, H-CL, and C-HL. Final approval: all authors.

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REVIEW
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Danger Signals and Graft-versus-host Disease: Current Understanding and Future Perspectives

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Graft-versus-host response after allogeneic hematopoietic stem cell transplantation (allo-HCT) represents one of the most intense inflammatory responses observed in humans. Host conditioning facilitates engraftment of donor cells, but the tissue injury caused from it primes the critical first steps in the development of acute graft-versus-host disease (GVHD). Tissue injuries release pro-inflammatory cytokines (such as TNF-α, IL-1β, and IL-6) through widespread stimulation of pattern recognition receptors (PRRs) by the release of danger stimuli, such as damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). DAMPs and PAMPs function as potent stimulators for host and donor-derived antigen presenting cells (APCs) that in turn activate and amplify the responses of alloreactive donor T cells. Emerging data also point towards a role for suppression of DAMP induced inflammation by the APCs and donor T cells in mitigating GVHD severity. In this review, we summarize the current understanding on the role of danger stimuli, such as the DAMPs and PAMPs, in GVHD.

Keywords: allogeneic hematopoietic stem cell transplantation, graft-versus-host-disease, danger signals, pathogen-associated molecular patterns, damage-associated molecular patterns

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HCT) has become widely used as a curative therapy for a variety of life-threatening hematological malignancies and congenital immune deficiencies (1). However, graft-versus-host disease (GVHD) remains as significant obstacle to improving the success of this treatment (2). The cause of GVHD reflects a complex process involving immune dysregulation in the context of recovering immunocompetent donor cells in recipients of allo-HCT. Donor T cells play a central role in the pathogenesis of acute GVHD. However, emerging data in the past 15 years have demonstrated a key role for donor, or recipient antigen presenting cells (APCs), derived from both hematopoietic and non-hematopoietic cells. Although current strategies of the prevention and treatment of acute GVHD mainly target T cells, modulating APC function represents a promising additional strategy for reducing acute GVHD. Therefore, a greater understanding of how APCs are activated and regulated is of significant interest. Myeloablative or reduced intensity conditioning regimens are a prerequisite for facilitating engraftment of donor hematopoietic cells, and for eliminating residual tumor cells, but they also cause significant host tissue damage. The impact of damage responses on APCs has become an active area of research. Host tissue injuries by conditioning regimens release

"danger signals" including pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS), and damage-associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB-1) as well as pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α from the inflamed tissues. These danger signals activate host or donor APCs that in turn present alloantigens *via* major histocompatibility complex (MHC) class I or class II to donor T cells. In addition, activated APCs produce an abundance of T-cell stimulating cytokines, such as IL-12, which further escalate the inflammatory response. In this review, we describe several encouraging investigations that have been conducted in both experimental bone marrow transplantation (BMT) models and humans over the last two decades. We further summarize the updated findings of how DAMPs and PAMPs amplify or mitigate GVHD and explore potential new strategies for the regulation of these "danger signals" in the regulation of GVHD.

DANGER SIGNALS IN GVHD DEVELOPMENT

PAMPs are non-host derive molecules derived from microbes and are recognized by pattern recognition receptors (PRRs) that initiate and sustain the innate immune responses for protecting host from foreign pathogens (3). DAMPs are host-derived molecules released by host tissue damages and binds to PRRs that initiate and sustain non-infectious immune responses (4). These DAMPs and PAMPs are released as a consequence of conditioning-related tissue damage after allo-HCT. They activate APCs that in turn stimulate donor T cell proliferation and differentiation into effector T cells that migrate to target organs and cause GVHD. Upon target tissue destruction, additional PAMPs and DAMPs are released that perpetuate and amplify GVHD (**Figure 1**). Therefore, our understanding of the release of PAMPs/DAMPs and ways to limit this potentially lethal immunologic cascade by ameliorating tissue damages by inhibiting danger signaling

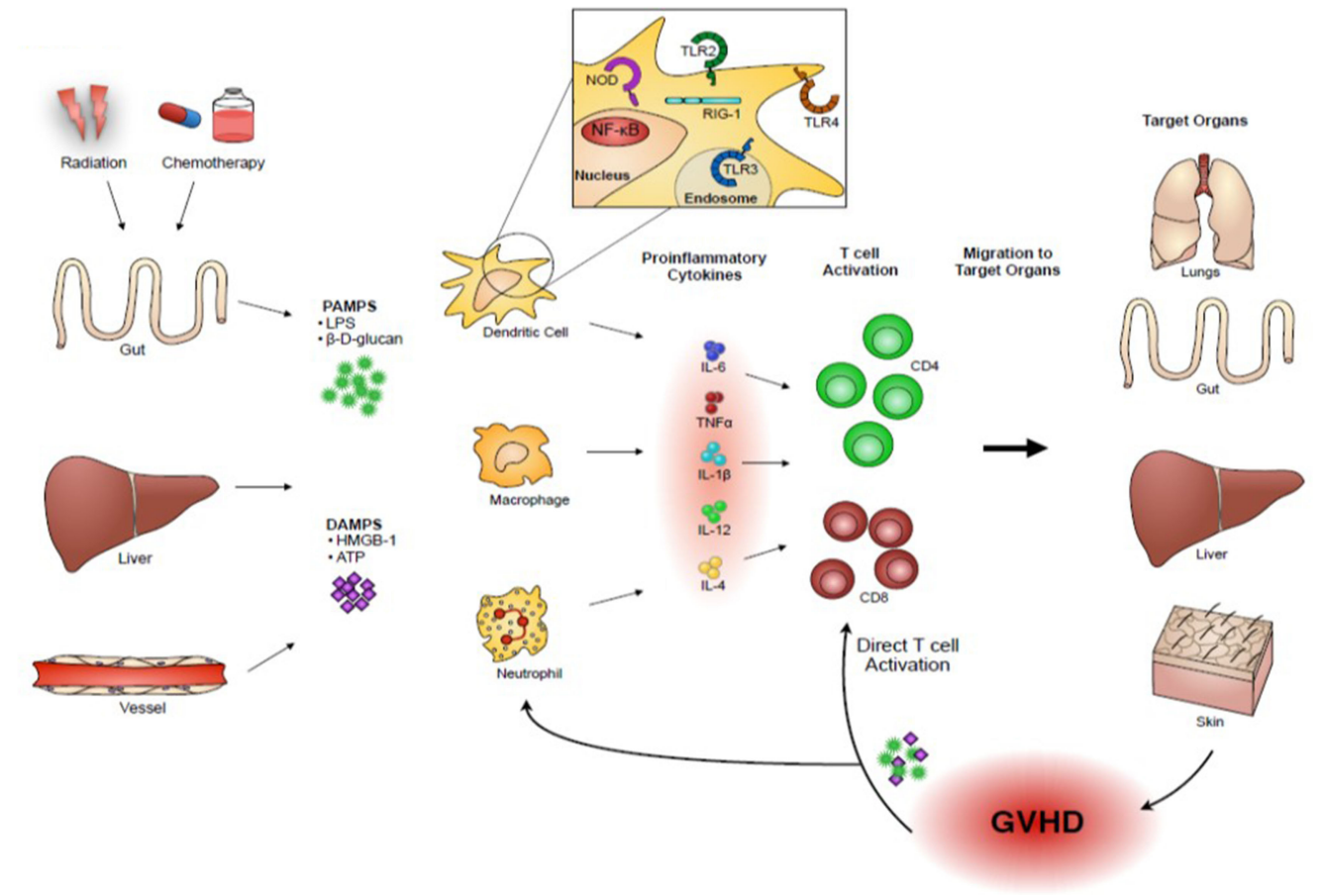

FIGURE 1 | **Danger signals play an important role in acute GVHD pathogenesis**. Host tissue injuries by conditioning regimens release "danger signals" including pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) and β-D-glucans, and damage-associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB-1) and adenosine triphosphate (ATP). These danger signals activate host or donor antigen-presenting cells (APCs), such as dendritic cells and macrophages, which in turn present alloantigens *via* major histocompatibility complex (MHC) class I or class II to donor T cells. In addition, activated APCs produce an abundance of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, and T-cell stimulating cytokines, such as IL-12, which further escalate the inflammatory response. Activated donor T cells proliferate and differentiate into effector T cells that migrate to target organs and cause GVHD. Upon target tissue destruction, additional PAMPs and DAMPs are released and they might perpetuate GVHD responses.

with specific inhibitors may be important for mitigating the intensity of GVHD.

ROLE OF SPECIFIC PRRs IN GVHD

Danger signaling is transmitted through PRRs when they bind PAPMs and DAMPs. Several signaling pathways, such as toll-like receptor (TLR), Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR), and retinoic acid-inducible gene 1 (RIG-I) signaling, are recognized. The detailed mechanisms are recently reviewed in several articles (5–7). In this review, we focus on some of these receptors that have been implicated in GVHD.

TOLL-LIKE RECEPTORS

Toll-like receptors are one of the PRRs and play a key role in innate immune responses by recognizing PAMPs as well as DAMPs (8). TLRs are expressed on a variety of cells derived from both hematopoietic and non-hematopoietic lineages (8). We discuss below the experimental studies of TLRs in the pathogenesis of acute GVHD. The studies are also summarized in **Table 1**.

TABLE 1 | The role of TLRs in the pathogenesis of acute GVHD.

BMT models	MHC	Conditioning	Donor cells	Results	Reference
TLR4					
B6 ($H2^b$) → C3H/HeJ ($H2^k$) (TLR4 mutant)	Mismatch	9 Gy	BM: 5×10^6 SP: 2.5×10^7	GVHD: ↑	(9)
BALB/c ($H2^d$) → B6-TLR4$^{-/-}$ ($H2^b$)	Mismatch	10.5 Gy	BM: 1×10^7 SP: 2×10^7	GVHD: →	(10)
B6-TLR4$^{-/-}$ ($H2^b$) → BALB/c ($H2^d$)	Mismatch	9 Gy	BM: 1×10^7 SP: 2×10^7	GVHD: ↓	(10)
C3H/Hej ($H2^k$) (LPS resistant) → (C3FeB6)F1 ($H2^{b/k}$)	Mismatch, haploidentical	11 Gy	TCD-BM: 5×10^6 Tcells: $0.25–1 \times 10^6$	GVHD: ↓	(11)
BALB/c (H-2^d) → B6-TLR2/4$^{-/-}$ (H-2^b)	Mismatch	Treosulfan + cyclophosphamide	BM: 5×10^6 SP: 3×10^6	GVHD severity: ↓, mortality: →	(12)
129S6 (H2b) → B6-TLR4$^{-/-}$ ($H2^b$)	Match, multiple minor antigen mismatch	11 Gy	BM: 5×10^6 SP: 30×10^6	GVHD: →	(13)
B6-TLR4$^{-/-}$ ($H2^b$) → 129 *Rag2*$^{-/-}$ ($H2^b$)	Match, multiple minor antigen mismatch	Anti-NK11 or anti-asialoGM1, ±7 Gy	SP: 30×10^6	GVHD: ↓	(13)
BALB/c ($H2^d$) → B6-TLR4$^{-/-}$ ($H2^b$)	Mismatch	9 Gy	BM: 1×10^7 SP: $4–5 \times 10^7$	GVHD: →	(14)
C3Hsw ($H2^b$) → B10ScNcr-TLR4$^{-/-}$ ($H2^b$)	Match, multiple minor antigen mismatch	10 Gy	BM: 1×10^7 $CD8^+$ T cells: 2×10^6	GVHD: →	(15)
MyD88/TRIF					
BALB/c ($H2^d$) → B6-MyD88$^{-/-}$ ($H2^b$)	Mismatch	Treosulfan + cyclophosphamide	BM: 5×10^6 SP: 3×10^6	GVHD severity: ↓, mortality: →	(12)
B6-MyD88$^{-/-}$ ($H2^b$) → B6D2F1 ($H2^{b/d}$)	Mismatch, haploidentical	11 Gy	TCD-BM: 5×10^6 Tcells: $1–2 \times 10^6$	GVHD: ↑	(16, 17)
B6-MyD88$^{-/-}$ ($H2^b$) → 129 *Rag2*$^{-/-}$ ($H2^b$)	Match, multiple minor antigen mismatch	Anti-NK11 or anti-asialoGM1, ±7 Gy	SP: 30×10^6	GVHD: ↓	(13)
BALB/c ($H2^d$) → B6-TRIF$^{-/-}$ (H-2^b)	Mismatch	Treosulfan + cyclophosphamide	BM: 5×10^6 SP: 3×10^6	GVHD severity: ↓, mortality: →	(12)
C3Hsw ($H2^b$) → B6 LPS2 (TRIF$^{-/-}$)($H2^b$)	Match, multiple minor antigen mismatch	10 Gy	BM: 1×10^7 $CD8^+$ T cells: 2×10^6	GVHD: →	(15)
B6-TRIF$^{-/-}$ ($H2^b$) → 129 *Rag2*$^{-/-}$ ($H2^b$)	Match, multiple minor antigen mismatch	Anti-NK11 or anti-asialoGM1, ±7 Gy	SP: 30×10^6	GVHD: →	(13)
TLR2					
B6-TLR2$^{-/-}$ ($H2^b$) → B6D2F1 ($H2^{b/d}$)	Mismatch, haploidentical	11 Gy	TCD-BM: 5×10^6 SP: 2×10^7	GVHD: →	(20)
B6-TLR2$^{-/-}$ ($H2^b$) → BALB/c ($H2^d$)	Mismatch	85 Gy	TCD-BM: 5×10^6 SP: 2×10^7	GVHD: →	(20)

(Continued)

TABLE 1 | **Continued**

BMT models	MHC	Conditioning	Donor cells	Results	Reference
TLR5					
B10BR ($H2^k$) → B6 ($H2^b$) with flagellin (50 μg)	Mismatch	11 Gy	TCD-BM: 5×10^6 SP: 5×10^6	GVHD: ↓	(28)
TLR9					
B6 ($H2^b$) → B10BR ($H2^k$) with CpG (100 μg)	Mismatch	8 Gy	BM: 5×10^6 SP: 25×10^6	GVHD: ↑	(40)
BALB/c($H2^d$) → B6 ($H2^b$) with CpG (100 μg)	Mismatch	8 Gy	BM: 5×10^6 SP: 15×10^6	GVHD: ↑	(40)
BALB/c ($H2^d$) → B6 ($H2^b$) with CpG (50–100 μg)	Mismatch	10 Gy	BM: 5×10^6 SP: 1×10^7	GVHD: ↑	(41)
BALB/c ($H2^d$) → B6-TLR9$^{-/-}$ ($H2^b$)	Mismatch	9 Gy	BM: 1×10^7 SP: 4×10^7	GVHD: ↓	(14)
BALB/c ($H\text{-}2^d$) → B6-TLR9$^{-/-}$ ($H\text{-}2^b$)	Mismatch	Treosulfan + cyclophosphamide	BM: 5×10^6 SP: 3×10^6	GVHD: ↓	(12)
TLR3					
C3Hsw ($H2^b$) → [TLR3$^{-/-}$ ($H2^b$) → B6 ($H2^b$)]	Match, multiple minor antigen mismatch	9 Gy	TCD-BM: 5×10^6 $CD8^+$ T cells: 0.5×10^6	GVHD: →	(48)

TLR4

TLR4 is a cell-surface receptor for PAMPs such as LPS and also for DAMPs. TLR4 is broadly expressed on many immune cells, such as dendritic cells (DCs). TLR4 signaling is transmitted through intracellular adaptor molecules myeloid differentiation primary response gene 88 (MyD88) and Toll/IL-1receptor (TIR)-domain-containing adaptor-inducing interferon β (TRIF) that activate NF-κB signaling that potently enhances expression of pro-inflammatory cytokines. The role of TLR4 in APCs for mediating acute GVHD remains controversial. Mutations in TLR4 are involved in the reduction of GVHD responses by hyporesponsiveness of APCs to LPS stimulation while over activation of TLR4 signaling results in exacerbation of GVHD (9). When TLR4$^{-/-}$ animals were used as either donor or recipient, acute GVHD severity and mortality were significantly ameliorated in MHC-mismatched B6 into BALB/c model by altering DC functions in TLR4$^{-/-}$ APCs (10). This finding was consistent with a previous report in which recipients receiving LPS resistant donor cells demonstrated less GVHD and prolonged survival in MHC-mismatched haploidentical BMT (11). TLR2/4$^{-/-}$ animals receiving MHC-mismatched BMT (BALB/c into B6) also showed significantly less intestinal GVHD, but reduction was appeared dependent on conditioning intensity (12). In minor mismatched BMT context, MyD88-mediated TLR4 signaling on donor, but not recipient cells, was required for mediating acute GVHD (13). In addition, when TLR4 signaling was impaired in host APCs, that is, in the case of the absence of MyD88, TRIF, or both MyD88 and TRIF expression, acute GVHD severity and mortality were equivalent to WT animals (14, 15). While there is an increasing understanding of the key role of TLR4 signaling in contributing to the initiating event of GVHD, such disparate findings indicate that the role of TLR4 signaling for mediating GVHD may differ depending on the strain, the cell type where TLR4 is mutated, and the conditioning.

MyD88/TRIF

As MyD88 is required for the signaling of many TLRs, when MyD88$^{-/-}$ animals were used as the host, acute GVHD was significantly improved (12). In contrast, the recipients that received MyD88$^{-/-}$ T cell depleted BM (TCD-BM) cells showed greater intestinal GVHD (16) but reduced hepatic GVHD. This was found to be dependent on myeloid-derived suppressor cells (MDSCs) (17). In addition, MyD88$^{-/-}$ donor T cells reduced graft-versus-tumor (GVT) activity through the expansion of Foxp3- and IL-4-producing T cells in MHC-mismatched haploidentical B6 into B6D2F1 model (18). However, donor MyD88 was shown to be required in minor mismatch GVHD (13). TRIF is also required to transmit TLR signaling, but its role seems to be negligible in the development of GVHD (12, 13, 15). Collectively, these studies suggest that MyD88 may have pleiotropic functions that are cell intrinsic during allo-HCT.

TLR2

TLR2 is a cell-surface receptor expressed on APCs as well as T cells. TLR2 recognizes cell-wall components such as peptidoglycan (PGN) from gram-positive bacteria as well as zymosan from yeast. Intriguingly, granulocyte-colony stimulating factor (G-CSF) mobilized donor grafts showed the increase level of TLR2 expression on myeloid cell populations (19), but upregulated TLR2 expression did not correlate with enhanced allogeneic responses (20). The studies utilizing TLR2$^{-/-}$ animals as either

donor or host demonstrated that TLR2 has little effect on acute GVHD (12, 20).

TLR5

TLR5 recognizes flagellin that is an essential component of bacterial flagella from both gram-negative and -positive bacteria and regulates immunity (21–26). CBLB502, a TLR5 agonist and a polypeptide drug derived from Salmonella flagellin, protects intestinal and hematopoietic cells from total body irradiation (TBI) in mice and primates (27). Consistent with this observation, when TLR5 agonist was administered before conditioning, acute GVHD was reduced with enhanced anti-CMV immunity in both MHC-mismatched and haploidentical murine models (28, 29). By contrast, TLR5 mRNA expression on peripheral blood, especially in the Lin(−)HLADR(−)CD33(+) CD16(+) and CD14(++)CD16(−) monocytes, was increased in the patients with GVHD after receiving adaptive Treg infusion for prevention of GVHD in human (30).

TLR7

TLR7 recognizes endosomal single strand ribonucleic acids (RNAs) leading to production of type I interferons (IFNs), pro-inflammatory cytokines, as well as regulatory cytokines (31, 32). TLR7 is critical for antiviral immunity and the development of autoimmune diseases (33–36). The contribution of TLR7 to acute GVHD is not well characterized. TLR7 ligand imiquimod was shown to increase alloreactivity of host-derived DCs and Langerhans cells (LCs) in the skin and to enhance donor lymphocyte infusions (DLIs)-mediated GVHD in MHC-matched multiple minor antigen-mismatched model of BMT (37). Because Type I IFNs are indispensable in the antitumor responses, whether TLR7 agonists increase GVT activities without enhancing GVHD after allo-HCT is of significant interest.

TLR9

TLR9 recognizes unmethylated cytosine-phophorothionate-guanine (CpG) dinucleatides in the bacterial DNA and triggers a Th1-mediated inflammatory response (38). CpG-mediated immune responses through TLR9 distinguish bacterial DNA from self-DNAs. TLR9 is expressed intracellularly in both immune and non-hematopoietic derived cells, such as endothelial and epithelial cells (39). Administration of CpG oligonucleotides (CpG ODNs), a synthetic TLR9 ligand, exacerbates acute GVHD in a host APC and IFN-γ dependent manner (40, 41). In addition, CpG ODNs enhances rejection donor HSCs in donor-derived APC dependent manner (40). When lethally or sub-lethally conditioned TLR9$^{-/-}$ animals were used as hosts, acute GVHD severity and mortality was ameliorated. This was dependent on the expression of TLR9 expression on the non-hematopoietic cells (12, 14). However, clinical studies exploring TLR9 polymorphisms in allo-HCT hosts suggested that those with homozygous CC gene variant of TLR9 (which correlates with lower expression of TLR9 mRNA) showed significantly improved overall survival (OS) and reduced relapse rate with no difference in acute GVHD when compared with patients having TC/TT gene variants (42, 43). Donor TLR9 gene tag single nucleotide polymorphisms (SNPs), +1174A/G (rs352139) and +1635 C/T (rs 352140), respectively, correlated increased severity of acute GVHD and CMV reactivation (44).

TLR3

TLR3 recognizes double-stranded RNA (dsRNA), which is produced by most of viruses. It signals through interferon regulatory factor (IRF)3 and activates NF-κB and enhances production of type I IFNs (45, 46). TLR3 also plays an important role in enhancing antigen presentation in APCs (47). Using chimeric recipients with TLR3$^{-/-}$ hematopoietic cells, we have already demonstrated that TLR3 deficiency in host APCs showed equivalent GVHD severity and mortality to WT animals but impaired GVT activity in MHC-matched multiple minor mismatched BMT model. Activation of TLR3 by polyinosine-polycytidylic acid (Poly I:C) improved GVT activity without enhancing GVHD (48). Because type I IFNs are essential role in antitumor immune responses (49, 50), TLR3 may have greater influence on GVT activity.

NLR Signaling

Nucleotide-binding oligomerization domain-like receptors are subtype of PPRs that function as cytoplasmic sensors of PAMPs and DAMPs. NLRs are expressed by majority of immune cells and some non-immune cells. NLRs have been extensively studies for their role in innate immunity. NOD1 and NOD2 are the most widely investigated NLRs in GVHD. NOD1 and NOD2 recognize different kinds of PGN fragments from bacterial cell wall. NOD1 binds to diaminopimelate-containing N-acetyl glucosamine-N-acetylmuramic acid (GluNAc-MurNac) tripeptide from gram-negative bacterial PGN (51, 52), while NOD2 binds to muramyl dipeptide (MDP) that is produced by all bacteria (53). Once NOD1 and NOD2 are ligated, NF-κB and mitogen-activated protein kinase (MAPK) pathways are activated through the caspase recruitment domain (CARD)-containing serine/threonine kinase, receptor-interacting protein 2 (Rip2) (54), and induce pro-inflammatory cytokines. NOD1 and NOD2 signaling is also involved in endoplasmic reticulum (ER) stress induced inflammation through the IRE1α/TRAF2 signaling pathway (55) and production of antimicrobial peptides in the intestinal tract (56). The role of NOD2 signaling pathway in allo-HCT is somewhat controversial. NOD2 polymorphism in both donor and recipient was associated with increased transplant-related mortality in humans after HLA-identical sibling HCT or T cell depleted HCT as well as increased GVHD severity (43, 57–64). However, other studies demonstrated that NOD2 had no impact on outcome including GVHD severity and mortality (44, 65–69). In addition, intriguingly, NOD2 polymorphism was associated with increased relapse of leukemia after unrelated HCT (70). In experimental models, NOD2$^{-/-}$ recipient animals showed exacerbated GVHD severity and morality, particularly intestinal GVHD. This was due to host APC activation in experimental BMT (71). By contrast, donor NOD$^{-/-}$ BM cells reduced GVHD-related mortality in MHC-mismatched haploidentical BMT model (72).

Inflammasomes

Inflammasomes are multiprotein molecules that are in the cytoplasm of immune cells, such as APCs, as well as non-hematopoietic cells. They consist of an adaptor protein, apoptosis

associated speck-like protein containing a caspase recruit domain (CARD) (ASC), which has pyrin domain (PYD) and CARD, pro-caspase 1, and certain receptor proteins, such as NLR family members (NLRP1, NLRP3, NLRC4, NLRP6, NLRP7, and NLRP12), the protein absent in melanoma 2 (AIM2) (73, 74). Once inflammasomes are activated, they produce inflammatory cytokines, specifically IL-1β and IL-18, and induce pyroptosis, a highly inflammatory form of programed cell death (73, 74). This is called canonical inflammasome pathway, which in contrast to the non-canonical pathway activates caspase 11 in mouse and caspases 4 and 5 in humans (75). The detailed molecular and activation pathways of inflammasomes have recently been summarized in excellent reviews (73, 74). Studies exploring the role of inflammasomes in acute GVHD have recently been published. NLRP3 activation by the intestinal commensal bacteria and uric acid released after conditioning, enhanced GVHD severity and mortality by increasing levels of caspase-1, IL-1β, and TH17 cells (76). Another mechanism of enhanced GVHD by NLRP3 was shown to be associated with microRNA-155 dependent host DC migration toward sites of ATP release (77). In addition, inflammasome activation in the inflammatory milieu ameliorated the immune suppressive function of MDSCs and exacerbated GVHD (78). Relevant to clinical translation, the addition of TBI or busulfan and cyclophosphamide (BU/CY) conditioning is capable of mediating NLRP3 activation in the liver and enhancing inflammation (79). As therapeutic strategy, inhibiting NLRP3 activation by nucleotide reverse transcriptase inhibitors decreased GVHD severity and mortality (80). Further, NLRP3 inflammasome in human $CD4^+$ T cells promotes IFN-γ production and Th1 differentiation by enhancing caspase 1-dependent IL-1β secretion mediated through intracellular C5 activation (81). These data suggest that NLRP3 inflammasome contribute to the functional important mediators of GVHD: donor T cells, APCs, and non-hematopoietic cells in target tissue. Consistent with experimental models, donor polymorphisms in the NLRP3 inflammasome have been associated with outcomes after allo-HCT. TT genotype at rs10925027 in NLRP3 was associated with disease relapse and donor GG genotype at rs1043684 in NLRP2 was associated with non-relapse mortality (NRM) and OS. Also, patient AA genotype at rs5862 in NLRP1 was associated with NRM and OS after HLA-matched sibling HCT (82). We recently found that a related but distinct inflammasome, NLRP6, expressed in intestinal epithelial cells regulates innate immune responses and intestine homeostasis though the regulating normal commensal bacteria (83, 84). Absence of NLRP6 improved GVHD contrary to models of inflammatory bowel disease (IBD). Intriguingly, $NLRP6^{-/-}$ animals showed enhanced mucin family protein MUC2 expression in epithelial cells after allo-BMT (85). These results suggest that depending on the context, NLRP6 may exert opposite effects in various inflammatory disorders.

RIG-I Signaling

RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) are known as RIG-I-like receptors (RLRs). RIG-I and MDA5 contain a DExD/H box RNA helicase domain and CARD, but LGP2 has no CARD-like domain. These receptors bind to intracellular dsRNA or ssRNA and trigger innate antiviral responses by producing type I IFNs (86–91). Therefore, RIG-I pathways also play an important role in PAMPs and DAMPs-mediated inflammatory responses. However, whether RIG-I pathways facilitate GVHD development is presently unknown. Preliminary study suggests RIG-I-induced type I IFNs promote the regeneration of intestinal stem cells during acute tissue damage may ameliorate GVHD severity with preserving GVL activities in mouse model (92, 93).

C-Type Lectin Receptors

C-type lectin receptors (CLRs) are expressed on myeloid-derived APCs as soluble or transmembrane embedded proteins. They directly activate NF-κB through spleen tyrosine kinase (SYK) (94) or indirectly, by cooperating with other PRRs such as TLRs (95–97). Stimulation of CLRs promotes the production of pro-inflammatory cytokines, effector T cell differentiation into Th1 and Th17 (98). CLRs are divided into two groups; group 1 CLRs belong to the mannose receptor family and group 2 CLRs belong to the asialoglycoprotein receptor family which has subfamilies, the DC-associated C-type lectin1 (Dectin-1) and DC immunoreceptor (DCIR) subfamily including Dectin-2 (95). These recognize mannose, fucose, and glucan carbo-hydrate structures of bacteria, fungi. Both Dectin1 and Dectin2 activate NF-κB by enhancing SYK signaling through either the cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) in Dectin1 (94) or the ITAM-containing adaptor molecules, such as Fc receptor γ-chain (Fcr γ) or DAP12 in Dectin2 (99). Clinical studies have suggested that the incidence of acute GVHD increases with candida colonization in dectin1 gene dependent manner (100, 101). In murine model, α-mannan, which is a major component of fungal cell wall, mediated Th17 dependent pulmonary GVHD in a host dectin2 dependent manner (102).

ROLE OF SPECIFIC PAMPs AND DAMPs PROTEINS IN GVHD

Both exogenous and endogenous danger signal proteins are released from damaged tissues and abnormal intestinal microbial colonies after conditioning. In addition, blood stream infection (BSI) caused by gut translocation of colonized bacteria is another critical source of PAMPs after allo-HCT (103). Experimental data showed that individual PAMPs and DAMPs proteins can function either independently or cooperatively to initiate GVHD.

Lipopolysaccharides

Lipopolysaccharides (endotoxin) are membrane component of many gram-negative bacteria representing one of the earliest and most investigated PAMPs in GVHD. The role of LPS in GVHD is complex and controversial. LPS translocation due to gastrointestinal (GI) tract damage is correlated with conditioning intensity (104) and shown to contribute to GVHD in select model systems. LPS activates APCs including DCs and macrophages (MFs) triggering production of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 (105, 106). These events contribute idiopathic lung injuries after allo-BMT (107). Persistent exposure of LPS precipitates pulmonary GVHD pathogenesis because recipient mice directly exposed to repeated inhaled LPS after allo-BMT showed

pulmonary GVHD in hematopoietic donor-derived C-C motif ligand 2 (CCL2) and C-C motif receptor (CCR2) dependent manner (108, 109). In addition to host-derived cells, sensitivity to LPS in donor non-T cells has been suggested to be involved in GVHD severity (11). LPS is one of the ligands of TLR4, which plays a key role in innate immune responses (110), and its signaling is transmitted through the common MyD88 and TRIF pathway that can nuclear translocation of NF-kB to induce expression of inflammatory cytokine genes (111–113). TLR4 mutations lead to LPS hyporesponsiveness (110). As noted above, role of TLR4 and MyD88 in GVHD seems to depend on the model system.

Flagellin

The recipient animals treated with flagellin before allo-HCT demonstrated reduced GVHD mortality and enhanced immune reconstitution with preservation of antiviral and GVT effects after allo-HCT (28). Modulating TLR5 functions with flagellin enhanced GVT without exacerbating GVHD in $CD8^+$ T cell and NK cell dependent model (114). Tumor reactive T cells engineered to produce flagellin along with expression of a melanoma-specific antigen-augmented antitumor responses. Contrary to enhanced T cell-mediated antitumor responses, TLR5-dependent commensal bacteria promote tumor development by expanding MDSCs and dampen antitumor immunity in TLR5- and IL-6-dependent manner (115). In addition, mesenchymal stem cells (MSCs), pretreated by flagellin showed increased Foxp3 expression, enhanced IL-10 production, and suppressed GVHD (116). Interestingly, TLR5 stimulation with flagellin protects gut mucosal tissue from damages caused by irradiation (27, 117).

Damage-Associated Molecular Patterns

HMGB1

HMGB1 is a ubiquitous DNA-binding nuclear protein of all eukaryotic cells, binds to nucleosome and regulates gene transcriptions (118). By contrast, HMGB1 plays an important role in initiating innate immune responses because endogenous HMGB-1 that is located in nucleus in resting cells is acetylated and is released from damaged tissues. HMGB-1 binds to TLRs (TLR2 or TLR4) or receptor for advanced glycation endproducts (RAGE) and activates NF-κB or MAPK signaling to produce pro-inflammatory cytokines in especially DCs or MFs (119–123). Therefore, extracellular HMGB1 functions as a DAMP. In addition, the inflammatory milieu with abundant IFN-γ, TNF-α, as well as LPS may promote further HMGB1 release from DCs and MFs (124). Inflammasome and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) 1 pathways are involved in molecular mechanisms of HMGB-1 release, which requires its acetylation and translocation from nucleus to cytoplasm and released to extracellular space through unique protein releasing pathway, such as pyroptosis. Recent reports suggests that HMGB1 promotes not only immune suppressive function through the facilitating MDSCs proliferation in cancer (125) but also protection from tissue injury in IBD by regulating cellular autophagy and apoptosis (126). Patients with HMGB1 polymorphism, the 2351insT minor allele, showed reduced grade II to IV acute GVHD following myeloablative allo-HCT (127). Increased serum levels of HMGB1 were observed in acute GVHD patients and donors treated with granulocyte-colony-stimulating factor (G-CSF) (128, 129). Myeloablative conditioning such as TBI or cyclophosphamide + TBI also increased serum HMGB1 levels consistent with its function as a DAMP (130).

Adenosine Triphosphate

All cells generate adenosine triphosphate (ATP) as the primary energy source *via* glycolysis and oxidative phosphorylation (OXPHOS) that is stored within cytoplasm and mitochondria (131). Once cells are exposed to stress or injury, ATP is released from damaged cells and the concentration of ATP in extracellular space is increased. Released ATP binds to purinergic receptor families, such as P2X expressed on the hematopoietic and non-hematopoietic cells, and can function as a potent DAMP (132, 133). In GVHD, extracellular ATP is dramatically increased after TBI and binds to P2X7R on host APCs. After its ligation, host APCs expressed greater co-stimulatory molecules, such as CD80 and CD86, and enhanced stimulation of donor $CD4^+$ T cells and production of IFN-γ, and decreased Tregs. These results were associated with reduction of STAT5 phosphorylation and enhanced GVHD (134). Another purinergic receptor, P2Y2, in host hematopoietic derived APCs was shown to enhance GVHD (135). Increased extracellular ATP is regulated by ecto-nucleotidases, such as CD39, which phosphohydrolyzes ATP to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) and then dephosphorylate into adenosine by CD73, also known as ecto-5′-nucleotidase (136). In line with this, agonists of the adenosine receptors (AR) decreased GVHD (137). Loss of this regulatory mechanism by $CD73^{-/-}$ T cells or in APCs exacerbated GVHD (138, 139). The recent study showed that inhibiting Notch 1 signaling by inducing expression of A2A receptor in CD73 dependent manner is a critical mechanism of Treg-induced GVHD suppression (140). The immunosuppression of BM-derived MSCs in GVHD was also shown to be partially dependent on CD73 activity (141).

Uric Acid

Uric acid is a metabolite of purine nucleotide and hyperuricemia is known to lead gout (142). Uric acid is also released from injured cells, stimulates DC maturation, activates $CD8^+$ T cell cytotoxic functions, and is recognized as an endogenous DAMP (143). Recent data show that uric acid contributes to GVHD severity by stimulating with NLRP3 inflammasome (76). Patients with acute GVHD show a high level of serum uric acid during the pretransplantation period and the patients received a recombinant urate oxidase appeared to show significantly reduced GVHD in phase I study. These results are consistent with a study that blood uric acid homeostasis may be altered after allo-HCT by conditioning and using cyclosporine A (144). However, a recent study showed low serum level of uric acid was associated with GVHD severity (145).

Heat Shock Proteins

Heat shock proteins (HSPs) work as molecular chaperones that enhance protein folding and intracellular transportation (146). HSPs have been demonstrated the association with chronic inflammatory diseases as well as autoimmune disease (147, 148).

HSPs bind to TLR2/4 and mediate inflammatory responses. In GVHD, expression of HSP70 in lymphoid and target organs is increased and correlated with severity in both human and experimental GVHD (149–151). HSP70 homogene polymorphism (+2763 A/A) was associated with the development of GVHD (152). Another study demonstrated that antibodies to HSP70 and HSP90 increased in the patients with GVHD after allogeneic peripheral blood stem cell transplantation (allo-PBMCT) (153). HSP90 expression is increased in activated T cells and facilitates effector function and survival in activated T cells (154). HSP90-specific inhibitor decreased allogeneic T cell responses *in vitro* (155), but the *in vivo* effects on GVHD were not studied.

Heparan Sulfate Proteoglycans

Heparan sulfate proteoglycans (HSPGs) are component of extracellular matrix and play fundamental role in cell development, metabolism, and immunity (156, 157). HSPGs are crucial role in enhancing innate immune responses by stimulating DCs to enhance production of pro-inflammatory cytokines through TLR4 pathway (158). HSPGs promote neutrophils recruitment into the site of inflammation (159, 160) while enhance neutrophil infiltration exacerbates GVHD (161). The serum level of syndecan-1, which is one of the HSPGs, and heparin sulfate are increased in patients with GVHD (162, 163). In experimental models, heparin sulfate activates TLR4 signaling on DCs and leads to enhanced DC maturation and allogeneic T cell proliferation and increased GVHD severity (163). On the other hand, the absence of syndecan-4, which is one of the HSPGs and a ligand of DC-HIL that functions as a co-inhibitory pathway of donor T cell immune responses worsened GVHD (164).

Alpha-Mannan

The alpha-mannan (α-mannan) is a component of fungal cell wall as a known DAMP. The α-mannan is recognized by dectin1 and dectin2, which is one of the CLRs and activates NF-κB signaling through SYK and produces pro-inflammatory cytokines and effector T cell differentiation (96). α-mannan stimulated macrophages through dectin2, enhanced Th17 differentiation, and worsened lung GVHD (102). Colonization of *candida species* exacerbated GVHD in clinical studies (100, 101).

THERAPEUTIC STRATEGIES THROUGH MODULATING DANGER SIGNALING

"Danger signals" are indispensable role in initiating and developing acute GVHD. Regulating danger signal in an efficient manner in early phase of allo-SCT would ameliorate GVHD and have a great therapeutic strategy. Herein, we summarize potential therapeutic strategies for prevention and treatment of GVHD through modulating this signaling pathway. The studies are also summarized in **Table 2**.

Purinergic Receptor Antagonist

P2X7 receptor on APCs binds to extracellular ATPs, which released from damaged tissues by conditioning, and activates APCs to produce pro-inflammatory cytokines. Therefore, P2X7R plays a key role in DAMPs-mediated inflammatory responses. Systemic administration of the broad-spectrum P2X7R antagonist, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), or an ATP diphosphohydrolase, apyrase attenuated GVHD by suppressing APC activation (134). Additionally, another P2X7R receptor antagonist, brilliant blue G (BBG) improved liver function by regulating the infiltration of donor MFs and neutrophils in liver and attenuated GVHD (165). Beside P2X7R antagonist, adenosine A2A receptor agonist, ATL146e, decreased GVHD severity by modulating T cell activation and Treg function in experimental model (137, 166). Whether modulating other purinergic receptors ameliorates GVHD in the context allo-HCT needs to be studied.

Alpha-1 Antitrypsin

Serine protease inhibitor alpha-1 antitrypsin (AAT) attenuates GVHD by inhibiting HS, one of the DAMPs, and reducing HS mediated allogenic T cell responses in murine model (163). Clinical investigation demonstrates that the patients who have GVHD increased the serum level of HS after allo-HCT (163).

TABLE 2 | Targeting danger signals to ameliorate GVHD.

Drug	Function	Results of preclinical model	Results of clinical trials	Reference
PPADS Brilliant blue G (BBG)	P2X7R antagonist	FVB → BALB/c: GVHD↓ B6 → BALB/c: GVHD↓ B6 → BALB/c: GVHD↓	Not tested	(134, 165)
Apyrase	ATP diphosphohydrolase	FVB → BALB/c: GVHD↓ B6 → BALB/c: GVHD↓	Not tested	(134)
ATL146e ATL370 ATL1223	Adenosine A2A receptor agonist	B6 → B6D2F1: GVHD↓ B6 → B6D2F1: GVHD↓ B6 → BALB/c: GVHD↓	Not tested	(137, 166)
Alpha-1 antitrypsin (AAT)	Serine protease inhibitor (targeting heparin sulfate, IL-32)	B10.D2 → BALB/c: GVHD↓ C3H.sw → B6: GVHD↓ B6 → B6D2F1: GVHD↓ B6 → C3H.sw: GVHD↓	Phase I/II: GVHD↓ w less toxicity	(163, 167–169, 175, 176)
CD24 fusion protein	CD24 agonist (Siglec-G agonist)	BALB/c → B6: GVHD↓ B6 → BALB/c: GVHD↓	Phase IIa (to be initiated in 2016)	(181, 182)

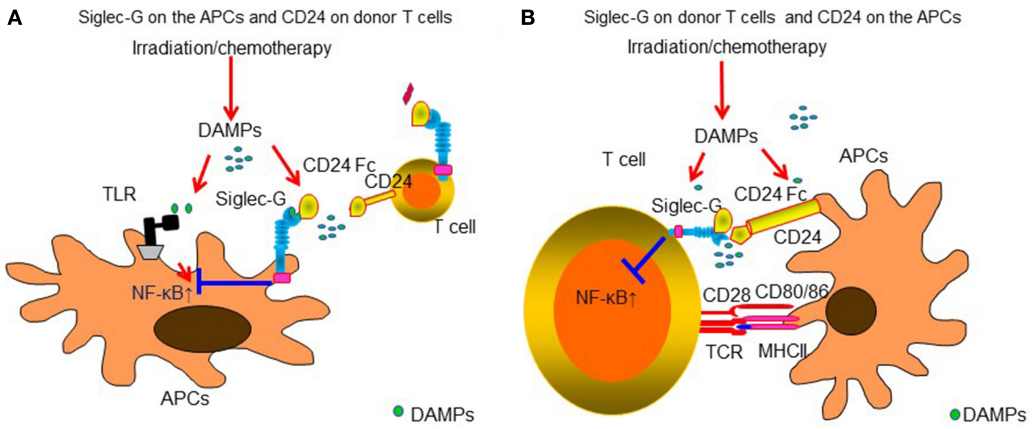

FIGURE 2 | Siglec-G-CD24 axis is critical for regulating acute GVHD. Sialic acid-binding immunoglobulin-like lectins (Siglecs) have an immunoreceptor tyrosine-based inhibitory motifs (ITIM) or ITIM-like regions in their intracellular domains and negatively regulate DAMPs-mediated innate inflammatory responses. Siglec-G expression in host APCs plays an important role in protecting from DAMPs-mediated GVHD following conditioning-mediated tissue damage. Interaction of Siglec-G with CD24, a small glycosyl-phosphatidyl-inositol (GPI)-anchored glycoprotein on T cells that is recognized as a ligand of Siglec-G was critical for protection from GVHD. Enhancing Siglec-G-CD24 axis by a novel CD24 fusion protein (CD24Fc) mitigated GVHD **(A)**. In addition, enhancing the interactions between Siglec-G on T cells and CD24 on APCs with CD24Fc mitigated GVHD **(B)**.

In addition, we and others found that AAT attenuates GVHD with reducing serum levels of pro-inflammatory cytokines but increasing IL-10 levels by modulating function of donor and host APCs as well as altering the ratio of donor effector T cells to Tregs (167, 168). AAT also inhibits IL-32 activation mediated by proteinase-3, which is a neutrophil granule serine proteinase (169, 170). AAT homeostasis after allo-HCT may be important for regulating allogeneic responses because elevated AAT clearance in stool was correlated with the severity of GI-GVHD and steroid resistant GVHD (SR-GVHD) in some studies (171–173), but not others (174). Consistent with this, we and others have demonstrated that AAT treatment for SR-GVHD-improved GVHD manifestations without significant adverse effects or increased rates of infection in a multicenter prospective or single institution phase I/II study (175, 176). This data indicates that AAT may be a rational first-line therapy for SR-GVHD or other high risk GVHD, which is associated with high mortality. Although the mechanism how AAT suppresses SR-GVHD has not been clearly elucidated, these encouraging findings warrant further prospective, randomized, and multi-centered study.

Siglec-G: A Potential Negative Signaling for DAMPs-Mediated Inflammation

Sialic acid-binding immunoglobulin like lectins (Siglecs) have an immunoreceptor tyrosine-based inhibitory motifs (ITIM) or ITIM-like regions in their intracellular domains and negatively regulate DAMPs-mediated innate inflammatory responses (177, 178) or induce B-cell tolerance by suppressing NF-κB pathways (179, 180). We observed that Siglec-G expression in host APCs plays an important role in protecting DAMPs-mediated GVHD following conditioning mediated tissue damage. Interaction of Siglec-G with CD24, a small glycosyl-phosphatidyl-inositol (GPI)-anchored glycoprotein on T cells that is recognized as a ligand of Siglec-G (181), was critical for protection from GVHD. Enhancing Siglec-G-CD24 axis by a novel CD24 fusion protein (CD24Fc), consisting of the extracellular domain of mature human CD24 linked to the human immunoglobulin G1 (IgG1) Fc domain, mitigated GVHD in multiple experimental BMT models (181). We also found that enhancing the interactions between Siglec-G on T cells and CD24 on APCs with CD24Fc mitigated GVHD while preserving GVT effects in experimental models as well as human PBMCs (182). The summarized mechanism of Siglec-G-CD24 axis for controlling GVHD is shown in **Figure 2**. Based on these preclinical studies, a prospective, randomized multi-centered phase IIa study is currently investigating whether the addition of CD24Fc to standard immune-prophylaxis can limit the incidence and severity of acute GVHD following myeloablative allo-HCT.

CLOSING REMARK

Danger signals mediate inflammatory responses through a multitude of PRRs that play a key role in the pathogenesis of GVHD. Once danger signals are released after conditioning, multiple innate immune signaling pathways are activated and amplified. Therefore, regulating danger signaling pathways in an effective manner is complex. Preclinical data suggest that targeting one specific signaling pathway or molecule may have only limited effects in reducing GVHD. In addition, the specific timing of regulation by blockade using antagonists may be a critical factor to consider. However, it is plausible that stimulating the negative regulating pathway that is commonly employed by several DAMPs may be a more rational way to mitigate GVHD. Thus exploring novel mechanisms of negative regulation of danger DAMP signaling that mediate lethal inflammatory responses should be carefully examined as new strategy of the prevention and treatment of GVHD. One potential benefit of regulating danger signaling is that GVT responses may be preserved due to selective attenuation

of APCs with presumably limited effects on donor tumor-specific T cells necessary for mediating GVT responses. Clinical trials that investigate critical mediators of the danger response hold promise in the prevention of GVHD without affecting GVT responses.

AUTHOR CONTRIBUTIONS

The review article was designed and written by TT and PR. Both NM and JM helped with designing figures. together with the help of co-authors NM and JM. All of them performed literature review and critically discussed the published literature.

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The Role of Purine Metabolites as DAMPs in Acute Graft-versus-Host Disease

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Acute graft-versus-host disease (GvHD) causes high mortality in patients undergoing allogeneic hematopoietic cell transplantation. An early event in the classical pathogenesis of acute GvHD is tissue damage caused by the conditioning treatment or infection that consecutively leads to translocation of bacterial products [pathogen-associated molecular patterns (PAMPs)] into blood or lymphoid tissue, as well as danger-associated molecular patterns (DAMPs), mostly intracellular components that act as pro-inflammatory agents, once they are released into the extracellular space. A subtype of DAMPs is nucleotides, such as adenosine triphosphate released from dying cells that can activate the innate and adaptive immune system by binding to purinergic receptors. Binding to certain purinergic receptors leads to a pro-inflammatory microenvironment and promotes allogeneic T cell priming. After priming, T cells migrate to the acute GvHD target organs, mainly skin, liver, and the gastrointestinal tract and induce cell damage that further amplifies the release of intracellular components. This review summarizes the role of different purinergic receptors in particular P2X7 and P2Y2 as well as nucleotides in the pathogenesis of GvHD.

Keywords: graft-versus-host disease, P2X7, P2Y2, ATP, ectonucleotidase, inflammasome, uric acid, neutrophils

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INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) is a potentially curative therapeutic option mainly for patients with acute leukemias or malignant lymphomas but also for selected non-malignant diseases. Initial transplantation attempts remained ineffective due to the lack of knowledge regarding human leukocyte antigen (HLA) compatibility between donor and recipient and the lack of adequate immunosuppressive drugs. Today, more than 60 years later, immunologic reactions between donor and host still remain one of the major causes of morbidity and mortality after allo-HCT. Here, we discuss the impact of purines as danger-associated molecular patterns (DAMPs) in the context of acute GvHD. We decided to focus on purines and their receptors in GvHD because other danger signals in the context of allo-HCT have been discussed in a previous review (1). Nucleosides and nucleotides bind to the P1 and P2 family of purinergic receptors. Whereas adenosine activates four receptors belonging to the P1 family, UDP (Uridine-5′-diphosphate), uridine-5′-triphosphate (UTP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) activate the large family of P2 receptors with a variable affinity. The P2 receptor family is divided into two subfamilies, the ligand-gated ion channels P2X receptors and the G-protein-coupled P2Y receptors. Purinergic signaling is regulated by the expression of cell surface enzymes known as ectonucleotidases, most

prominently CD39 and CD73 that convert ATP/UTP to ADP/UDP and ultimately to the respective nucleosides adenosine and uridine. Purinergic signaling modulates inflammation on multiple levels and contributes to the pathogenesis of a broad variety of diseases besides GvHD. P1 and P2 receptors show a variable distribution among different tissues that ensures a broad spectrum of effects. For instance, the P2Y2 receptor is expressed on immune cells but also on epithelial and endothelial cells and osteoblasts. Expression of the P2X7 receptor is predominant on immune cells, such as antigen-presenting cells, but is also found in the skin and pancreas (2). Expression of the P2Y12 receptor on platelets is a key feature for the use of P2Y12 receptor antagonists in the clinic.

PURINERGIC SIGNALING IN CARDIOVASCULAR DISEASE

Purinergic signaling has a well-established role in cardiovascular disease on multiple levels. For instance, nucleotides play a role in the formation of atherosclerotic plaques as a result of lipid metabolism dysregulation. In a murine model of atherosclerosis with apolipoprotein E-deficient mice, lack of the P2Y12 receptor was linked to a reduced plaque lesion area, decreased monocyte infiltration, and enhanced fibrous content of the plaque (3). In this same model, deficiency of the P2Y1 receptor significantly decreased the expression of vascular adhesion molecules P-selectin, VCAM-1, and ICAM-1 leading to diminished recruitment of leukocytes to lesion sites (4). Furthermore, endothelial cell cytoskeleton, motility, and adhesion are regulated via activation of the P2Y2 receptor following ATP or UTP binding (5). This is of particular importance due to the fact that the P2Y2 receptor is upregulated in the neointima of injured arteries in rats (6). Nucleotide binding to the P2Y2 receptor results in co-localization of the P2Y2 and VEGFR2 with subsequent upregulation of VCAM-1 that facilitates leukocyte adhesion (7). Endothelial cell migration is enhanced upon binding of ADP to the P2Y1 receptor via activation of the mitogen-activated protein kinase pathways (8). Last but not least, purinergic signaling has long been known to modulate platelet aggregation (9), mostly by ADP binding to the P2Y12 receptor (10). This fact led to the utilization of P2Y12 receptor antagonists, such as clopidogrel for inhibition of platelet aggregation for multiple cardiovascular diseases in patients (**Table 1**). Taken together, these data indicate that release of nucleotides with subsequent activation of purinergic receptor is a pro-inflammatory stimulus that enhanced leukocyte binding to the endothelium, platelet aggregation, and subsequent atherosclerotic plaque formation. Given the fact that multiple receptors have been implied to play a role, a more general purinergic receptor blockade might be required in order to achieve optimal protection.

PURINERGIC SIGNALLING IN AIRWAY INFLAMMATION

In the context of airway inflammation, purinergic signaling also plays a significant role in the activation of immune cells (**Table 1**). For instance, increased ATP levels following allergen challenge recruit airway-specific myeloid cells and induce Th2 cell polarization and eosinophilic airway inflammation, which are major features of allergic asthma in humans. Neutralization of ATP signaling abrogated airway inflammation in response to allergens (11). Further studies emphasized the role of P2X7 receptor expression on dendritic cells (DCs) in this context (12). However, purinergic receptor expression is not limited only to the immune cell compartment. The P2Y6 receptor was found on airway epithelial cells with abundant expression upon allergen challenge and its inhibition by synthetic antagonists or the genetic deletion reduced IL-6 and IL-8 secretion by epithelial cells and improved disease outcome in a murine model (13). More recent studies suggest that purinergic signaling

TABLE 1 | Purinergic signaling and ectonucleotidases in inflammatory diseases.

Disease context	Mechanism	Reference
Airway inflammation	ATP triggers airway inflammation via P2X7 expression on dendritic cells	(11, 12)
Airway inflammation	P2Y6 receptor expressed on lung epithelial cells mediates IL-6 and IL-8 secretion upon allergen challenge	(13)
Airway inflammation	ATP activation of the P2Y2 receptor contributes to eosinophilic lung inflammation	(44, 45)
Cardiovascular disease	P2Y12 receptor deficiency reduces monocyte infiltration and plaque lesion area	(3)
Cardiovascular disease	Lack of the P2Y1 receptor decreases leukocyte infiltration into atherosclerotic plaques	(4)
Cardiovascular disease	Neointima injury results in upregulation of the P2Y2 receptor in rats, which in turn promotes leukocyte adhesion	(6, 7)
GvHD	ATP released from damaged cells aggravates GvHD by activation of antigen-presenting cells	(23, 73)
GvHD	P2Y2 deficiency in monocytes reduces GvHD severity by abrogating ERK activation and ROS production	(50)
GvHD	CD73 deficiency increases T cell allo-reactivity and aggravates murine GvHD	(65, 66)
Inflammatory bowel disease	CD39 deletion aggravates chemically induced colitis in mice	(57, 60)
	CD39 expression on Tregs is associated with better therapy response in inflammatory bowel disease patients	
Inflammatory bowel disease	Lack of CD73 aggravates experimental inflammatory bowel disease in mice	(64)
Ischemia–reperfusion injury	CD39 plays a protective role by reducing vascular leakage	(56)
Lupus-associated nephritis	Inhibition of the P2X7 receptor reduces nephritis severity	(17)
Multiple sclerosis	ATP increases oligodendrocyte excitotoxicity and plaque formation via binding the P2X7 receptor	(15, 16)
	Gain of function polymorphisms of the P2X7 receptor are associated with increased MS risk	
Platelet aggregation	Inhibition of P2Y12 signaling blocks platelet aggregation	(10)

might be regulated by microRNAs. There is evidence that the immunomodulatory miR-155 is necessary for intact purinergic signaling. Lack of miR-155 resulted in impaired DC chemotaxis toward pro-inflammatory stimuli with subsequently reduced airway inflammation in mice (14).

PURINERGIC SIGNALING IN AUTOIMMUNITY

Purine nucleotide-mediated signaling has been implied in autoimmune diseases, including multiple sclerosis (MS), psoriasis, and nephritis among others (**Table 1**).

P2X7 receptor activation by ATP binding triggers oligodendrocyte excitotoxicity and increases MS plaque formation in an experimental autoimmune encephalomyelitis model. More importantly, P2X7 receptor upregulation is observed in healthy tissue of MS patients, suggesting that inhibition of purinergic signaling might be a novel therapeutic target (15). In line with these data, a signal nucleotide polymorphism in the human *p2x7r* gene that leads to a gain of function amino acid exchange, occurs more frequently in MS patients than in healthy controls (16). P2X7 receptor upregulation was also observed in lesional and non-lesional skin of psoriasis patients. In lupus-associated nephritis, P2X7 receptor antagonists reduced nephritis severity, pro-inflammatory serum cytokines, and NLRP3 inflammasome activation underlying once more the broad therapeutic potential of these pathways (17).

THE ROLE OF P2X7 IN GvHD

ATP is a molecule with a high intracellular concentration that is released upon cell stress. In the absence of tissue damage, the intracellular ATP concentration ranges from 3 to 10 mM, while extracellular ATP levels are as low as 10 nM. This balance is regulated by ectonucleotidases, such as CD39 and CD73, which dephosphorylate ATP to ADP, AMP, and ultimately generate adenosine (2, 18, 19). The P2X7 receptor is a cation channel activated by high concentrations of ATP (20). P2X7 plays a central role for IL-1β secretion via activation of the NACHT, LRR, and PYD domains-containing protein 3 (Nlrp3) inflammasome (21, 22). We observed that release of ATP from damaged cells after allo-HCT amplified acute GvHD (23) via enhanced maturation of APCs and reduced Treg numbers. Besides activation via P2X7, the Nlrp3 inflammasome can be activated by uric acid (24) and Syk signaling (25). We found that uric acid enhanced GvHD in the early phase after allo-HCT (26). Inhibition of Syk reduced GvHD-related mortality in the mouse model without impairing anti-MCV or anti-leukemia responses (27). After tissue damage due to chemotherapy of irradiation, neutrophil granulocytes (neutrophils) and inflammatory monocytes reach a site of inflammation particularly early and participate in the first line of defense. Different studies have shown the chemotactic role of ATP for neutrophil chemotactic recruitment (28, 29). It was shown that purinergic signaling causes strong activation of human neutrophils (30) and activated neutrophils can release ATP through pannexin-1 hemichannels by an active process, which means that the process consumes energy (30). Neutrophils release reactive oxygen species (ROS) upon activation with bactericidal activity and the potential to cause local tissue damage (31, 32) that was shown to enhance GvHD (33) (**Table 1**). The mechanism as to how DAMPs, PAMPs, and neutrophils may contribute to GvHD is depicted in **Figure 1**. Besides neutrophils (33–35), other myeloid cell populations in particular DCs (36), macrophages (37), and certain monocyte subsets (38, 39) were found to enhance or reduce GvHD. Different purinergic receptors were found to be expressed by these myeloid cells (2) and their activation modifies the immune response elicited by the respective myeloid cell type. Myeloid-derived suppressor cells (MDSC) that lack a function Nlrp3 inflammasome are more protective against GvHD compared to WT MDSC (40), indicating that a functional Nlrp3 inflammasome modifies the inflammatory phenotype of this myeloid cell type. Besides MDSCs, DCs were shown to be influenced by different signals from purinergic receptors. To present the antigen that was taken up at the site of inflammation by a DC, costimulation is required. ATP is involved in this process as it enhances the maturation of human monocyte-derived DCs with increased levels of costimulatory molecules (41, 42). Recently, the central role of donor-derived colonic $CD103^+$ DCs in Ag presentation to donor T cells that then induce GvHD was reported (43). These reports from different groups support the concept that P2X7 activation in myeloid cells enhances their inflammatory phenotype that then promotes T cell priming and inflammation that ultimately lead to GvHD.

THE ROLE OF P2Y2 IN GvHD AND INFLAMMATION

The activation of P2Y2 was shown to promote tissue damage in airway inflammation (44, 45) and acute liver injury (46). However, P2Y2 was also shown to have protective effects in a model of lung infection induced by pneumonia virus of mice (47). P2Y2 can be activated by different nucleotides, the P2Y2 ligand ATP was found in different inflammatory diseases, including inflammatory bowel disease (48), glomerulonephritis (49), asthma (11), and diabetes (48). We recently reported that *P2y2* deficiency of the recipient caused lower levels of myeloperoxidase in the intestinal tract of mice developing GvHD (50). Selective deficiency of P2Y2 in inflammatory monocytes lead to reduced GvHD severity (50) and $P2y2^{-/-}$ inflammatory monocytes had defective ERK activation and ROS production. Besides these results in the mouse model, histochemical analysis of patient samples revealed that the frequency of $P2Y2^+$ cells in inflamed GvHD lesions correlated with histopathological GvHD severity.

PURINERGIC SIGNALING AND THE GRAFT-VERSUS-TUMOR EFFECT

Transplanation of the donor immune system into the allo-HCT recipient provides the graft-versus-tumor (GvT) effect that ensures long-term control of the underlying malignancy. Due to the fact that most treatments reduce the activation of the immune

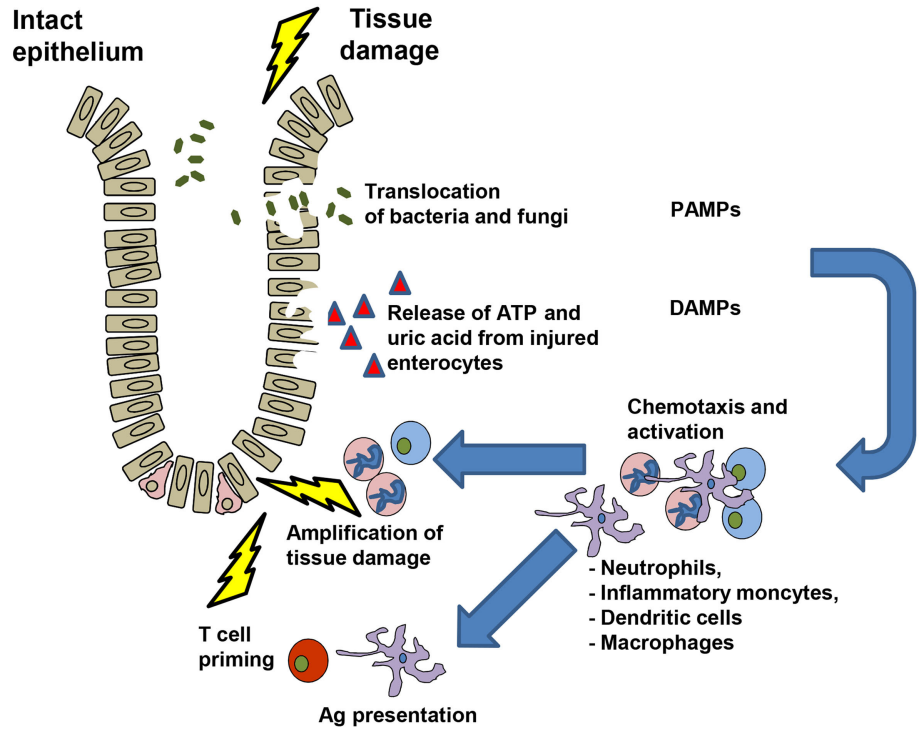

FIGURE 1 | **DAMPs, PAMPs and immune cell interactions during GvHD development**.

system, preserving the GvT effect is a major issue in post-transplant care. Interestingly, reduction of GvHD by application of the broad-spectrum P2R inhibitor PPADS did not interfere with the GvT effect, likely due to the fact that $CD8^+$ T cell function is independent of purinergic signaling (23). On the other hand, A2A-AR expression on T cells allows adenosine to reduce allo-reactivity in a murine allo-HCT model, so that blockade of adenosine production allows for a more potent GvT effect at the cost of aggravated GvHD (51). These data suggest that purinergic receptor expression on T cells is crucial for the modulation of the GvT effect and offers a few therapeutic perspectives. On the one hand, in cases where a functional GvT effect is required, such as high-risk malignancies, a P2R inhibition might be a successful approach that reduces GvHD but leaves the GvT effect untouched. On the contrary, when performing allo-HCT in a patient with a benign hematopoietic disease without a necessity for a GvT activity, adenosine signaling might be enhanced as a therapy strategy for GvHD.

MECHANISMS TO COUNTERBALANCE THE EFFECTS OF NUCLEOTIDES

Impact of the Ectonucleotidases CD39 and CD73 on Purinergic Signaling

Purine nucleotides are naturally metabolized by ectonucleotidases – cell surface enzymes with catabolic activity in the extracellular space. Two ectonucleotidases have been mainly proposed in the context of inflammation and GvHD so far – CD39 and CD73 (ecto-5′-nucleotidase). While CD39 catabolizes the first two steps in purine metabolization, mainly the dephosphorylation of ATP to ADP and AMP, CD73 is involved in the last step, namely the generation of adenosine from AMP. Adenosine itself is a potent anti-inflammatory mediator that binds to the four receptors belonging to the P1 receptor family and counteracts the effects of the pro-inflammatory ATP (51). Ectonucleotidase activity counterbalances the effects of nucleotides by regulating their concentration in the extracellular space. Concomitant expression of CD39 and CD73 is observed, for example, on regulatory T cells (52) and multipotent mesenchymal stromal cells (53). Activity of soluble recombinant CD39 removes ATP and ADP from the extracellular space and inhibits platelet aggregation *in vitro* (54). However, CD39 seems to play a dual role in hemostasis, as CD39-deficient mice exhibited prolonged bleeding times resulting from P2Y1 receptor desensitization (55). CD39 activity protects in the context of ischemia–reperfusion injury by modulation of vascular leakage (56). Furthermore, CD39 deletion rendered mice more susceptible to chemically induced murine colitis (57).

With regard to GvHD, recent studies demonstrate that CD39 activity on regulatory T cells induces the expression of the A2A-adenosine receptor on conventional T cells (58). Moreover, CD39-mediated adenosine signaling is important for the regulatory T cell-mediated inhibition of NOTCH1 signaling in conventional T cells (58), which is a known protective mechanism in the context of acute GvHD (59). Additionally, higher CD39 levels were found on regulatory T cells of inflammatory

bowel disease patients in clinical remission when compared to non-responders (60).

Generation of adenosine from AMP via CD73 is mostly known as an anti-inflammatory reaction that dampens the pro-inflammatory cascades following ATP accumulation. In rheumatoid arthritis, lack of CD73 enhanced disease development, including Th1 cell differentiation, cytokine production, and joint destruction, and this was reversed by administration of a selective A2A-adenosine receptor agonist (61). In addition, decreased levels of CD73 were found on the surface of synovial fluid mononuclear cells in children with juvenile idiopathic arthritis (62). The immunosuppressive role of CD73 is also shown by the fact that mice lacking this molecule are more prone to autoimmune glomerulonephritis (63) and inflammatory bowel disease (64).

In the context of allo-HCT, we and others have previously shown that CD73 and adenosine modulate the severity of GvHD but might also represent a target for the enhancement of the graft-versus-leukemia (GvL) effect (65, 66). In absence of CD73 and adenosine, alloreactive T cells show a stronger proliferation with increased secretion of pro-inflammatory cytokines and improved migration capacity. This more aggressive T cell phenotype translates into more pronounced GvHD severity, but also offers a target for enhancing the GvL effect in the context of allo-HCT (67).

CD73 and adenosine seem to play a differential role in inflammation, depending on the disease model, since recent studies suggest that CD73 might potentiate inflammation in the context of atherosclerotic plaque formation (68) and radiation-induced lung fibrosis (69).

P1 and P2 Receptor Modulation

The broad role of purinergic signaling in inflammation suggests a great therapeutic potential for compounds which modulate purinergic receptor signaling. P2Y12 receptor blockers, such as clopidogrel have now long been employed for inhibition of platelet aggregation.

Another receptor with a promising role in immune responses is the P2X7 receptor. Preclinical studies have suggested a beneficial role for P2X7 blockade in allograft vasculopathy (70) ischemia–reperfusion injury (71), acute lung injury (72), and GvHD (23, 73) among others. Also blocking downstream effects of P2X7, namely Nlrp3 inflammasome activation reduced GvHD in different models (26, 74, 75). However, first clinical trials with P2X7 receptor antagonists proved to be disillusioning. Phase II clinical trials with the compound AZD9056 could not show a reliable benefit in the treatment of patients with rheumatoid arthritis (76, 77) or Crohn's disease (78). These data indicate that inhibition of P2X7 receptor signaling might be a powerful target to modulate inflammation but there is still need for development of active compounds for the clinical setting.

Adenosine is the counterpart of the pro-inflammatory nucleotides ATP, ADP, UTP, and UDP. Adenosine is mostly generated among others by regulatory T cells and binds to the four receptors belonging to the P1 receptor family, A1, A2A, A2B, and A3 adenosine receptor. In the context of inflammation, the A2A receptor has been implied as anti-inflammatory in a wide spectrum of preclinical disease models.

A2A receptor agonists showed beneficial effects also in preclinical models of rheumatoid arthritis (79), encephalomyelitis (80), and allergic asthma (81). A2A receptor involvement in GvHD has also been shown by our group and others. A2A receptor expression on alloreactive T cells is critical for the integration of the protective CD73-mediated adenosine signaling (65). Treatment with a selective A2A receptor agonist, ATL146e inhibited T cell activation and reduced GvHD severity (82). These data were confirmed using other A2A receptor agonists with increased frequency of regulatory T cells in the GvHD target tissues (83). Numerous early clinical trials with adenosine receptor agonists are ongoing or have been completed recently, including indications such as psoriasis, rheumatoid arthritis, sickle cell anemia, myocardial reperfusion, and nerve injury (84) and hold promise to become part of the therapeutic arsenal against inflammatory diseases. To interfere with a broad activation signal as it is exerted by nucleotides the inhibition of the central signal is most promising and modification of TCR signaling can lead to Treg development (85). Besides purinergic receptor inhibition promising targets are the γc receptor (86) or Janus kinases (87, 88).

SUMMARY

Purinergic signaling belongs as a DAMP to the intrinsic mechanisms for inflammation regulation without pathogen exposure. Differential receptor expression is observed on various cell and tissue types, indicating distinct roles of purines depending on the particular disease context. In general, nucleotides as ADP, ATP, UDP, and UTP serve as "alarmins" and activate neutrophil granulocytes, macrophages, DCs, and platelets. On the other hand, adenosine produced by regulatory T cells or mesenchymal stem cells counteracts the effects of the nucleotides by binding to P1 receptors. The findings from multiple groups in different models of pathogenic inflammation indicate a central function of different purinergic receptors, such as P2X7 and P2Y2, in ATP-activated recipient myeloid cells during GvHD, which could be exploited when targeting danger signals to prevent GvHD. Current efforts are concentrating on the development of bioavailable and efficient compounds for the conduct of clinical trials.

AUTHOR CONTRIBUTIONS

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REVIEW
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Clinical Evidence for the Microbiome in Inflammatory Diseases

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Clinical evidence is accumulating for a role of the microbiome in contributing to or modulating severity of inflammatory diseases. These studies can be organized by various organ systems involved, as well as type of study approach utilized, whether investigators compared the microbiome of cases versus controls, followed patients longitudinally, or intervened with antibiotics, prebiotics, or bacterial introduction. In this review, we summarize the clinical evidence supporting the microbiome as an important mechanism in the onset and maintenance of inflammation.

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INTRODUCTION

The human body is host to hundreds of thousands of bacteria and other microorganisms, primarily colonizing our epithelial surfaces and attaining their highest densities within the lower gastrointestinal tract. These commensals play an important homeostatic role in a variety of our body systems, including the immune system, and can have both immune-stimulatory and immune-regulatory effects. Intriguingly, microbiota differences have been associated with a variety of inflammatory diseases, indicating that targeting or modulating the microbiota may be a novel therapeutic strategy that could nicely complement established treatments for inflammatory conditions.

At the extremes of age, humans show changes in microbiome composition. The degree of diversity observed in the fecal microbiota is highest at 2 years of age, and the gut microbiota undergoes dramatic changes during the first 3 years of life, after which it stabilizes and generally changes only slightly throughout adulthood (1). This pattern of early fluctuations in life followed by stabilization has been attributed to dietary changes as infants transition from breastfeeding or formula to more solid food (2).

After birth, the mode of delivery has been seen to impact on the microbial composition of the newborn, with infants born vaginally exhibiting bacterial communities of their own mother's vaginal microbiota, while infants born by cesarean section showed bacterial communities of skin origin (1). Moreover, the intestinal microbiota of the mother can shape the bacterial colonization of their infant's gut (3). Even the placenta seems to have a unique microbiome that correlates with those found in the oral cavity (4). This may raise a question whether these findings correlate with higher chances of preterm deliveries in mothers with periodontal diseases (5) and modulating the oral microbiota could potentially prevent such complications during pregnancy.

The Human Microbiome Project was a NIH initiative launched in 2008 on the heels of the Human Genome Project that reflected an increasing interest in the study of microbes as complex and dynamic communities rather than isolated species. The interdisciplinary collaboration set out to characterize the human microbiome and explore the relationship between population fluctuations and disease; to establish new computational tools and strategies to complement a resource repository for ongoing studies; and to study the ethical, legal, and social implications of the field's expansion. The 5-year initiative accelerated the progress of what had been a nascent discipline and facilitated our

in-depth and multi-omic understanding of human microbiome science (6).

This review summarizes clinical studies that have helped to elucidate the involvement of the microbiota in inflammatory diseases. In addition to describing findings, we have also made an effort to provide context regarding numbers of patients and types of studies performed in **Table 1** to allow the reader to better gage the quality of evidence being presented.

STRATEGIES OF GUT MICROBIOTA MODULATION

Multiple avenues for therapeutic modulation of the microbiota have been gleaned from the continued research into microbiota–host interactions. Most widely administered are probiotics, which can be administered by way of lyophilized or live single or combination of bacterial preparations or fermented foodstuffs (primarily milk products) containing known active cultures. The benefits of both single-strain preparations and synergistic bacterial mixtures are loosely attributed to any of three mechanisms: (1) interference with the growth or survival of pathogenic microorganisms in the gut lumen, (2) improvement of mucosal barrier function or mucosal immune system, and (3) influence beyond the gut through the systemic immune system and other organs (7). Administration of prebiotics, which are non-digestible food ingredients, is designed to enrich native beneficial populations. While a number of dietary carbohydrates show promise for such use, not all have been formally evaluated

TABLE 1 | Clinical studies of microbiome associations and interventions in inflammatory conditions.

Condition	Longitudinal cohort studies	Case–control studies	Cross-sectional studies	Interventional
Inflammatory bowel diseases		(14) (*n* = 227)		(61) (*n* = 31)
Ulcerative colitis				(60) (*n* = 59), (64) (*n* = 19), (66) (*n* = 29), (67) (*n* = 105), (17) (*n* = 120), (18) (*n* = 327), (19) (*n* = 120), (20) (*n* = 100), (21) (*n* = 90), (22) (*n* = 40), (23) (*n* = 26), (24) (*n* = 15), (26) (*n* = 29), (27) (*n* = 90), (29) (*n* = 144), (28) (*n* = 147), (30) (*n* = 40), (31) (*n* = 20), (32) (*n* = 30), (33) (*n* = 30), (34) (*n* = 32), (68) (*n* = 18), (69) (*n* = 41), (76) (*n* = 9), (77) (*n* = 11), (79) (*n* = 11), (80) (*n* = 8), (81) (*n* = 6), (82) (*n* = 5), (83) (*n* = 6), (88) (*n* = 3), (84) (*n* = 62), (89) (*n* = 75), (90) (*n* = 48), (78) (*n* = 1), (85) (*n* = 1)
Pouchitis				(65) (*n* = 20), (43) (*n* = 12), (45) (*n* = 20), (36) (*n* = 40), (38) (*n* = 40), (37) (*n* = 36), (39) (*n* = 31), (41) (*n* = 43), (42) (*n* = 16), (40) (*n* = 31)
Crohn's disease		(15) (*n* = 38), (16) (*n* = 21)		(59) (*n* = 22), (62) (*n* = 10), (63) (*n* = 103), (46) (*n* = 20), (47) (*n* = 32), (48) (*n* = 34), (49) (*n* = 165), (50) (*n* = 120), (54) (*n* = 119), (55) (*n* = 30), (52) (*n* = 11), (53) (*n* = 75), (51) (*n* = 37), (56) (*n* = 28), (57) (*n* = 98), (58) (*n* = 70), (70) (*n* = 30), (71) (*n* = 10), (72) (*n* = 4), (73) (*n* = 30), (74) (*n* = 4), (75) (*n* = 5), (86) (*n* = 8), (87) (*n* = 19)
Multiple sclerosis		(132) (*n* = 78), (133) (*n* = 67), (134) (*n* = 40), (135) (*n* = 15), (136) (*n* = 103), (137) (*n* = 24), (139) (*n* = 61)	(138) (*n* = 17)	(141) (*n* = 12), (142) (*n* = 5), (140) (*n* = 3)
Guillain–Barré syndrome		(145) (*n* = 171), (146) (*n* = 103), (147) (*n* = 212), (148) (*n* = 549)	(144) (*n* = 56)	
Fibromyalgia		(151) (*n* = 95), (152) (*n* = 114), (154) (*n* = 100), (155) (*n* = 233), (156) (*n* = 1,125), (157) (*n* = 251), (158) (*n* = 168)	(153) (*n* = 233)	
Systemic lupus erythematosus		(161) (*n* = 40), (162) (*n* = 35)		
Atherosclerosis	(98) (*n* = 2,595), (99) (*n* = 4,007)	(92) (*n* = 17), (91) (*n* = 2), (93) (*n* = 49), (96) (*n* = 7)		
Vasculitis		(94) (*n* = 38), (95) (*n* = 31), (100) (*n* = 17)		
Eczema		(103) (*n* = 23)		
Scleroderma		(112) (*n* = 4), (110) (*n* = 80)		
Psoriasis		(106) (*n* = 6), (107) (*n* = 22), (108) (*n* = 9,295)		
Rheumatoid arthritis		(114) (*n* = 30), (117) (*n* = 83), (116) (*n* = 156), (118) (*n* = 114), (113) (*n* = 212)		(115) (*n* = 46)
Asthma	(121) (*n* = 321), (124) (*n* = 411), (125) (*n* = 47), (126) (*n* = 319)	(120) (*n* = 43), (122) (*n* = 20), (123) (*n* = 51)		

to meet criteria for classification as a prebiotic compound. Such ingredients must (1) be neither hydrolyzed nor absorbed in the upper digestive tract, thus ensuring fermentation in the colon, (2) be a selective substrate for specific potentially beneficial commensal bacteria in the colon, stimulating growth and expansion or metabolic activation, and (3) thus be capable of effecting beneficial shifts in colonic bacterial communities (8). Live microbes are also administered with prebiotic substrates in preparations known as synbiotics. In addition to conferring the benefits of both probiotics and prebiotics, synbiotics are thought to improve the survival of the probiotic organism in the host by making its specific fermentable substrate readily available (9). In contrast, modulation of the intestinal microbiota has also been achieved through selective decontamination with narrow-spectrum antimicrobials. Removal or attenuation of undesirable bacterial species allows for reorganization of other populations within existing ecological niches (10). Newer treatment modalities include fecal microbiota transplant (FMT), introducing the complete microbial ecology of a healthy donor to restore phylogenetic diversity and synergistic function (11), and administration of bacterial metabolic byproducts such as short-chain fatty acids or conjugated bile acids (12). These strategies are summarized in **Figure 1**.

GASTROINTESTINAL–INFLAMMATORY BOWEL DISEASES (IBD)

The gastrointestinal tract includes several sites that harbor the highest densities of bacterial commensals, and thus much of the exploration of the potential of therapeutic modulation of the intestinal microbiota has been focused on patients with disorders of the digestive system. In inflammatory bowel disease (IBD), comprised largely by Crohn's disease (CD) and ulcerative colitis (UC), genetic susceptibilities are thought to predispose to development of heightened immunity against enteral "dysbiosis," a term used to describe a deviation or perturbation from a healthy microbiome (13). In patients with UC, inflammation is localized primarily to the colonic mucosa, while in CD, discrete portions of the entire gastrointestinal tract can be involved, particularly the terminal ileum. Currently, no specific microorganism has been implicated in the pathogenesis or pathophysiology of IBD, but differences in the abundance and biodiversity of the enteric flora of individuals affected by IBD have been observed. Twin studies have elucidated the role of the microbiota excluding genetic involvement and explored potential environmental triggers of disease onset. An examination of twin pairs with discordance in their disease status found that risk factors for IBD included early gastrointestinal infection, implicating

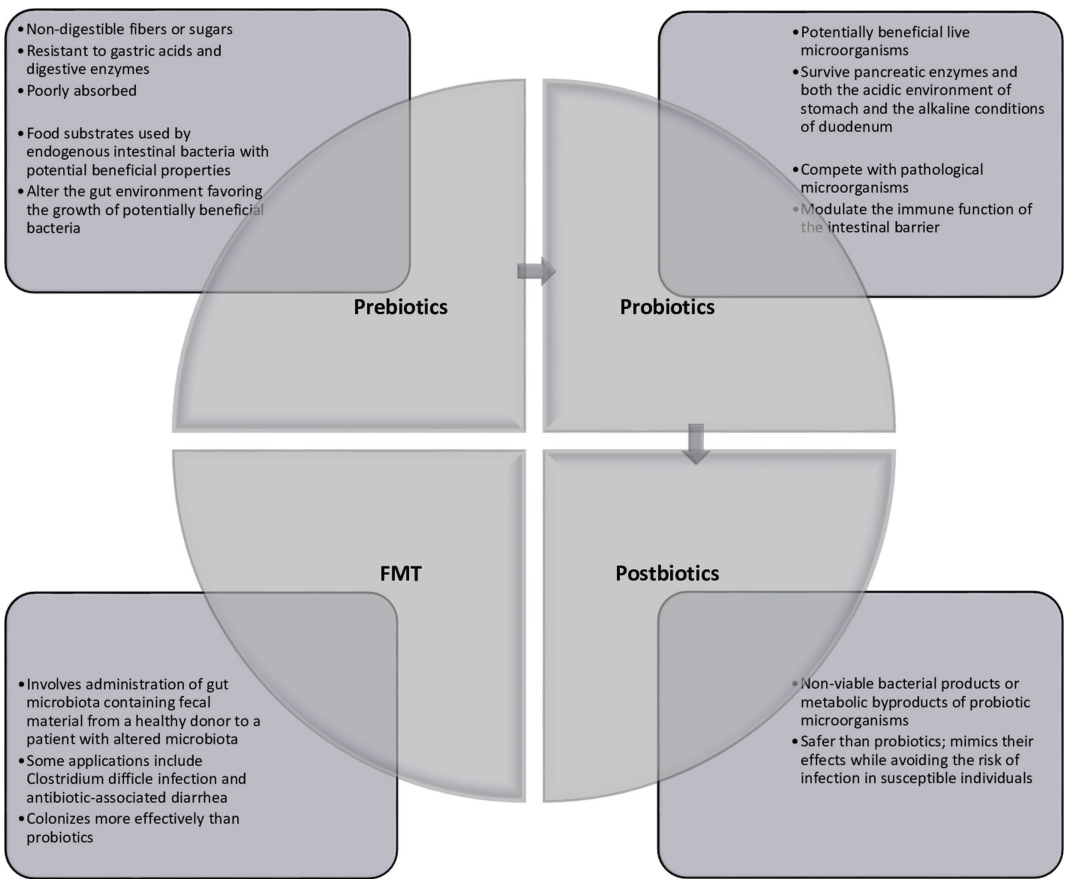

FIGURE 1 | **Current strategies of gut microbiota modulation**.

a potential role for different subsets of intestinal bacteria in maintaining or disturbing homeostasis of the gastrointestinal tract (14). Furthermore, compared to healthy and concordant twin pairs, discordant pairs were more likely to have disparities in microbiome parameters (15). An analysis of the mucosa-associated microbiota of CD patients identified that a reduction of Firmicutes member *Faecalibacterium prausnitzii* was associated with a higher risk of endoscopic and surgical disease recurrence (16). *F. prausnitzii* supernatant also reduced the transcription and secretion of pro-inflammatory molecules and mitigated symptom severity in murine colitis models both *in vitro* and *in vivo.*

Probiotics and UC

There is a sizeable body of research examining the clinical effects of introducing single bacterial preparations and probiotic mixtures to patients with IBD, overall with variable results on disease outcomes. The oral probiotic strain *Escherichia coli* Nissle 1917 (EcN) was found to be equivalent over both short-term and long-term periods to standard 5-aminosalicylic acid (5-ASA) treatment for UC in three controlled studies (17–19), but had no benefit when used as an adjunct to ciprofloxacin treatment (20). In a double-blind study of 90 patients with UC, EcN enemas led to no changes in remission rates of UC between the groups, although there was a dose-dependent trend where patients who received a higher volume of daily EcN enemas responded best (21). A number of other single probiotic species have been studied for their ability to induce UC remission, with varying results. Rectal administration of *Lactobacillus reuteri* had a significant effect on mucosal cytokines, including increased IL-10 secretion leading to remission in 31% of pediatric subjects (22). In contrast neither rectal nor oral administration of *Lactobacillus casei* resulted in improvement in clinical activity scores following a 2-month trial, although there was a decrease in inflammatory cytokine activity and increased secretion of IL-10 seen with oral *L. casei* (23).

Administration of probiotic product VSL#3, a cocktail of eight different bacteria (four species of lactobacilli, three species of bifidobacteria, and *Streptococcus thermophilus*) has been quite extensively evaluated in patients with UC and found to have some promise for induction and maintenance of remission. An open-label study showed significantly decreased disease activity and increased mucosal alkaline sphingomyelinase activity, which, through hydrolysis of sphingomyelin and resultant ceramide production, induces epithelial differentiation and apoptosis and can inhibit proliferation (24). Another study found that VSL#3 treatment reduced levels of platelet-activating factor, a pro-inflammatory compound implicated in the development and activity of IBD (25). A randomized controlled trial in the pediatric population observed an effect on both induction and maintenance of remission over a 1-year period, with significantly lower endoscopic and histologic measures of disease activity in the treatment group even at time of relapse (26). When administered in combination with VSL#3, the 5-ASA prodrug balsalazide, formulated for targeted colonic release of 5-ASA, was superior to either balsalazide monotherapy or traditional 5-ASA in inducing remission (27). Administration of VSL#3 alone resulted in significant improvement in both disease parameters and remission rate compared to placebo (28). A second VSL#3 study also observed a significant decrease in disease activity, although the remission rate was not significantly improved compared to placebo (29). Finally, a study explored the immunomodulatory effects of the mixture by comparing the probiotic to corticosteroid treatment, placebo-treated patients, and healthy controls. The authors observed that both corticosteroids and VSL#3 enhanced IL-10 expression in dendritic cells (DCs) and reduced expression of TLR-2 and IL-12, although VSL#3 patients showed a non-significant improvement in clinical responses (30).

Other probiotic mixtures have been evaluated in UC with varying success. A randomized placebo-controlled trial studying the effects of bifidobacteria-fermented milk on UC observed improvement in disease activity, which was associated with significant increases in fecal butyrate, propionate, and short-chain fatty acid concentrations in the treatment group compared to the placebo group (31). A mix of *Bifidobacterium* species was found to positively influence relapse rate and demonstrated a variety of anti-inflammatory effects (32). Treatment with a mixture of *Lactobacillus delbrueckii* and *Lactobacillus fermentum* significantly reduced mucosal leukocyte infiltration. Expression of IL-6, TNF-α, and NF-κB and fecal calprotectin levels were shown to be associated with neutrophil infiltration of intestinal tissues when compared to the placebo treatment (33). In contrast, a trial of a mixture of *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis,* in which no other concomitant UC treatments were permitted, found no significant clinical benefit (34).

Probiotics and Pouchitis

Colectomy is a common surgical treatment modality for UC, after which pouchitis, or inflammation of the surgically created ileal pouch, is a common complication. Pouchitis is clinically associated with frequent relapses and appears to be linked to intestinal bacterial composition given the results of several probiotic studies (35).

Mixed probiotic agents, including VSL#3, have been studied for efficacy in secondary prevention of pouchitis (36). Among patients who underwent probiotic treatment to maintain antibiotic-induced remission, only 15% relapsed compared to all patients in the control group ($p < 0.001$). Microbial components of the supplement could be detected in fecal samples during the trial period, but returned to baseline levels within a month of discontinuation, and all remissions relapsed within 4 months after completing treatment. These findings were supported by a similar study of patients with chronic or relapsing pouchitis (37). VSL#3 was also found to prolong time before onset of primary pouchitis, which was again correlated with an increase in fecal colonization of the probiotic species (38). Treatment has been associated with an increase in regulatory T-cells and a decrease in expression of pro-inflammatory cytokines in the intestinal mucosa of treated subjects (39). However, a study of long-term remission maintenance, modeling more realistic use in actual clinical practice, found no benefit, and subclinical evidence of mild-to-moderate disease was found on endoscopy in the 19% who remained in the study for the full 8 months (40).

Other studies of mixed probiotics have been generally positive. One study evaluated a subset of VSL#3 components in treatment for pouchitis (41). After 9 months of probiotic treatment, the

number of patients with pouchitis was reduced when compared to the control group, concluding that long-term probiotic use can serve as an effective method of pouchitis prevention. Another study found that a fermented milk product containing lactobacilli and bifidobacteria altered fecal microbiota composition and significantly improved superficial measures of pouchitis compared to the control (42).

Studies that focus on a single bacterial species have also been reported to have some efficacy for pouchitis. A small 6-month trial of *Bifidobacterium longum* found a significant difference in clinical, endoscopic, histological disease, and microbiota parameters (43). A small double-blind study of *Clostridium butyricum MIYAIRI* found a non-significant trend toward developing pouchitis in the placebo group, but endoscopic and microbial data were inconclusive (44). In contrast, administration of *Lactobacillus rhamnosus GG* to maintain pouchitis remission was ineffective despite shifts in microbial community profiles observed during treatment (45).

Probiotics and CD

Compared to the large number of studies performed in patients with UC, less evidence exists to support the efficacy of either single-strain or multistrain probiotics in inducing or maintaining remission in patients with CD. Several small randomized controlled trials of *Saccharomyces boulardii* observed significant reduction in symptoms (46), significant effect on relapse rate (47), and decreased intestinal permeability (48); however, a 52-week trial of 165 patients found no effect of the yeast on either latency to relapse or relapse rate (49). VSL#3 was found in one study to display a trend toward benefit in maintenance of surgical remission, as well as significant reduction in IL-1β, TNF-α, and IFN-γ and increase in TGF-β, which correlated with decreased endoscopic disease activity (50). Several other studies of VSL#3, however, found no significant effect of the supplement on relapse rate or cytokine profiles (51–54) and one investigation found administration to be associated with an increase in symptom flares (55). Other trials of single-species probiotics were similarly unencouraging, with EcN administration over a 1-year maintenance period demonstrating a non-significant trend to benefit (56), and two large trials of *Lactobacillus johnsonii* demonstrating no effect on disease activity or recurrence (57, 58).

Prebiotics and IBD

Dietary interventions designed to provide intestinal bacteria with metabolic substrates are termed prebiotics and can include fibers, resistant starches that are difficult for the small intestine to completely digest, and poorly absorbed monosaccharides, oligosaccharides, and polysaccharides. A study of CD patients asked subjects to quickly transition from a low-residue diet that is commonly recommended for IBD to a high-fiber diet rich in vegetables and found that all achieved disease remission within 2 months, which was sustained in 92% of patients at 2 years without scheduled maintenance pharmaceutical therapy (59). Another study found promising results with germinated barley foodstuff for maintenance of remission and potentially decreased steroid burden with reduced risk of relapse (60). In contrast, lactulose had no significant effect on clinical, endoscopic, or immunohistochemical parameters in either UC or CD (61). Fructooligosaccharide (FOS) supplementation, however, during active CD increased the abundance of fecal bifidobacteria and also led to increased secretion of IL-10 by intestinal DCs (62). A subsequent randomized, double-blind trial of FOS compared to a placebo for 4 weeks found a similar augmentation of IL-10 production by DCs, but unfortunately neither significant clinical benefit nor differences in fecal concentration of potentially beneficial commensals were found (63). Another study of FOS in combination with inulin found no change in inflammatory mediators IL-8 and PGE-2 or disease activity, but there was a significant reduction in calprotectin levels (64). Administration of single-agent inulin to patients with pouchitis reduced *Bacteroides fragilis* concentration in feces, increased fecal butyrate concentrations, and attenuated pouchitis disease activity index (65). Finally, prebiotic maintenance treatment of UC with *Plantago ovata*, also known as psyllium, was found to be more effective than placebo over 4 months (66), but was no better than traditional 5-ASA treatment in a long-term study of 1 year (67).

Synbiotics and IBD

The results of trials with synbiotics, or matched combinations of probiotics and prebiotics, in the management of IBD have been inconsistent. In UC, a randomized controlled study of *B. longum* + FOS + inulin demonstrated no significant disease mitigation (68); however, bifidobacteria + galactooligosaccharide significantly reduced endoscopic disease parameters, as well as levels of fecal Bacteroidaceae (69). A 2-year study in CD patients on the efficacy of synbiotics for maintaining surgical remission found no effect on any disease parameters, although the trial ended prematurely due to poor enrollment (70).

FMT and IBD

Fecal microbiota transplant has gained traction as a successful therapy for chronic, recurrent, or resistant *Clostridium difficile* infection. Although the pathophysiology of IBD is likely more complex than that of *C. difficile* infection, several case and cohort studies and two randomized controlled trials have begun the investigation of FMT as a treatment methodology. We have divided our summary of these studies into single-arm studies with either upper or lower introduction approaches, followed by controlled studies.

Single-arm studies of upper FMT introduction have been reported. Two studies in CD patients looked at the effects of nasogastric or gastroscopic FMT administration over a follow-up period of 1–3 months. FMT administration in a pediatric cohort induced remission within 2 weeks in 7 of 10 patients, all of whom demonstrated microbial engraftment, and remission was sustained by 5 patients at 6 weeks (71). However, a second pediatric study of an additional four patients by the same group found the same procedure to have no effect over the 3-month trial period (72). In a larger study of 30 patients receiving 1–3 treatments, clinical improvement and steroid-free maintenance were observed in 8 patients and a complete remission in another 4 patients (73). However, series of three nasojejunal transplants in four CD patients was associated with only transient changes in microbiota composition and no improvement in symptoms (74),

while a second trial of nasojejunal in five UC patients reported only one instance of clinical improvement at the end of 12 weeks and the deterioration of two subjects within a month (75).

Single-arm studies of lower FMT introduction have also been reported for UC. Treatment with a series of five once-daily transplants induced remission that was sustained throughout the month-long trial period in three of nine subjects, and clinical response was observed in another five patients by the conclusion of the trial (76). A study of single transplants in 11 patients showed a benefit in both disease activity and quality of life measures at the end of 4 weeks (77). A case report of a patient with steroid-dependent UC who was treated shortly after primary onset of the disease reported that a series of 3 transplants 4–6 weeks apart led to complete mucosal healing (78), and a case series of 11 patients similarly observed reductions in disease activity in all 11 patients (79). In a trial of eight patients, two achieved remission after 2 weeks and another by the end of the 12-week period, while four others showed clinical improvement (80). Six of six patients demonstrated clinical improvement within 2 weeks of a single transplant, with sustained improvement at 3 months observed in two patients (81). The group also reported a shift in fecal microbial profile toward donor structure in three patients, although evidence of a shift did not correlate with symptomatic response. Another study of single transplants in UC patients surprisingly noted no correlation between microbial engraftment and clinical response to therapy, but nevertheless observed response in three of five subjects (82). A subsequent 3-month study by the same group noted remission in only one of six patients at the 1-month time point, and all patients displayed worsening symptoms by the end of the trial (83). Again, achieving remission was not associated with success of donor flora engraftment; interestingly, however, loss of similarity achieved over time did correlate with the reemergence of disease activity. Finally, a single-institutional review of all FMTs administered to UC patients found that complete clinical remission was observed in 42 of 62 patients and partial response and failure in 15 and 5 patients, respectively (84). This group has also described a case of complete and unexpected reversal of concomitant immune-mediated thrombocytopenia, as well as gastrointestinal symptom attenuation, in a woman undergoing the procedure in an effort to control her chronic, relapsing disease (85).

Single-arm studies of lower FMT in CD patients have also been performed. Two single-transplant studies conducted showed preliminary promise for CD patients, with one showing remission in half of the 8 subjects after 2 months (86) and the other reporting response in 11 of 19 subjects (87), over half had maintained their stability at 12 months, although 7 experienced a worsening of symptoms. They also noted significant species-level similarity between donor and recipient in responders compared to non-responders, as well as increased microbial diversity. A small study of the long-term effects of colorectal FMT was carried out in 3 patients, who received a tapering course of 22–30 transplants over 6–12 weeks (88). All three achieved immunotherapy-free remission for over 2 months until symptom return motivated a return to conventional therapies.

Finally, two controlled studies of FMT for UC have been reported with opposing results. One trial randomized 75 UC patients to receive either 6 weekly FMTs or water enemas and found that those in the treatment group had a superior rate of achieving remission (89). Greater efficacy was seen in those patients who had been diagnosed within a year, perhaps indicating an association between outcome and degree of mucosal damage. Interestingly, the source of FMT may be an important factor, stool from one of six donors induced remission in nearly 40% of recipients, significantly higher than the 10% achieved by each of the other five donors. In contrast, a study of 48 patients randomized individuals to receive two nasoduodenal FMTs from either healthy donors or their own stool and found no significant differences between the treatment and placebo groups (90). The conflicting results of these two studies could be attributed to a number of differences in methodology, including site of administration (upper versus lower gastrointestinal tract introduction), type of control treatment, and number of treatments per patient, and so to date, the efficacy of FMT for UC and IBD in general remains to be fully elucidated.

Overall, clinical and microbial responses to FMT in patients with IBD appear to be mixed and when positive unfortunately are often transient. A handful of impressive clinical responses, however, have been reported. As the bulk of the studies thus far have been small, short, and uncontrolled, further research into optimal donor and patient characteristics, exploration of underlying immunomodulatory mechanisms, and substantiation of long-term efficacy are warranted.

CIRCULATORY

Many studies have investigated the role of microorganisms in the pathophysiology of vascular inflammatory conditions, including atherosclerosis (91, 92), aortic aneurysms (93), and systemic vasculitides such as Behçet's syndrome (94) and polyarteritis nodosa (95). Several studies have identified bacteria within the diseased vasculature, primarily in the setting of atherosclerosis, while derangements in intestinal bacterial composition have been seen in patients with systemic vasculitis.

Atherosclerosis

Inflammation is an important contributor to the pathogenesis of atherosclerosis. Thought to arise from endothelial dysfunction, the resulting inflammation in the form of an accumulation of macrophages leads to deposition of low-density lipoproteins and eventual formation of fatty streaks. A role for bacteria in the pathogenesis of atherosclerosis was identified when the respiratory tract pathogen *Chlamydia pneumoniae* was detected within atherosclerotic vessels (96). Interestingly, infection of macrophage precursors (monocytes) by *C. pneumoniae* increased their adherence to endothelium, and chlamydial lipopolysaccharide was considered a key player in this process (97). T cell responses against *C. pneumoniae* have also been demonstrated within atherosclerotic plaques (92), suggesting an additional potential contribution from the adaptive immune system. Besides *C. pneumoniae*, other bacteria such as *Porphyromonas gingivalis* have also been implicated in the inflammatory process that leads to atherosclerosis (91). More recently, the intestinal microbiota have been implicated in the pathogenesis of atherosclerosis by altering

the metabolism of ingested dietary compounds. L-carnitine, a trimethylamine and a compound found in red meat, is metabolized by intestinal bacteria to trimethylamine *N*-oxide (TMAO), which was found in higher concentrations in non-vegan humans than in vegans (98). TMAO was found to enhance calcium release from platelets stores in response to various stimuli, thus increasing their thrombogenic activity and accelerating atherosclerosis in animal models (99).

Vasculitis

Many systemic vascular diseases have been historically associated with a specific microorganism; an example is the link between polyarteritis nodosa and hepatitis C virus (95). Another systemic vasculitic disease, Behçet syndrome, may have an intestinal microbiota contribution to its pathogenesis. Patients with Behçet syndrome have a significant dysbiosis of their gut microbiota and also a decrease in the short-chain fatty acid bacterial metabolite butyrate compared to healthy controls (94). Giant cell arteritis has been found to be associated with several viruses and bacteria, but a recent unbiased DNA sequencing study of temporal artery biopsy samples was unable to confirm these associations (100).

INTEGUMENTARY

The skin is the largest organ in the body. In addition to providing barrier function, it also harbors a reservoir of numerous populations of microbiota. Recent advances in molecular approaches toward culturing microbiota on the skin have revealed a huge topographical variability of skin microbiota (101). Researchers have begun to examine this variability and determine whether it could contribute to a predilection for developing certain skin conditions at particular body sites, for example, psoriasis tends to affect hairy scalp and extensor surfaces, while eczema tends to affect flexor surfaces.

Atopic Dermatitis (AD)

Atopic dermatitis, also known as eczema, is a common chronic inflammatory skin disease characterized by frequent flares of inflammation with subsequent pruritus, dryness, and scaling. Two mechanisms were historically hypothesized to explain eczema: one mechanism considers the trigger an epithelial barrier dysfunction secondary to an intrinsic defect in epithelial cells and the other mechanism suggests that IgE sensitization occurs secondary to an immunological disturbance and inflammation, which then leads to epithelial barrier dysfunction (102).

Recent studies have revealed a relationship between the severity of AD flares and the type of bacterial communities colonizing the skin and overall diversity (103). A longitudinal examination of antecubital and the popliteal regions of children with eczema found that diversity was significantly lower during the no-treatment flares, and *Staphylococcus aureus* was the predominant bacteria, while there was no significant difference in diversity during the baseline, intermittent treatment, postflare, and controls. These findings suggest that dysbiosis of skin microbiota occurs during AD, although whether this dysbiosis contributed to inflammation remains unclear. Interestingly, however, treatment of AD with topical steroids and antimicrobials resulted in restoration of bacterial diversity by decreasing *S. aureus* predominance in favor of *Streptococcus*, *Corynebacterium*, and *Propionibacterium* species.

The initial mechanisms or triggers that induce inflammation in AD are unknown. Some evidence suggests that AD may begin independently of IgE and that IgE-mediated sensitization occurs after the first lesion appears (102). Mast cell degranulation occurs after the development of antigen-specific IgE. *S. aureus* produces several toxins, including alpha toxin that targets cell membranes and induces mast cell degranulation, supporting a potential immunological link between *S. aureus* colonization and development of AD (104).

Psoriasis

Psoriasis is an inflammatory skin disorder characterized by hyperproliferation of keratinocytes. This is thought to be a response to an unknown trigger with possible interactions between the host microbiota and the immune system (105). One study examined the difference in bacterial composition between psoriatic lesions, unaffected skin of psoriatic patients, and healthy participants using the 16S rDNA PCR on skin swabs (106). In contrast to normal participants and healthy skin samples of psoriatic patients where Actinobacteria (predominately *Propionibacterium acne*) were the most abundant and diverse, psoriatic lesions showed an abundance of Firmicutes such as *Streptococcus*. Similarly, Proteobacteria were detected less frequently in psoriatic lesions compared to healthy controls. A related study of skin biopsies found a similar loss of *Propionibacterium* and an increased abundance of *Streptococcus* and *Staphylococcus* in psoriatic lesions (107). However, unlike the swab study described above, Proteobacteria in biopsies were found at much higher levels in psoriatic lesions than in controls.

A weak association between development of psoriasis and preceding antibiotics has been reported, potentially indicating a link between microbiota perturbation and disease. However, in this study, a history of prior skin infections was also associated with having a diagnosis of disease regardless of whether patients received antibiotics, leading the authors to conclude that infections could be associated with the development of pediatric psoriasis but antibiotics do not appear to contribute substantially to that risk (108). An additional caveat includes the possibility that misdiagnoses of skin infections were occurring in children at times prior to the making of a clear diagnosis of psoriasis.

Systemic Sclerosis (SSc) (Scleroderma)

The hallmark of SSc is pathological fibrosis of skin and internal organs, which is thought to arise from abnormalities in the vascular and immune system. An autoimmune disease that typically affects middle-aged women, SSc can also occur in men and children. Its pathogenesis is multifactorial and includes genetic, autoimmune, and environmental factors. The presence of autoantibodies further supports the autoimmunity aspect of this disease and are utilized in categorizing SSc into diffuse and limited forms. However, to date, there is no animal model of SSc that successfully induces the disease by immunization against a specific autoantigen or *via* transfer of immune cells. Thus, the role of the immune system in the pathogenesis of this disease remains unclear (109).

Infectious agents that have been previously shown to be associated with the pathogenesis of SSc include parvovirus B19, Epstein–Barr virus, endogenous retroviruses, *Helicobacter pylori*, and chlamydial species (110, 111). In addition, antibodies against cytomegalovirus, hepatitis B virus, and toxoplasmosis have been detected at higher frequencies in patients with SSc, suggesting a role for these infectious agents in the initiation or progression of disease (110). It remains unknown whether the association between SSc and infectious agents is causal or if, alternatively, the immune system in these patients is altered leading to increased exposure to microorganisms.

Rhodotorula glutinis, a member of the fungal microbiome, was found to be overrepresented in the skin of a subset of SSc patients with an early diffuse presentation subtype (112). An environmental yeast that is known to cause fibrosis in the setting of opportunistic infections such as dialysis-associated fungemia, *Rhodotorula* has been hypothesized to contribute to inflammation-mediated fibrosis in this subset of SSc patients.

Although neither the mechanisms nor the pathogens responsible for the development of SSc have been definitively proven (111), four potential mechanisms have been studied: (1) molecular mimicry that may induce antibody production against vascular antigens, (2) endothelial cell damage that could be mediated by the direct toxic effects of the microorganisms or as a reaction of immune response against them, (3) superantigens derived from bacteria, and (4) microchimerism, where the presence of cells or DNA can be found in an individual but originated from a different organism (most commonly a fetus), and patients with diffuse SSc have been found to harbor more CD4+ microchimeric T cells than controls (111).

MUSCULOSKELETAL

Rheumatoid Arthritis (RA)

Of the various arthritic diseases, a relationship with the microbiome has been best characterized in patients with RA. RA is a systemic disease that affects multiple organs at different stages. Thus, studies of dysbiosis of the microbiota in RA patients have focused on a variety of sites to better understand its multisystemic manifestations. One study examined the bacterial composition of oral (dental and salivary) and fecal samples in patients with RA in comparison to healthy controls (113). Notably, RA patients were noted to have both oral and fecal flora that were distinct from that of controls, characterized by reduced *Haemophilus* spp. and increased *Lactobacillus salivarius*. The increase in *L. salivarius* could be interpreted as consistent with a prior study that found increased and more diversity in bacteria from the *Lactobacillus* genus in RA patients, including *L. salivarius* (114). However, not all lactobacilli may be pro-inflammatory in the setting of RA, as an interventional study with *L. casei* capsules showed reduced inflammation and improved severity of disease in patients receiving the probiotic (115). RA patients also had generally an increase in Gram-positive bacteria in the intestinal tract compared to controls, as well as an increased in oral anaerobic bacteria. Interestingly, levels of anticitrullinated protein autoantibodies, which are used in the clinical diagnosis of RA, were positively correlated with oral *Actinomyces* spp. and negatively correlated with oral *Haemophilus* spp. (113).

Porphyromonas gingivalis has also been reported to be associated with RA severity, although results have been mixed. *P. gingivalis* DNA was significantly more likely to be found in the synovial fluid of patients with RA than in controls (116), and interestingly the severity of periodontal disease (quantified by the number of missing teeth) in patients with RA was positively correlated with the number of affected joints. However, another study found only an association between *P. gingivalis* and the likelihood of developing and severity of periodontitis but no association with RA (117), and a third study found that *P. gingivalis* was more abundant in the oral cavity of controls compared to cases (113).

Prevotella copri has been linked with disease activity in RA patients (118). It was found in increased abundance in fecal samples from untreated newly diagnosed RA patients, while both treated chronic RA patients and controls had reduced amounts. The study went on to identify *Prevotella*-derived genes that were also associated with disease activity and also demonstrated exacerbation of disease by *P. copri* in a murine model of colitis. Thus, intestinal *P. copri* appears to exacerbate inflammation and may be a viable target in patients with autoimmunity.

RESPIRATORY

Asthma

Asthma has been described as a triad of airflow obstruction, bronchial hyperresponsiveness, and lower airway inflammation. The inflammatory trigger is thought to be multifactorial, although in genetically susceptible individuals, the immune system has been particularly implicated, specifically in the way it interacts with environment exposures (119). Both airway and intestinal sites of commensal bacteria have been associated with asthma outcomes. Respiratory airways are not sterile and in fact harbor significant populations of microbiota. Changes in the composition of airway bacteria have been found to be associated with asthma and emphysema, as well as with exacerbations of these diseases (120).

In a prospective cohort study, culture-based assays of the hypopharynx found that asymptomatic neonates at 1 month of age who were colonized with *Streptococcus pneumoniae*, *Hemophilus influenzae*, or *Moraxella catarrhalis*, or a combination of these organisms, were more likely to have manifestations of asthma at 5 years of age (121). *Hemophilus* and other Proteobacteria have also been implicated in studies comparing the airway flora of asthma patients with controls that utilized sequencing-based approaches (120, 122) and have been associated with more severe (corticosteroid-refractory) disease (123). Whether colonization with these organisms can help cause asthma, and potential mechanisms for how they may do this, remains to be fully explored.

The intestinal microbiome may also help contribute to asthma or other allergic diseases, particularly early life exposures to intestinal dysbiosis, although each study has had slight inconsistencies. In a large study of over 400 infants, intestinal flora diversity in the first year of life was associated with protection from development of allergic sensitization, allergic rhinitis, and peripheral blood eosinophilia, but not asthma or AD (124). In contrast, a smaller study of just fewer than 50 infants found that samples collected within 1 month of age showed that intestinal flora diversity was associated with protection from later development of asthma, but was not associated with protection from

other allergic manifestations (125). A third recent study of over 300 children who were at a higher risk for developing asthma was able to identify 4 bacteria taxa that were associated with protection in the first 100 days of life: *Lachnospira, Veillonella, Faecalibacterium, and Rothia*. An examination of urinary and fecal metabolites found that reduced urinary urobilinogen and increased fecal acetate were also associated with protection, suggesting that bacterial involvement in the metabolism of bile and short-chain fatty acids could be contributing to modulation of asthmatic risk. Interestingly, administering a cocktail of bacteria from these taxa led to reduced disease severity in a gnotobiotic mouse model of asthma, providing additional evidence for potential causality (126).

NEUROMUSCULAR

A potential interaction between the nervous system and the gut has only recently expanded to incorporate enteric flora (127). While the preponderance of current research is preclinical, knowledge of the gut–brain axis and the underlying neurologic, immunologic, and endocrine processes that facilitate communication between the two distal sites, as well an increasing understanding of the central role of the microbiome in intraintestinal and extraintestinal immunity, have indicated probable microbial involvement in human neuromuscular disorders such as multiple sclerosis (MS), Guillain–Barré syndrome (GBS), and fibromyalgia (FM).

Both MS and GBS are demyelinating diseases; MS is characterized by a vast range of neurological symptoms stemming from damage to the central nervous system (CNS) (128) and GBS presenting as rapid-onset sensory changes and ascending muscle weakness as a result of damage to peripheral nerve cells (129). Although MS has also been linked to genetic variation (130), both MS and GBS are thought to occur in large part due to environmentally acquired triggers that catalyze autoimmunity (128, 129). However, a murine model of MS has demonstrated a role for commensal enteric flora to drive T cell responses against a myelin autoantigen in gut-associated lymphoid tissue, which then condition and drive proliferation of antigen-specific B cells, resulting in spontaneous autoimmune demyelination (131). This demonstration of the immunomodulatory contribution of the gut flora in the pathogenesis of CNS disorders, as well as clinical evidence of dysbiosis or infection associated with MS and GBS, supports exploration of potential prognostic biomarkers in the microbiota as well as the effects of therapeutic microbial intervention.

Multiple Sclerosis

Compared to healthy controls, the flora of patients with MS is notable for a relative decrease in bacteria belonging to Clostridia and Bacteroidetes (132, 133), as well as an overall decrease in species richness in relapse-remitting (RRMS) patients. RRMS was further associated with significant intestinal permeability as measured by lactulose/mannitol urinary ratio (134). A different study of vitamin D therapy for MS found an increase in abundance of *Akkermansia*, *Faecalibacterium*, and *Coprococcus* genera in patients with previously untreated MS (135). Because strains of *Faecalibacterium* and *Coprococcus* are producers of the anti-inflammatory short-chain fatty acid butyrate, and experimental colonization with *Akkermansia* implicated members of the genera in immune tolerance of commensal gut microbes, these results suggested that intestinal bacterial shifts could help mediate beneficial effects of vitamin D therapy in MS patients. Intestinal dysbiosis has also been associated with modulation of gene expression in several immune cell populations, including maturation of DCs and activation of T cell and monocytes (136). A pediatric study of patients within 2 years of primary disease onset observed two associations between abundance of a bacterial subset and an immune parameter that were seen in the control cohort but not the case population (137). *Fusobacteria* was strongly positively associated with Tregs, and Firmicutes abundance was inversely associated with pro-inflammatory Th1. A prior study by the same group observed a significant inverse relationship between *Fusobacteria* abundance and MS relapse risk (138).

Pathogenic bacteria have also been reported to be associated with development or maintenance of MS. In one patient, *Clostridium perfringens* type B colonization could be detected shortly after primary disease onset and was associated with actively enhancing lesions on brain MRI (139). The group found evidence that epsilon toxin (ETX), secreted by *C. perfringens*, disrupts the blood–brain barrier and selectively binds to CNS myelin and white matter, which supports its involvement in lesion formation. Although identified only in a small minority of samples, possibly due to known difficulties in maintaining humoral ETX immunity, reactivity to ETX was found to be 10 times more prevalent in patients with MS versus healthy controls. While this is the only known report of *C. perfringens* type B in a human, the human commensal type A was found in significantly more healthy controls than in MS patients, suggesting that the absence of this commensal could lead to an open ecological niche for non-commensal *C. perfringens* toxinotypes.

A few studies have reported efforts at microbial modulation in the treatment of MS. One three-patient series found that FMT performed to address MS-associated constipation resulted in progressive improvement of neurological symptoms and eventual sustained remission (140). Two small studies treated RRMS orally with ova from the non-pathogenic helminth *Trichuris suis* (141, 142), which has been successfully applied in cases of IBD. Both studies observed that *T. suis* was associated with an increase in anti-inflammatory and immunoregulatory serum cytokines and a decrease in MRI-detectable brain lesions. In addition to its documented immunomodulatory effects, helminth colonization was also associated with greater fecal species richness in an otherwise healthy population (143), although the microbiome was not examined in these trials. This is evidence that the correlated clinical improvement could be effected directly by the organism or indirectly by alterations in community structure and humoral immunity.

Guillain–Barré Syndrome

The presence of pathogenic bacteria at disease onset is particularly associated with GBS, in which approximately 30–40% of patients show serological evidence of recent infection with *Campylobacter jejuni* (144–148). Many *C. jejuni*-positive patients demonstrate signs of enteric infection just prior to onset of neuropathy with the median interval of 9 days to onset of neurological symptoms (147). The latency period suggests that GBS may not be the

direct effect of the pathogen or its toxins but rather could result from a mounted immune response against its presence. Current understanding of infection-associated dysbiosis (149) thus lends additional support to the role of the microbiota in demyelinating diseases.

Fibromyalgia

The etiology of FM is less understood, but hypothesized to involve aberrations in the processing of pain signals, resulting in chronic diffuse pain and stiffness (150). An increase in serum IL-6 and IL-8 has been reported in FM patients, the concentrations of which were positively correlated to duration of disease (151). Although no comparisons of healthy and FM microbial community structure have been reported, intestinal permeability was significantly increased compared to controls in a cohort of 40 FM patients ($p < 0.0003$), suggesting that luminal antigens may be able to access and modulate immunocompetent cells as seen in the hepatic disorders detailed above (152). Furthermore, FM is often comorbid with IBD (153), IBS (154–158), and SIBO (158). Lactulose breath test administration to diagnose SIBO demonstrated abnormal results more commonly in FM patients than in controls, and the degree of somatic pain was significantly correlated with breath test hydrogen level, supporting further exploration of the dynamics of the enteric flora and FM.

SYSTEMIC

Systemic Lupus Erythematosus (SLE)

The hypothesized pathogenesis of SLE is similar to that of the other autoimmune diseases discussed herein, with genetic, hormonal, endocrine, and environmental factors all thought to play a role (159). SLE is associated with the largest number of detectable antibodies, resulting in the involvement of nearly every organ and a diversity of clinical manifestations (160). SLE-associated dysbiosis has been characterized in a few studies, highlighting changes in community structure, metabolics, and gene expression. SLE patients have been noted to have a relative increase in intestinal Bacteroidetes, resulting in a significant decrease in the Firmicutes/Bacteroidetes ratio (161). This was associated with a functional increase along oxidative phosphorylation pathways, suggesting possible compensatory adaptations to intestinal oxidative stress underlying the observed changes in community structure. Intestinal metabolomic analysis of SLE patients compared to controls found alterations in mediators of cell signaling, quorum sensing, and cell wall synthesis (162), and it was recently reported that dysbiotic microbiota from SLE patients elicited different *in vitro* immune responses from naive CD4+ lymphocytes than exposure to microbiota from healthy controls, promoting greater lymphocyte activation and Th17 differentiation (163). This response has the potential to exacerbate and sustain preexisting inflammation linked to the disease, which could therefore be mitigated by restoration of a balanced flora.

Graft-versus-Host Disease (GVHD)

Graft-versus-host disease is an immune-mediated condition that commonly occurs in patients who have undergone allogeneic hematopoietic cell transplantation (HSCT), a procedure that can mediate curative long-term remissions for many hematological malignancies as well as benign hematological conditions. The presentation of GVHD can be largely categorized into two forms, acute and chronic, with acute GVHD often occurring within the first 3 months and typically affecting the skin, liver, gastrointestinal, and hematopoietic systems, while chronic GVHD typically occurs beyond the first 3 months and can present with a variety of manifestations including exocrine dysfunction and systemic fibrosis. Whether chronic GVHD may be associated with changes in the microbiome has not been well investigated. Acute GVHD, however, has long been thought to be strongly modulated by the microbiome, particularly intestinal bacteria. Based on animal studies that indicated intestinal bacteria contributed to acute GVHD, a randomized study in the early 1980s of protective isolation including gut-decontaminating antibiotics and skin disinfection showed a benefit with reduced acute GVHD (164). However, later studies did not reproduce this finding, possibly due to inconsistent success of gut decontamination (165). As a result, improved pharmacologic prevention of GVHD, particularly with calcinuerin inhibitors, has largely replaced gut decontamination for GVHD prophylaxis, though one clinical trial did demonstrate a benefit for suppressing intestinal obligate anaerobes with metronidazole (166). More recent efforts have re-examined the relationship between intestinal bacteria and acute GVHD using culture-free methods such as 16S deep sequencing. Development of acute GVHD produces striking changes in patient intestinal bacterial composition (167–170). Furthermore, microbiota damage either prior to HSCT (169) or early post-HSCT (171, 172) may increase the subsequent risk of developing GVHD. Much of this microbiota damage is manifested as loss of *Blautia* and other Clostridia (171, 172) or loss of Bacteroidetes (169). Perhaps not surprisingly, antibiotics are a common cause of microbiota damage in this patient population, and treatment with piperacillin-tazobactam (173), carbapenems (173), metronidazole (174), and clindamycin (170) have all been identified as associated with increased acute GVHD. Given these results, interest is building in targeting the microbiota for either the prophylaxis or treatment GVHD. A study of daily *Lactobacillus plantarum* administration in pediatric HSCT patients was found to be safe and well tolerated, even in the setting of neutropenia. In addition, a study of fecal transplantation as therapy for acute GVHD was recently reported, with three of four patients have a complete response and the last patient having a partial response, an overall promising result in the steroid-refractory setting, which typically has a poor prognosis (175).

CONCLUSION

The causes of inflammatory diseases are multifactorial and include age, genetics, and environment. Microorganisms are crucial in maintaining gastrointestinal homeostasis and can potently modulate systemic immunity, and differences in the microbiota have been observed in patients with inflammatory diseases compared to healthy controls. There is a growing amount of clinical research being done to better understand the role that the microbial community can play in inflammatory diseases, and some progress has been made evaluating the effects of targeting the microbiome, particularly in the setting of IBD. Some

intriguing responses suggest that this may be a viable strategy, but conflicting and inconsistent results leave open questions. One possibility may be that patient disease heterogeneity is a source of statistical noise, and it is likely that in a subset of patients with inflammatory conditions, alterations in the microbiome contribute significantly to drive disease pathogenesis and maintenance, while for other patients, the microbiome may be only a minor contributing factor. Developing strategies to distinguish these patient subsets will likely be critical to advancing the growing field of clinically targeting the microbiome for patients with inflammatory diseases.

AUTHOR CONTRIBUTIONS

AES, ZS, and RRJ wrote the manuscript. DHW prepared the table. All authors revised the manuscript and approved it for publication. AES and ZS have contributed equally to this work.

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Increased Toll-Like Receptors Activity and TLR Ligands in Patients with Autoimmune Thyroid Diseases

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Objective: Autoimmune thyroid disease (AITD) is an organ-specific disorder due to the interplay between environmental and genetic factors. Toll-like receptors (TLRs) are pattern recognition receptors expressed abundantly on monocytes. There is a paucity of data on TLR expression in AITD. The aim of this study was to examine TLR expression, activation, ligands, and downstream signaling adaptors in peripheral blood mononuclear cells (PBMCs) extracted from untreated AITD patients and healthy controls.

Method: We isolated PBMC of 30 healthy controls, 36 patients with untreated Hashimoto's thyroiditis, and 30 patients with newly onset Graves' disease. TLR mRNA, protein expression, TLR ligands, and TLR adaptor molecules were measured using real-time PCR, Western blot, flow cytometry, and enzyme-linked immunosorbent assay (ELISA). PBMC was simulated with TLR agonists. The effects of TLR agonists on the viability of human PBMC were evaluated using the MTT assay. The supernatants of cell cultures were measured for the pro-inflammatory cytokines, interleukin (IL)-6, tumor necrosis factor alpha (TNF-α), and IL-10 by ELISA.

Results: TLR2, TLR3, TLR9, and TLR10 mRNA were significantly increased in AITD patients compared with controls. TLR2, TLR3, TLR9, high mobility group box 1 (HMGB1), and RAGE expression on monocytes was higher in patients than control at baseline and TLR agonists' stimulation. The release of TNF-α and IL-6 was significantly increased in PBMCs from AITD patients with TLR agonists, while IL-10 was significantly decreased. Downstream targets of TLR, myeloid differentiation factor 88 (MyD88), and myeloid toll/IL-1 receptor-domain containing adaptor-inducing interferon-β were significantly elevated in AITD patients. Levels of TLR2 ligands, HMGB1, and heat shock protein 60 were significantly elevated in AITD patients compared with those in controls and positively correlated with TgAb and TPOAb, while sRAGE concentration was significantly decreased in AITD patients.

Conclusion: This work is the first to show that TLR2, TLR3, and TLR9 expression and activation are elevated in the PBMCs of patients with AITD and TLRs may participate in the pathogenesis of AITD.

Keywords: autoimmune thyroid disease, innate immunity, toll-like receptor, signaling, pathogenesis

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INTRODUCTION

Autoimmune thyroid disease (AITD), which includes Hashimoto's thyroiditis (HT) and Graves' disease (GD) (1), is an organ-specific autoimmune condition that affects approximately 2–3% of the population in China. HT is characterized by substantial infiltration of thyroid-specific T and B lymphocytes and thyroid gland damage, eventually leading to hypothyroidism (2). GD is an autoimmune disorder characterized by the presence of autoantibodies that bind to and stimulate the thyroid stimulating hormone receptor, resulting in hyperthyroidism and goiter (3). Other than genetic and environmental factors, immune malfunction may also be involved in the development of AITD (4). Although the production of autoantibodies destroys self-tolerance and agitation of the adaptive immune system, the fundamental mechanism remains unclear.

Recent studies have demonstrated that aberrant activation of the innate immune response may have a significant effect on the pathogenesis of AITD. Due to an improved understanding of pattern recognition receptors, insight into innate immunity has expanded dramatically. Toll-like receptors (TLRs) are conserved pattern recognition receptors expressed on multiple types of cells, including monocytes, dendritic cells, B cells, and macrophages, and play a vital role in modulation of the innate immune system (5). TLRs recognize conserved molecules termed pathogen-associated molecular patterns (PAMPs), such as bacteria, viruses, and fungi, which act as ligands of specific TLR-induced signal transduction pathways (6), resulting in the activation of innate immune response and the release of inflammatory mediators, including interleukin (IL)-6, IL-12, IL-18, and tumor necrosis factor alpha (TNF-α). TLRs can also bind to certain endogenous molecules called damage-associated molecular patterns (DAMPs) and activate the innate immune response. Accordingly, TLRs have the ability to stimulate the production of monocytes and other antigen-presenting cells via activation of TLRs signaling pathways. To date, nothing is known regarding the ligands or cellular localization of TLR10, which is expressed only in humans (7). It is possible that TLR10 serves as a co-receptor for TLR2 (8). Ligands of TLR2 and TLR4 include heat shock protein 60 (HSP60), HSP70, hyaluronan, high mobility group box 1 (HMGB1), and advanced glycation end products (9). HSP60, the first endogenous ligand to be discovered, is an intramitochondrial chaperone that facilitates protein folding and stability (10). HSP60 also induce autoimmune responses by acting as a decoy to stimulate autoantibodies (11). HMGB1 has been described as a late inflammatory cytokine in several autoimmune diseases, such as rheumatoid arthritis (RA) (12) and systemic lupus erythematosus (SLE) (13). HMGB1 also plays a vital role in the initiation and progression of allograft rejection by acting as a "danger signal" (alarmin) (14). HMGB1 can interact with TLR2, TLR4, TLR9, and the receptor for advanced glycation end products (RAGE), thus inducing activation of TLR signaling. Once stimulated by ligands, TLRs recruit downstream adaptor molecules, including toll/IL-1 receptor-domain containing adaptor-inducing interferon-β (TRIF) and myeloid differentiation factor 88 (MyD88), resulting in signaling pathway activation and in the production of pro-inflammatory cytokines, such as IL-6 and TNF-α (15).

Toll-like receptors play an important role in the development of inflammatory disease. Previous studies have shown that TLRs are involved in the development of several autoimmune diseases, such as SLE, type 1 diabetes, and RA (16). TLR7 has been demonstrated to be involved in the production of autoantibodies and thus plays a vital role in the pathogenesis of SLE (17). TLR3, TLR7, and TLR9 are elevated in RA synovial fibroblasts (18), and a significant increase in TLR2 and TLR4 and their ligands has been described in T1DM patients (19, 20). In contrast, there is a paucity of studies regarding the role of TLR in AITD. Nonetheless, it has been demonstrated that TLR3 is highly expressed in thyrocytes from patients with HT (21), and increased expression of TLR3 was also observed in thyroid follicular epithelial cells from patients with GD (22). TLR3 signaling is activated by viral and bacterial molecules, dsRNA and poly (I:C), a synthetic viral analog, which results in the production of pro-inflammatory cytokines and interferons (IFNs) (22). It has also been demonstrated that TLR4 is expressed in FRTL-5 cells, leading to activation of NF-κB via recognition of LPS (23).

Although such findings based on animal and human thyroid gland investigations suggest that TLRs may play an important role in AITDs, it remains unclear whether activation of TLR pathways results in inflammation in AITD patients. The aim of the current study was to analyze TLR expression, activation, ligands, and downstream signaling adaptors in peripheral blood mononuclear cells (PBMCs) extracted from untreated AITD patients and healthy controls.

MATERIALS AND METHODS

Study Groups

In this particular study, 4 mL fresh blood was obtained from 36 patients with HT, 30 patients with GD, and 30 age- and sex-matched controls. PBMCs were prepared from all samples. Serum samples were obtained from these patients. All patients were recruited from the First Affiliated Hospital of China Medical University. All participants provided a detailed history and underwent a complete physical examination as a condition of participation. None of the patients were taking evothyroxine or any other antithyroid drug. Subjects with any chronic disease, infectious disease, cancer, diabetes, or a family history of diabetes were excluded from this study. Levels of serum thyrotropin (TSH), free T3 (FT3), free T4 (FT4), TPOAb, TgAb, and thyrotrophin receptor antibody were analyzed using an electrochemiluminescent immunoassay with Cobas Elesys 601 (Roche Diagnostics Ltd., Switzerland). These samples were stored until use.

This experiment was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University, and written informed consent was provided by each subject.

Peripheral Blood Mononuclear Cell Isolation and TLR Agonist Stimulation

Peripheral blood mononuclear cells were obtained from freshly collected blood in heparinized tubes, isolated by Ficoll-isopaque

density gradient centrifugation (Gibco BRL, Life Technologies Ltd., Paisley, UK), as described previously (24), and washed free of platelets and Ficoll. PBMCs were harvested and washed twice in RPMI 1640 (Gibco BRL, Life Technologies Ltd., Paisley, UK). The cells were diluted with complete medium consisting of RPMI 1640 supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin G, and 100 U/mL streptomycin to 1×10^6/mL. Cells were then dispensed in a 200-μL tube in 96-well plates. Pam3CSK4, Poly I:C, and CpG ODN were used as TLR2, TLR3, and TLR9 agonists, respectively. Each volume containing 1×10^6 isolated PBMCs were incubated with medium, 1 μg/mL Pam3CSK4, 10 μg/mL poly I:C, and 1 μg/mL CpG ODN for 2, 6, 8, 12, 24 and 48 h at 37°C in 5% CO_2. The supernatants were harvested and collected at −20°C until further use.

Quantitative mRNA Expression of TLRs in PBMCs

RNA was extracted from PBMC, using TRIzol (Invitrogen Corp., CA, USA). The first strand of cDNA was synthesized using total RNA. RT-PCR was performed using TagMan gene expression assays, with glyceraldehydes-3-phosphatedehydrogenase (GAPDH) as a control (R&D Systems, Minneapolis, MN, USA). The sequences of primers for TLR1-10 and GAPDH were designed using the ABI PRISM system (Applied Biosystems). Sequences of the human primers for polymerase chain reaction were as summarized in **Table 1**. The primers were examined with BLAST software against the National Center for Biotechnology Information database. All PCRs were performed in a total volume of 20 μL, and single transcript of genes expression was determined using SYBR green master mix. Relative expression of TLR1-10 was calculated using the comparative cycle threshold method. TLR1-10 mRNA was expressed as a ratio to GAPDH.

In Vitro Studies on PBMC from AITD Patients and Controls Cell Viability Assay

The effect of TLR agonists on the viability of human PBMC was analyzed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide] assay. PBMC were maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin, under 37°C, 95% humidified air and 5% CO_2. MTT was added to each well to a final concentration of 10 mg/L and the cells were continuously incubated for 5 h. After centrifugation for 5 min at 3,000 × *g*, the supernatant was removed and the wells were washed three times with PBS before addition of 100 mL isopropyl alcohol to dissolve MTT crystals and optical density (OD) was determined at 550 nm using micro enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad, Hercules, CA, USA). All measurements were performed in triplicate. Stimulation index was calculated as mean ratio of OD of the stimulated cells divided by OD of untreated cells.

TLR2, TLR3, TLR9, HMGB1, and RAGE Expression in the Resting State and upon Specific Agonist Activation

Peripheral blood mononuclear cells were analyzed under resting and after 24 h stimulation with TLR2, 3, or 9 agonists. PBMCs were resuspended in PBS and stained for surface mAbs CD14-FITC (eBioscience, San Diego, CA, USA) and TLR2-APC (eBioscience, San Diego, CA, USA). After 15 min of incubation at room temperature in the dark, cells were washed and fixed in 1% paraformaldeyde in PBS. Cells were then permeabilized with saponin 0.5% (Sigma-Aldrich, St. Louis, MO, USA) and mAbs for TLR3-APC and TLR9-APC, with unconjugated mouse anti-human HMGB1 monoclonal antibody (Abcam, Cambridge, UK), unconjugated mouse anti-human RAGE polyclonal antibody (Millipore, MA, USA) or its corresponding mouse IgG isotype control (BD Biosciences Corp., San Jose, CA, USA) with subsequence incubation with APC-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, CA, USA). Cells were incubated for 45 min at 4°C in the dark, washed, and fixed in 1% paraformaldeyde in PBS. Samples were then analyzed using BD FACS Array (Becton-Dickinson, Fullerton, CA, USA) equipped with software Cellquest.

Results are expressed as percentages of $CD14^+$ $TLR2^+$, $CD14^+$ $TLR3^+$, $CD14^+$ $TLR9^+$ cells, $CD14^+$ $HMGB1^+$, and $CD14^+$ $RAGE^+$. The intra- and inter-assay CVs for TLR2, TLR3, TLR9, HMGB1, and RAGE expression were found to be <5, <5, <15, <5, <10, and <15%, respectively.

Cytokine Production

Interleukin-6, IL-10, and TNF-α were examined in the supernatants of the cultured PBMCs in AITD patients and healthy subjects after TLR agonist stimulation using commercially available ELISA kits (R&D, Minneapolis, MN, USA) according to the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm using a microplate reader to analyze

TABLE 1 | Sequence of primers used to amplify.

Genes	Forward primer	Reverse primer
TLR1	GGATGCAGAAGGAGATCACTG	TTTCAAAAACCGTGTCTGTTAAGAGA
TLR2	GGAGGCTGCATATTCCAAGG	GCCAGGCATCCTCACAGG
TLR3	CAGTGTCTGGTACACGCATGGA	TTTCAAAAACCGTGTCTGTTAAGAGT
TLR4	AGTTTCCTGCAATGGATCAAGG	CTGCTTATCTGAAGGTGTTGCA
TLR5	GGCTTAATCACACCAATGTCACTATAG	TTAAGACTTCCTCTTCATCACAACCTT
TLR6	CATCCTATTGTGAGTTTCAGGCAT	GCTTCATAGCACTCAATCCCAA
TLR7	TGGAAATTGCCCTCGTTGTT	GTCAGCGCATCAAAAGCATT
TLR8	AGCGGATCTGTAAGAGCTCCATC	CCGTGAATCATTTTCAGTCAAGAC
TLR9	AGCGGATCTGTAAGAGCTCCATC	CCGTGAATCATTTTCAGTCAAGA
TLR10	AAGAAAGGTTCCCGCAGACTT	TGTTATGGCATAGAATCAAAACTCTCA
GAPDH	CAA AGACCTGTACGCCAACA	GAAGCATTTGCGGTGGAC

the intensity of color development in each well. The levels of cytokines were presented as picogram per milliliter.

Western Blot Analysis

Western blot was used to detect protein in PBMC of AITD patients and controls. Frozen cells were homogenized in PBS (pH 7.4) supplemented with 0.05% Triton X-100 and protease inhibitor cocktail (Sigma). The protein concentration was determined using the Bradford method with BSA as the standard. SDS-PAGE was performed using 30% acrylamide (Sigma). After electrophoresis, gels were equilibrated for 20 min in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol). Proteins were transferred onto polyvinylidene difluoride membranes (0.5 h, 30 V), which were then incubated with a blocking solution [5% dried skim milk in 100 mM Tris (pH 7.5) with 140 mM NaCl and 0.01% Tween 20 for a minimum of 1.5 h]. The blots were then incubated overnight at 4°C, either with a polyclonal rabbit anti-TRIF antibody (1:800, Abcam, UK), a polyclonal rabbit anti-MyD88 antibody (1:800, Abcam, UK), or a polyclonal rabbit anti-β-actin (1:1,000) on the same membrane. The blots were washed three times with the blocking solution and incubated with diluted horseradish-peroxidase-conjugated secondary antibodies (1:1,000) (Bio-Rad) for 1.0 h at room temperature. Blots were washed extensively and developed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Western immunoblot bands were quantified by means of a Bio-Rad calibrated densitometer (GS-800) using the vendor's software (Bio-Rad Laboratories) and β-actin served as an internal control for all analyses.

Serum Levels of HMGB1, HSP60, and sRAGE

Serum levels of HMGB1, HSP60, and sRAGE were measured using commercially available ELISA kits (R&D, Minneapolis, MN, USA). The procedures were performed in accordance with the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm using a microplate reader to analyze the intensity of color development in each well. The levels of HMGB1 and HSP60 were presented as nanograms per milliliter, whereas sRAGE was presented as picograms per milliliter.

Statistical Analysis

All statistical analyses were performed using SPSS Software 20.0 (SPSS, Inc., Chicago, IL, USA). Results are displayed as mean ± SD. The normally distributed data were analyzed by independent-samples *t*-test. Meanwhile, the analysis of abnormally distributed data was performed with Kruskal–Wallis test followed by the Mann–Whitney *U* test. One-way ANOVA was used to compare three experimental groups. The correlation between serum levels and clinical variables was analyzed using the Spearman's method. *P*-values <0.05 were considered statistically significant.

RESULTS

Elevated Expression of TLR2, TLR3, TLR9, and TLR10 in PBMCs of AITD Patients

Blood samples were obtained in 66 AITD patients and 30 healthy controls. The clinical features of enrolled subjects are shown in **Table 2**. In this study, the levels of expression of TLR1–TLR10 in PBMC samples from AITD patients (30 with untreated GD and 36 with HT) and 30 healthy subjects were assessed by RT-PCR were examined. As shown in **Figure 1**, the mRNA expression of TLR1, 4, 5, 6, 7, and 8 was equivalent between in AITD patients and healthy controls. The expression of TLR2, TLR3, TLR9, and TLR10 were significantly increased in both with HT ($P < 0.05$, $P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively) and those with GD subjects ($P < 0.05$, $P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively) than in controls, but no difference was found between HT and GD patients for all of these TLR.

Proliferative Response to TLR Agonists from PBMCs with AITD and HC

As shown in **Figure 2**, we found that PBMC from AITD patients significantly proliferated compared to controls ($P < 0.05$ for HT and GD, respectively). Increased OD was also found in AITD patients' *in vitro* stimulation with TLR2 agonist ($P < 0.01$ for HT and GD, respectively). Similarly, there are increased OD in AITD patients when cultured with TLR3 and TLR9 agonists ($P < 0.01$ and $P < 0.01$ for Poly I:C and $P < 0.05$ and $P < 0.01$ for CpG ODN, respectively).

TABLE 2 | The clinical characteristics of patients with Hashimoto's thyroiditis, Graves' disease, and healthy controls.

Variable	HC	HT	GD	Normal range
No.	30	36	30	–
Age (years)	37 ± 8	34 ± 9	30 ± 9	–
Gender (M/F)	4/26	4/32	5/25	–
Thyrotropin (mIU/L)	1.3 ± 0.2	14.2 ± 3.16	0.007 ± 0.01	0.35–4.94
FT4 (ng/dL)	1.4 ± 0.1	0.9 ± 0.2	46.02 ± 14.7	9.01–19.05
FT3 (pg/mL)	2.6 ± 0.5	3.7 ± 0.2	34.3 ± 6.9	2.63–5.70
Thyrotrophin receptor antibody (IU/L)	–	–	37.6 ± 9.5	0–1
TPOAb (IU/mL)	5.03 ± 0.7	343.27 ± 126.2	167.5 ± 23.2	0.11–5.23
TgAb (IU/mL)	2.54 ± 0.5	433.45 ± 102.7	192.28 ± 17.32	0.81–3.83

Data are expressed as mean ± SD. The "–" represents that the experiment was not performed, or the data are not applicable.
M, male; F, female; HT, Hashimoto's thyroiditis; GD, Graves' disease; HC, healthy controls.

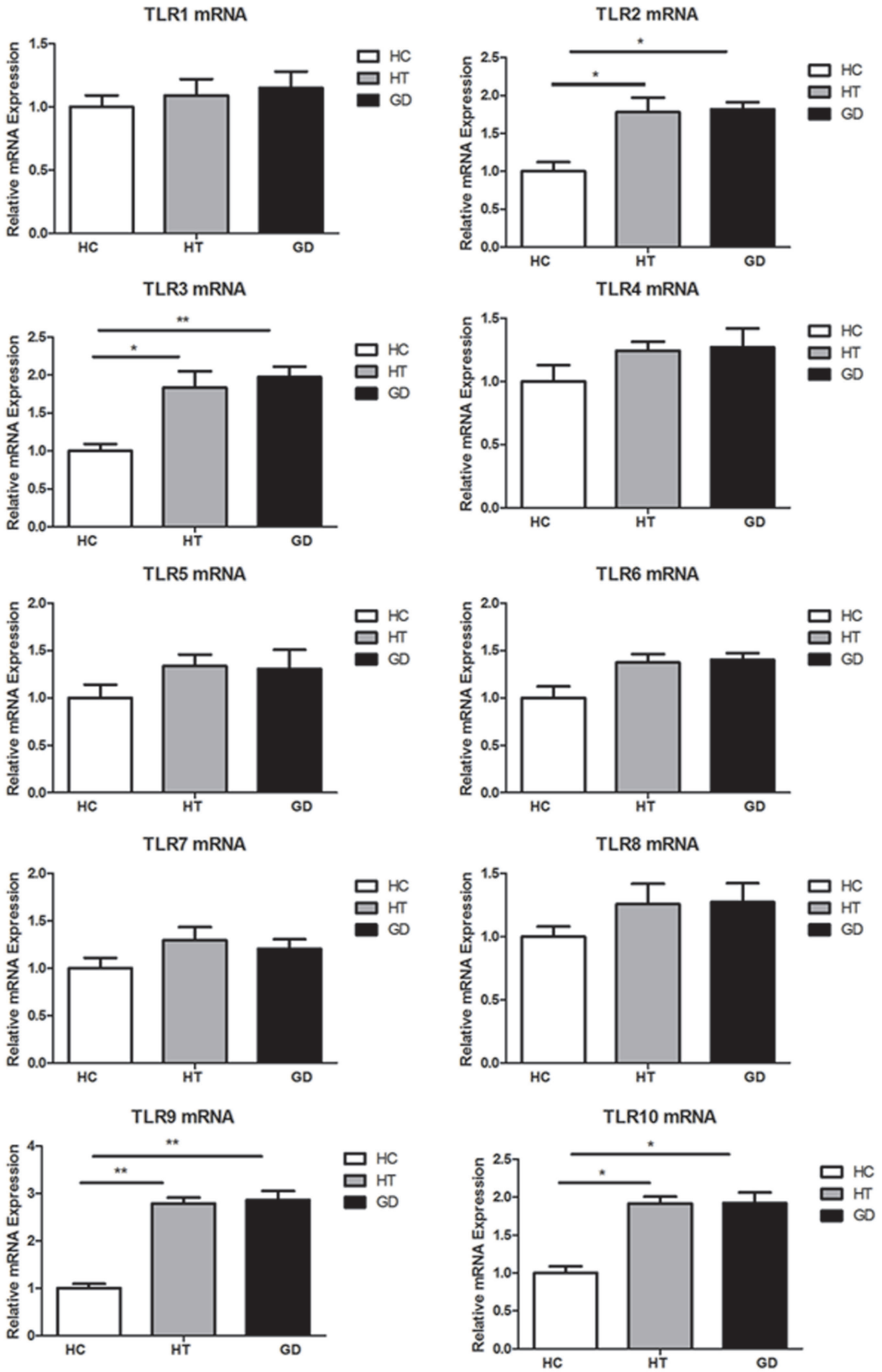

FIGURE 1 | Toll-like receptor (TLR) mRNA expression in peripheral blood mononuclear cells (PBMCs).

(*Continued*)

FIGURE 1 | Continued

Bar graphs comparing TLR1–TLR10 mRNA expression levels in PBMCs from patient with Hashimoto's thyroiditis, Graves's disease, and healthy controls. TLRs expression was measured by real-time PCR using GAPDH as endogenous control. Results are shown as fold change regarding TLRs gene expression in AITD patients relative to healthy subjects. Each bar represents the 1 ± SEM of three groups. One-way ANOVA was used to compare the differences of expression between AITD patients and healthy controls. AITD, autoimmune thyroid disease; HT, Hashimoto's thyroiditis; GD, Graves' disease; HC, healthy controls. Level of significant is $^{*}P < 0.05$ and $^{**}P < 0.01$.

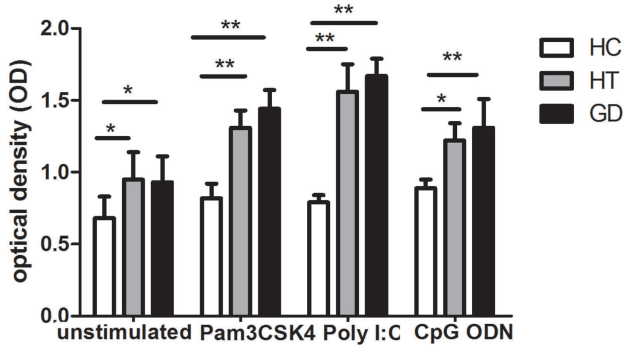

FIGURE 2 | Cell viability in peripheral blood mononuclear cells after toll-like receptor (TLR) agonists stimulation. Bar graphs comparing of cell viability optical density between unstimulated and stimulated cells treated with either unstimulate or stimulate of TLR agonists in both AITD patients and controls by MTT assay. Each bar represents the mean ± SD of three groups. One-way ANOVA was used to compare the differences of expression between AITD patients and healthy controls. AITD, autoimmune thyroid disease; HT, Hashimoto's thyroiditis; GD, Graves' disease; HC, healthy controls. Pam3CSK4, Poly I:C, and CpG ODN were used as TLR2, TLR3, and TLR9 agonists, respectively. Level of significant is $^{*}P < 0.05$ and $^{**}P < 0.01$.

Time-Dependent Modulation of TLR2, TLR3, TLR9, HMGB1, and RAGE Expression in PBMCs by Specific Agonist Activation

To further characterize regulation of TLR expression by specific agonists, a time–response study was conducted. In PBMCs from healthy controls stimulated with specific TLR agonists for 2, 6, 8, 12, 24, and 48 h, the maximal response for TLR2, TLR3, TLR9, HMGB1, and RAGE protein expression was all observed at 24 h (all $P < 0.05$, **Figure 3**), and the expression significant reduced at 48 h, suggesting that 24 h is the optimal time for the PBMCs by TLR agonists stimulation.

Responses of CD14^{+} Cells from AITD and HC to TLR2, TLR3, and TLR9 Stimulation with Pam3CSK4, Poly I:C, and CpG ODN

Intracellular and surface expression upon TLR stimulation in CD14^{+} cells were analyzed in PBMCs from AITD and control subjects. The expression of TLR2 was analyzed in HT, GD, and HC groups of CD14^{+} monocytes cells after cultured for 24 h in the presence or absence of Pam3CSK4. As shown in **Figure 4A**, the percentage of CD14^{+} TLR2 monocytes was significantly higher in AITD patients than in controls in resting and Pam3CSK4 activation ($P < 0.01$ and $P < 0.01$, respectively). Similarly, in both resting and agonist-stimulated cultured cells, TLR3 and TLR9 expression on peripheral CD14^{+} monocytes expression in AITD patients was significantly higher than in the control group ($P < 0.05$ and $P < 0.05$; $P < 0.01$ and $P < 0.01$ respectively, **Figures 4B,C**). TLR2, TLR3, and TLR9 expression did not differ between GD and HT patients (**Table 3**).

Responses of CD14^{+} Cells from AITD and HC to HMGB1 and RAGE Stimulation with CpG ODN

The expression of HMGB1 and RAGE were analyzed in HT, GD, and HC groups of CD14^{+} monocytes cells when cultured with TLR9 agonist, CpG ODN. As shown in **Figure 5A**, the percentage of CD14^{+} HMGB1 monocytes was significantly higher in AITD patients than in controls in resting (11.3 ± 2.0 for HT, 10.6 ± 1.8 for GD Vs 6.5 ± 1.2 for HC) and CpG ODN activation (14.7 ± 2.2 for HT, 13.8 ± 2.6 for GD Vs 10.5 ± 2.1 for HC; all $P < 0.01$, respectively). Similarly, the percentage of CD14^{+} RAGE^{+} cells in AITD patients was significantly higher than those in control group in both resting (4.7 ± 0.7 for HT, 4.3 ± 0.5 for GD Vs 2.3 ± 0.4 for HC) and agonist-stimulated cultured cells (4.9 ± 0.8 for HT, 5.0 ± 0.7 for GD Vs 3.0 ± 0.5 for HC; $P < 0.01$ for HT and $P < 0.01$ for GD, respectively, **Figure 5B**).

The Expression of TNF-α, IL-6, and IL-10 in PBMCs Stimulated with TLR2, TLR3, and TLR9 Agonists Pam3CSK4, Poly I:C, and CpG ODN

To determine whether TLR activation can induce cytokine upregulation in AITD patients, we examine the cytokine in TLR agonist activation PBMCs from AITD patients and healthy controls. TNF-α, IL-6, and IL-10 expression were evaluated in the culture supernatant of PBMCs after stimulation with Pam3CSK4, Poly I:C, and CpG ODN for 24 h. As shown in **Figure 6A**, the TNF-α expression in AITD patients, including GD and HT patients, were found to be significantly higher than those for control subjects when stimulated with either Poly I:C (TLR3) or CpG ODN (TLR9) ($P < 0.01$ and $P < 0.01$ for Poly I:C and CpG ODN, respectively). However, the expression of TNF-α in response to Pam3CSK4, a ligand of TLR2, appeared to be the same in all three groups. As shown in **Figure 6B**, the IL-6 secretion levels from PBMC samples stimulated with either Pam3CSK4, Poly I:C, or CpG ODN in the AITD groups were also found to be significantly higher than those in the control group ($P < 0.05$, $P < 0.05$, and $P < 0.01$ for Pam3CSK4, Poly I:C, and CpG ODN, respectively), while IL-10 expression, were significantly decreased in AITD groups under stimulation ($P < 0.01$,

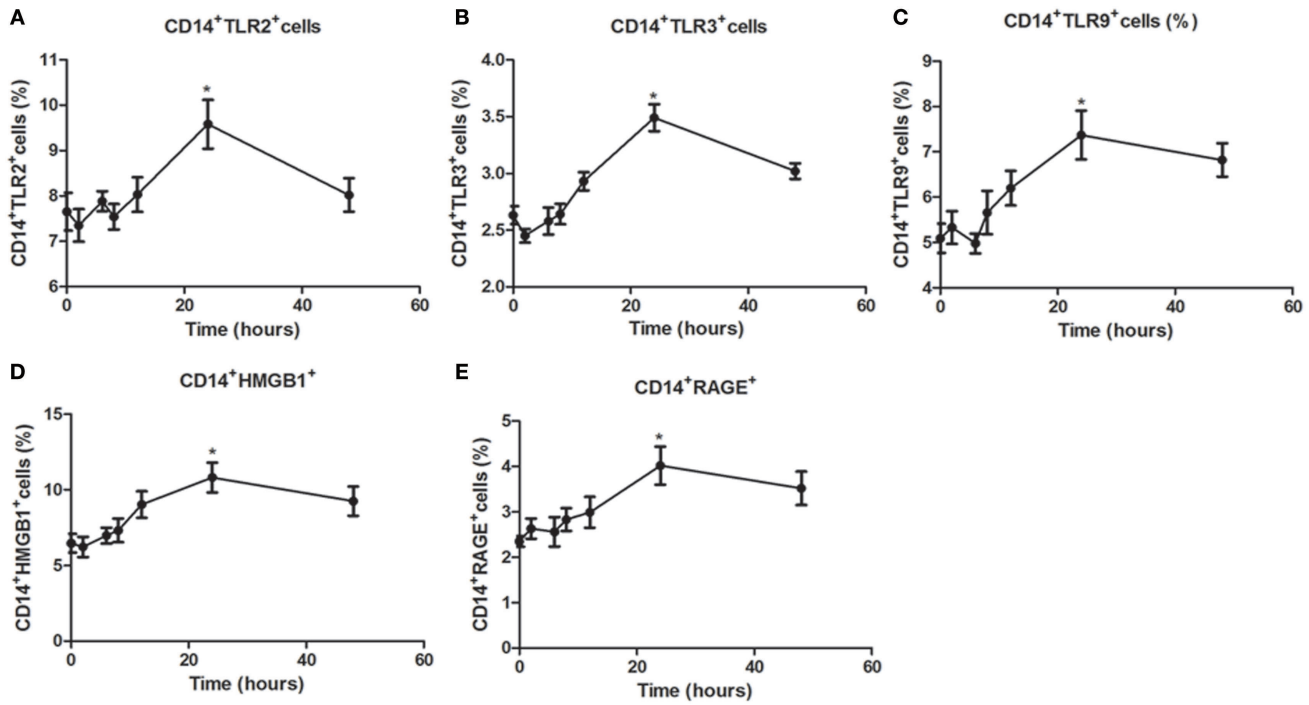

FIGURE 3 | Time-dependent modulation of TLR2, TLR3, TLR9, high mobility group box 1 (HMGB1), and receptor for advanced glycation end products (RAGE) expression in peripheral blood mononuclear cells (PBMCs) by specific agonist activation. PBMCs from healthy controls were treated with a TLR2, TLR3, or TLR9 agonist for 0, 2, 6, 8, 12, 24, and 48 h stained with anti-CD14 and anti-TLR2, TLR3, TLR9, HMGB1, and RAGE mAbs for flow cytometry. Percentages of $CD14^+TLR2^+$ **(A)**, $CD14^+TLR3^+$ **(B)**, $CD14^+TLR9^+$ **(C)**, $CD14^+HMGB1^+$ **(D)**, $CD14^+RAGE^+$cells **(E)** from HC was measured by flow cytometry. Results are shown as mean ± SD of control group. Level of significant is $^*P < 0.05$. Statistical analyses were conducted using one-way ANOVA. Pam3CSK4, Poly I:C, and CpG ODN were used as TLR2, TLR3, and TLR9 agonists, respectively.

$P < 0.01$, and $P < 0.01$ for Pam3CSK4, Poly I:C, and CpG ODN, respectively, **Figure 6C**).

Downstream Proteins under Western Blot Analysis

The downstream proteins TRIF and MyD88 were analyzed by Western blot analysis. As shown in the **Figures 7A,B**, the expression of TRIF was significantly higher in PBMC samples from HT and GD patients than in those from healthy subjects ($P < 0.01$ and $P < 0.01$, respectively). There was no significant difference between the HT and GD subjects.

MyD88 expression in HT and GD groups was also significantly higher than in HC group ($P < 0.01$; $P < 0.01$, respectively. **Figures 7A,C**). No significant difference was found between HT and GD groups.

Elevated HMGB1, HSP60, and sRAGE in the Serum of AITD Patients

The serum levels of HMGB1 were significantly higher in patients with HT (37.81 ± 12.26 ng/mL) and in patients with GD (40.03 ± 13.40 ng/mL) than in control subjects (17.54 ± 4.94 ng/mL, $P < 0.01$, and $P < 0.01$, respectively, **Figure 8A**). Similarly, the HSP60 levels were also higher in HT (135.33 ± 18.63 ng/mL) and GD groups (140.55 ± 19.81 ng/mL) than in control subjects (68.99 ± 18.34 ng/mL, $P < 0.01$, and $P < 0.01$, respectively, **Figure 8B**). In addition, the serum levels of HMGB1 and HSP60 did not differ between GD and HT patients. In contrast, there was a significantly decreased expression of sRAGE in HT (1,276.67 ± 98.47 pg/mL) and GD patients (1,278.08 ± 92.54 pg/mL) than in healthy subjects (1,433.80 ± 92.48 pg/mL, $P < 0.01$, and $P < 0.01$, respectively, **Figure 8C**).

Correlation of HMGB1, HSP60, and sRAGE with Clinical Variables

No significant correlation was found between either HMGB1, HSP60, or sRAGE levels and serum levels of TSH, FT3, or FT4 in HT patients. However, HMGB1 showed a significant association with TPOAb and TgAb in HT patients ($r = 0.5716$, $P < 0.001$, **Figure 9A**; $r = 0.5914$ $P < 0.001$, **Figure 9B**). There was also a significant correlation between HSP60 and TPOAb ($r = 0.4074$, $P < 0.001$, **Figure 9C**) and TgAb ($r = 0.4815$ $P < 0.001$, **Figure 9D**) in HT patients. However, no significant correlations were found between sRAGE expression and clinical variables.

DISCUSSION

Autoimmune thyroid diseases are characterized by production of pathogenic thyroid autoantibodies and lymphocyte infiltration

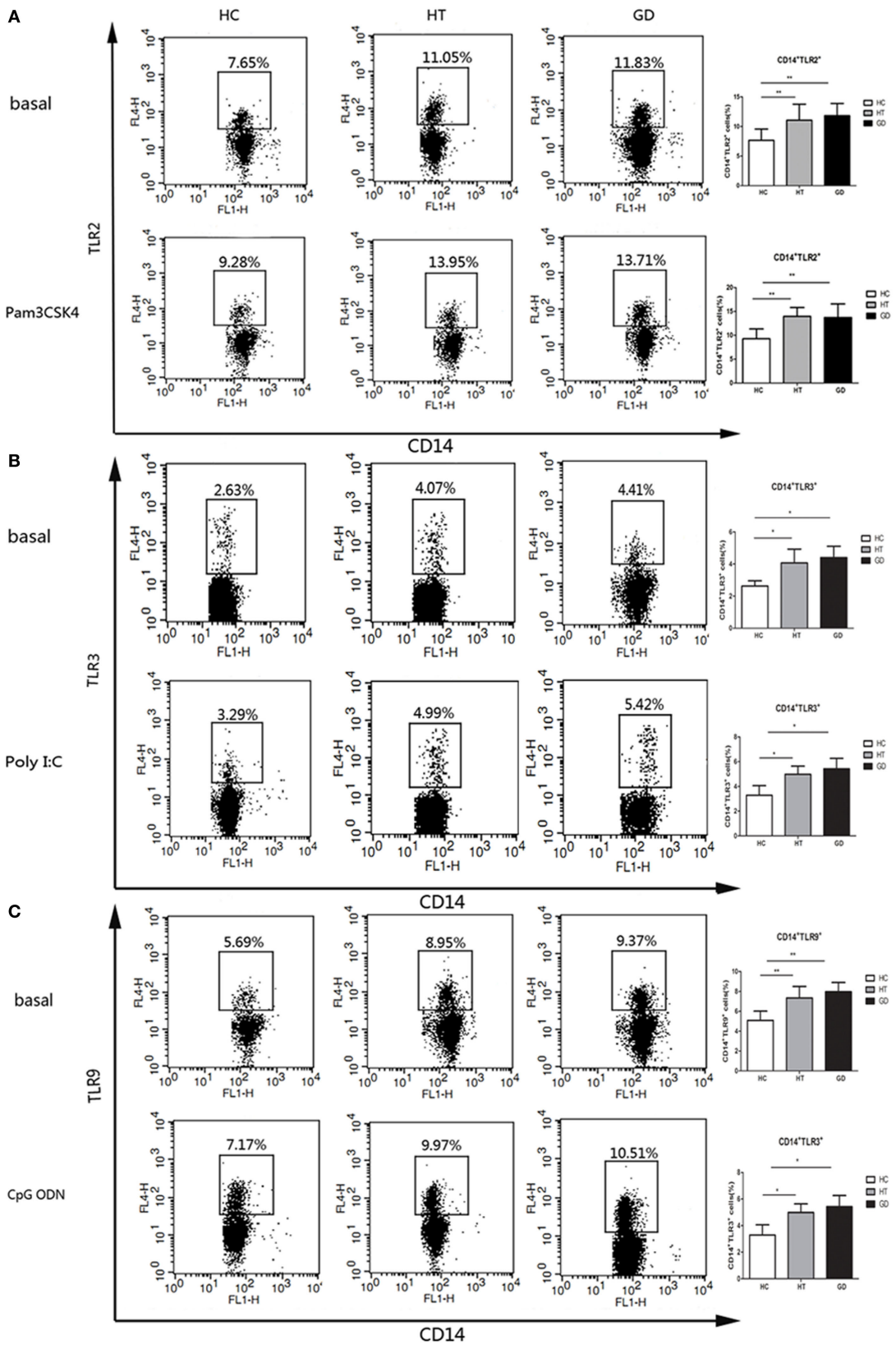

FIGURE 4 | TLR2, TLR3, and TLR9 surface protein expression in monocytes after toll-like receptor agonists stimulation.

(*Continued*)

FIGURE 4 | Continued

Peripheral blood mononuclear cells from AITD patients and healthy controls were treated with a TLR2, TLR3, or TLR9 agonist or PBS as basal for 24 h and stained with anti-CD14 and anti-TLR2, TLR3, and TLR9 mAbs for flow cytometry. We first selected singlets using forward scatter area Vs forward scatter height parameters. From the singlet population, we removed dead cells from total events by gating on LIVE/DEAD events. Then, we selected total monocytes from the living using the pan-monocyte marker CD14, then TLR2 positive, TLR3 positive, and TLR9 positive, respectively. **(A)** Flow cytometry dot plots from AITD and HC depicting TLR2 expression by CD14 enriched monocyte cells (left panels). The number in the right quadrants represents the percentage of $CD14^+TLR2^+$ between AITD and healthy controls. **(B)** Flow cytometry dot plots from AITD and HC depicting TLR3 expression by CD14 enriched monocyte cells (left panels). Percentages of $CD14^+TLR3^+$ cells were compared between HC and AITD patients (right panels). **(C)** Flow cytometry dot plots from AITD and HC depicting TLR9 expression by CD14 enriched monocyte cells (left panels). Percentages of $CD14^+TLR9^+$ cells were compared between HC and AITD patients (right panels). Each bar represents the mean ± SD of three groups. Level of significant is $^*P < 0.05$ and $^{**}P < 0.01$. AITD, autoimmune thyroid disease; HT, Hashimoto's thyroiditis; GD, Graves' disease; HC, healthy controls. Statistical analyses were conducted using one-way ANOVA. Pam3CSK4, Poly I:C, and CpG ODN were used as TLR2, TLR3, and TLR9 agonists, respectively.

TABLE 3 | Percentage of TLR2, TLR3, and TLR9 expressing $CD14^+$ cells in basal condition and after stimulation of AITD and HC PBMCs with Pam3CSK4 (TLR2), Poly I:C (TLR3), and CpG ODN (TLR9) for 24 h.

	Basal			Pam3CSK4	Poly I:C	CpG ODN
	TLR2	TLR3	TLR9	TLR2	TLR3	TLR9
HC	7.6 ± 2.0	2.6 ± 0.3	5.1 ± 1.0	9.3 ± 2.7	3.2 ± 0.7	6.6 ± 1.2
HT	11.8 ± 2.1	4.1 ± 0.7	7.4 ± 1.2	13.8 ± 1.8	5.0 ± 0.6	9.8 ± 2.0
GD	11.0 ± 2.7	4.4 ± 0.8	7.9 ± 0.9	13.5 ± 2.9	5.3 ± 0.8	10.4 ± 1.9

Results are shown as mean ± SD of three groups.

AITD, autoimmune thyroid disease; HT, Hashimoto's thyroiditis; GD, Graves' disease; HC, healthy controls.

Pam3CSK4, Poly I:C, and CpG ODN were used as TLR2, TLR3, and TLR9 agonists, respectively.

(1). TLRs play a vital role in the increased inflammation observed in AITD, which may be mediated in part through activation of innate immune pathways by TLRs. However, no study to date has examined TLR expression in PBMCs from AITD patients or their contribution to the production of pro-inflammatory cytokines.

In the current study, mRNA expression of TLR2, -3, -9, and -10 was significantly increased in patients with HT and GD compared with controls. Despite the lack of data regarding TLR in AITD, TLR3 has been found to be highly expressed by thyrocytes and thyroid follicular epithelial cells of HT and GD patients (21). TLR3 is also expressed in both thyroid follicles and inflammatory cells of iodine-induced NOD.H-2^{h4} mice (25), a classic model of spontaneous autoimmune thyroiditis (26). In addition, poly (I:C) administration aggravates inflammation of excessive iodine-induced thyroiditis in NOD mice, and evidence of the severity of thyroid follicle destruction and amount of lymphocyte infiltration has been reported (25). DNA microarray analysis has shown that stimulation of cultured human thyroid follicles with dsRNA [poly (I:C)] can cause activation of inflammatory responses and upregulation of a variety of related genes, including pro-inflammatory cytokines (IL-6) and chemokines (27). The results of the present study are consistent with previous studies suggesting that TLR3 may be involved in the development of AITD. In our study, mRNA expression of TLR2 was found to be elevated in AITD patients. It has been reported that FRTL-5 cells express TLRs, including TLR2, TLR3, TLR4, and TLR9, and stimulate the expression of pro-inflammatory cytokine (27). Thyrocytes produce pro-inflammatory cytokines and type I IFNs in response to PAMPs and DAMPs, thereby activating the innate immune response (28). Here, we present evidence that TLR9 was more highly expressed in PBMCs from HT and GD patients compared to controls. Although TLR9 has been demonstrated to be associated with SLE (29) and type 1 diabetes (30), there are limited data regarding the levels of TLR9 in AITD patients. A previous study demonstrated that TLR9 single-nucleotide polymorphisms (SNPs) play an important role in males with thyroid-associated ophthalmopathy (31). TLR9 recognizes the unmethylated CpG motif of microbial DNA and endogenous DNA, which acts as a PAMP and binds to TLR9 to stimulate signal transduction via the MyD88 pathway against viral pathogens. A recent study has shown a significant association between TLR10 SNPs and the mechanism of AITD in patients (32). TLR10 is expressed by immune-related cells and is more likely to interact with TLR1 and TLR6 than other TLRs. Although the ligands and the signaling of TLR10 remain unknown (7), it has been reported that TLR10 functions as a co-receptor of TLR2 (33). The underlying mechanism of TLR10 in AITD remains unknown. Further studies are needed to confirm the role of TLR10 in thyrocytes.

A remarkable increase in viability was found for PBMCs from AITD patients in the resting state and after TLR stimulation. This finding indicates that activation of TLR signaling triggers abnormal activation of lymphocytes and stimulates and amplifies the immune response in AITD patients, which may lead to persisting autoimmunity.

TLR2, TLR3, and TLR9 surface expression is increased on monocytes from AITD subjects compared with controls. In addition, following specific agonist stimulation, the percentage of TLR2-expressing $CD14^+$ cells increased in the PBMCs of both HT and GD patients, suggesting further activation of signaling pathways. Similarly, increased TLR3 and TLR9 expression in AITD patients was also found under TLR3 and TLR9 stimulation. HMGB1 and RAGE expression was found to be upregulated in PBMCs from AITD patients in the resting state and under TLR9 stimulation. HMGB1, a nuclear

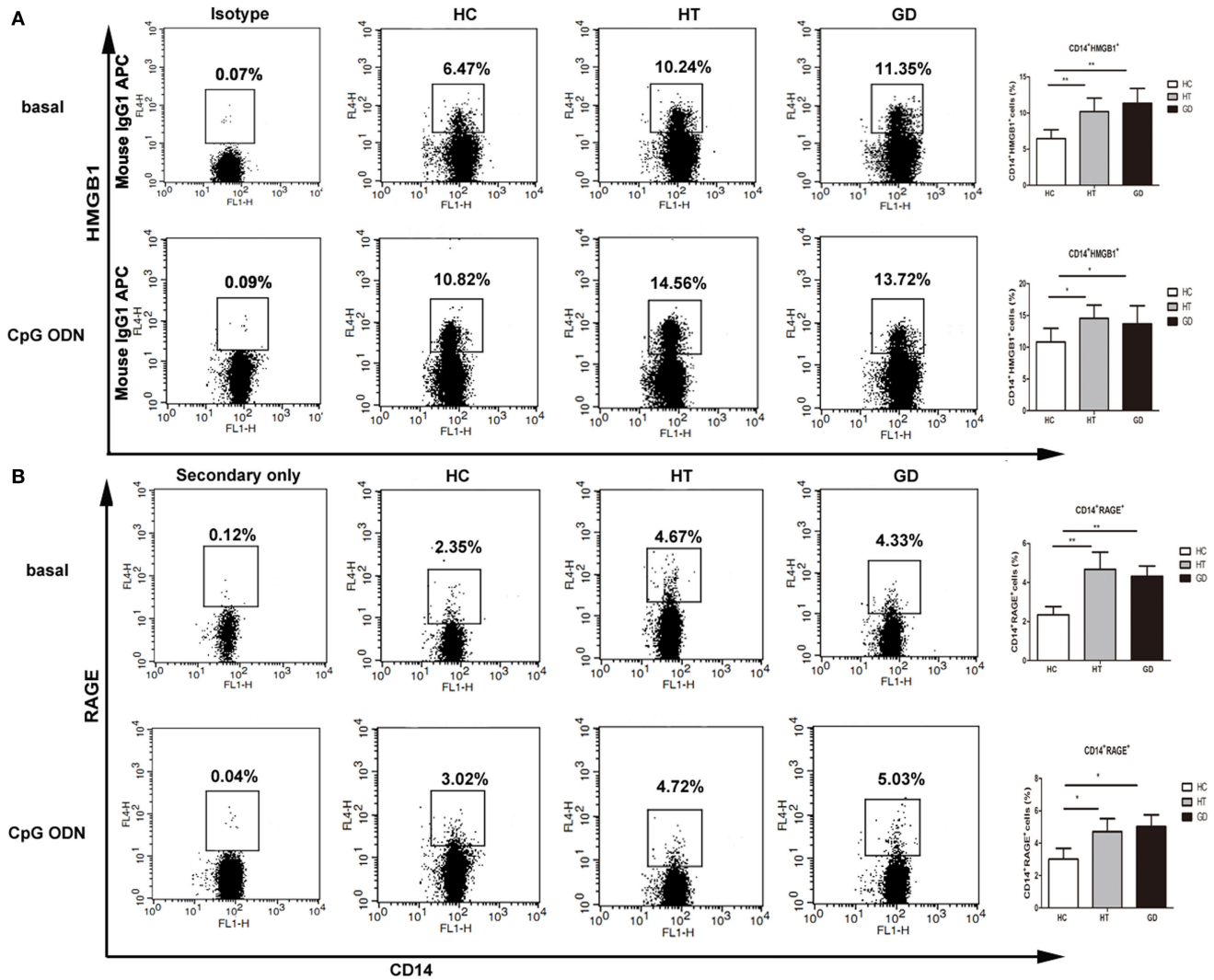

FIGURE 5 | High mobility group box 1 (HMGB1) and receptor for advanced glycation end products (RAGE) surface protein expression in monocytes after TLR9 agonist stimulation. Peripheral blood mononuclear cells from AITD patients and healthy controls were treated with a TLR9 agonist or PBS as basal for 24 h and stained with anti-CD14 and anti-HMGB1, and RAGE mAbs for flow cytometry. We first selected singlets using forward scatter area Vs forward scatter height parameters. From the singlet population, we removed dead cells from total events by gating on LIVE/DEAD events. Then, we selected total monocytes from the living using the pan-monocyte marker CD14, then HMGB1 positive and RAGE positive cells selected, respectively. **(A)** Flow cytometry dot plots from AITD and HC depicting HMGB1 or match isotype control expression by CD14 enriched monocyte cells (left panels). The number in the right quadrants represents the percentage of $CD14^+HMGB1^+$ between AITD and healthy controls. **(B)** Flow cytometry dot plots from AITD and HC depicting RAGE expression by CD14 enriched monocyte cells (left panels). Percentages of $CD14^+RAGE^+$ cells were compared between HC and AITD patients (right panels). Control cells were stained with the secondary antibodies without using the anti-RAGE primary antibody. Each bar represents the mean ± SD of three groups. Level of significant is $^*P < 0.05$ and $^{**}P < 0.01$. AITD, autoimmune thyroid disease; HT, Hashimoto's thyroiditis; GD, Graves' disease; HC, healthy controls. Statistical analyses were conducted using one-way ANOVA. CpG ODN was used as a TLR9 agonist.

protein released from damaged or necrotic cells, is involved in many chronic inflammatory diseases. The HMGB1–DNA complex binds to RAGE and then activates the TLR9-mediated inflammatory response (34), and a previous study showed that extracellular HMGB1 could bind to CpG ODN and stimulate its transfer to TLR9 in inflammatory disease (35). Our finding suggested that HMGB1 and RAGE play an important role in the development of chronic inflammation in AITD through the activation of TLR9 pathway.

Toll-like receptor activation has been demonstrated to have the ability to regulate the production of pro-inflammatory cytokines (36, 37). Our results showed increased IL-6 and decreased IL-10 production in PBMCs from AITD patients in response to specific ligands for either TLR2, TLR3, or TLR9 and increased TNF-α expression in response to TLR3 and TLR9 ligands compared to healthy controls. Conversely, TNF-α secretion after TLR2 agonist stimulation in PBMCs from AITD patients and controls was equivalent, suggesting that TNF-α production may not be

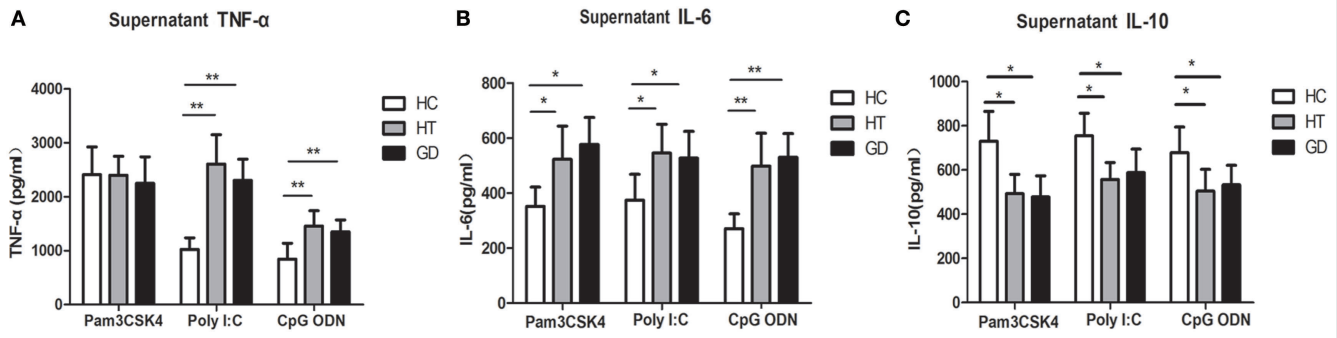

FIGURE 6 | Release of tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-10 in the cultured supernatants from AITD patients and healthy controls. **(A)** Peripheral blood mononuclear cells (PBMCs) isolated from subjects were stimulated with Pam3CSK4, poly I:C, and CpG ODN for 24 h, TNF-α were measured by enzyme-linked immunosorbent assay (ELISA). **(B)** PBMCs were treated as in **(A)** and the release of IL-6 was measured by ELISA. **(C)** PBMCs were treated as in **(A)** and the release of IL-10 was measured by ELISA. Level of significant is $^{*}P < 0.05$ and $^{**}P < 0.01$. Values were shown as mean ± SD and expressed as picograms per milliliter. AITD, autoimmune thyroid disease; HT, Hashimoto's thyroiditis; GD, Graves' disease; HC, healthy controls. Statistical analyses were conducted using one-way ANOVA. Pam3CSK4, poly I:C, and CpG ODN were used as TLR2, TLR3, and TLR9 agonists, respectively.

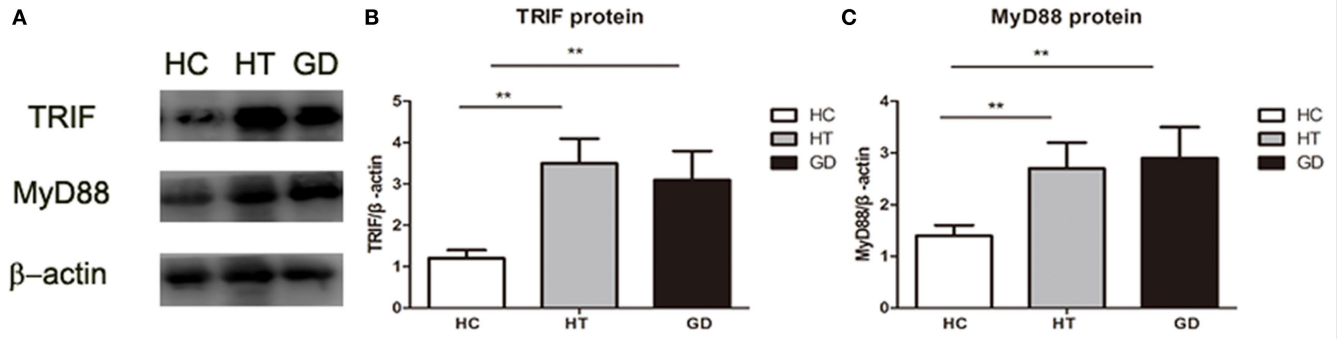

FIGURE 7 | Toll-like receptor (TLR) signaling proteins in peripheral blood mononuclear cell from patients with Hashimoto's thyroiditis, Graves's disease, and healthy controls. **(A)** Representative Western blotting results of TLR downstream signaling proteins MyD88 and toll/IL-1 receptor-domain containing adaptor-inducing interferon-β (TRIF) was performed using specific antibodies to the respective proteins as described in methods. β-actin was used as loading and internal control for MyD88 and TRIF. **(B)** A ratio of TRIF/β-actin was determined to represent a mean net density. **(C)** A ratio of MyD88/β-actin was determined to represent a mean net density. Level of significant is $^{*}P < 0.05$ and $^{**}P < 0.01$. Values were shown as mean ± SD. HT, Hashimoto's thyroiditis; GD, Graves' disease; HC, healthy controls. One-way ANOVA was used to compare the differences of TRIF and MyD88 proteins of these three groups.

connected to TLR2 activation in cultured PBMCs. This finding appears to be consistent with a previous study in gout patients, whereby TNF-α secretion after Pam3CSK4 stimulation by PBMCs from gout patients was not significantly different from controls (38). These findings indicate that PBMCs from patients with AITD may be more sensitive to response induced by the agonists of TLRs, resulting in the secretion of pro-inflammatory cytokines.

Toll-like receptors act mainly through the adaptor molecule MyD88, which results in transcription of the NF-κB gene and activation of pro-inflammatory cytokines, such as IL-6 and TNF-α (39, 40). TRIF is also important to the TLR3 and TLR4 signaling pathways. In the present study, we found increased expression of downstream adapter proteins MyD88 and TRIF in PBMCs from patients with AITD compared with controls. Increased mRNA expression of MyD88, TRAF6, and IRAK4 has been demonstrated in PBMCs from SLE patients, which correlated with disease activity (41). Increased expression of MyD88 in B lymphocytes was also found in SLE patients but unlikely to be affected by disease activity (42). Increased expression of MyD88, TRIF, and IRAK has been found in monocytes from patients with T1DM (20, 43). The results of these studies are consistent with our results, suggesting that MyD88-dependent and -independent pathways are activated in the development of AITD.

Expression of endogenous ligands (HSP60 and HMGB1) for TLR2 and TLR4 and sRAGE was also examined in this study. We found HSP60 and HMGB1 levels to be significantly increased in the serum of AITD patients compared with controls. Moreover, the serum levels of sRAGE were significantly decreased in AITD patients. A previous study has shown that HMGB1 is overexpressed in the thyroid of HT patients, and HMGB1 expression is very low in healthy thyroids (44). Recent results also revealed increased numbers of HMGB1-positive monocytes in GD patients compared to healthy controls; with expression decreased by approximately 50% under antithyroid drug treatment (45).

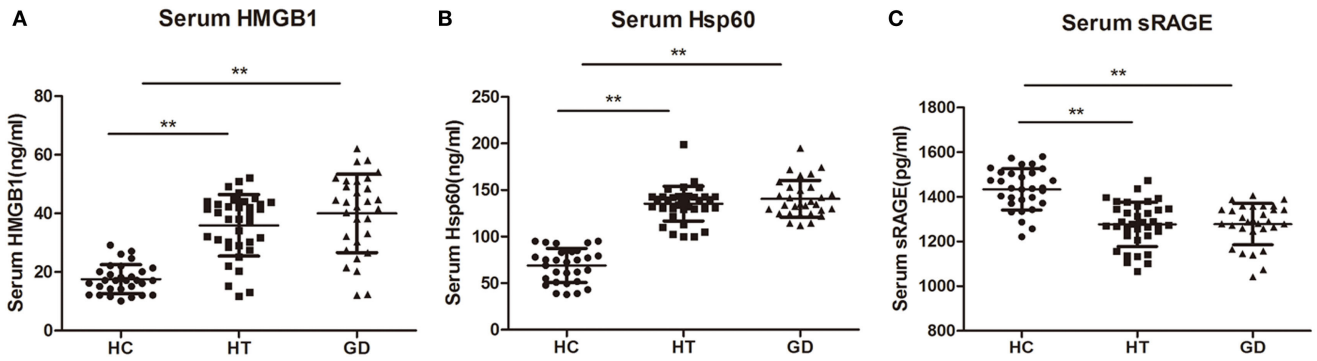

FIGURE 8 | Dot plots showing serum levels of high mobility group box 1 (HMGB1), heat shock protein 60 (HSP60), and sRAGE in patients with Hashimoto's thyroiditis, Graves's disease, and healthy controls. Serum levels of HMGB1 **(A)**, HSP60 **(B)**, and sRAGE **(C)** were measured by using enzyme-linked immunosorbent assay. Results are shown in the graphic. HT, Hashimoto's thyroiditis; GD, Graves' disease; HC, healthy controls. Level of significant is $^{*}P < 0.05$ and $^{**}P < 0.01$. Values were shown as mean ± SD. Statistical analyses were conducted using one-way ANOVA.

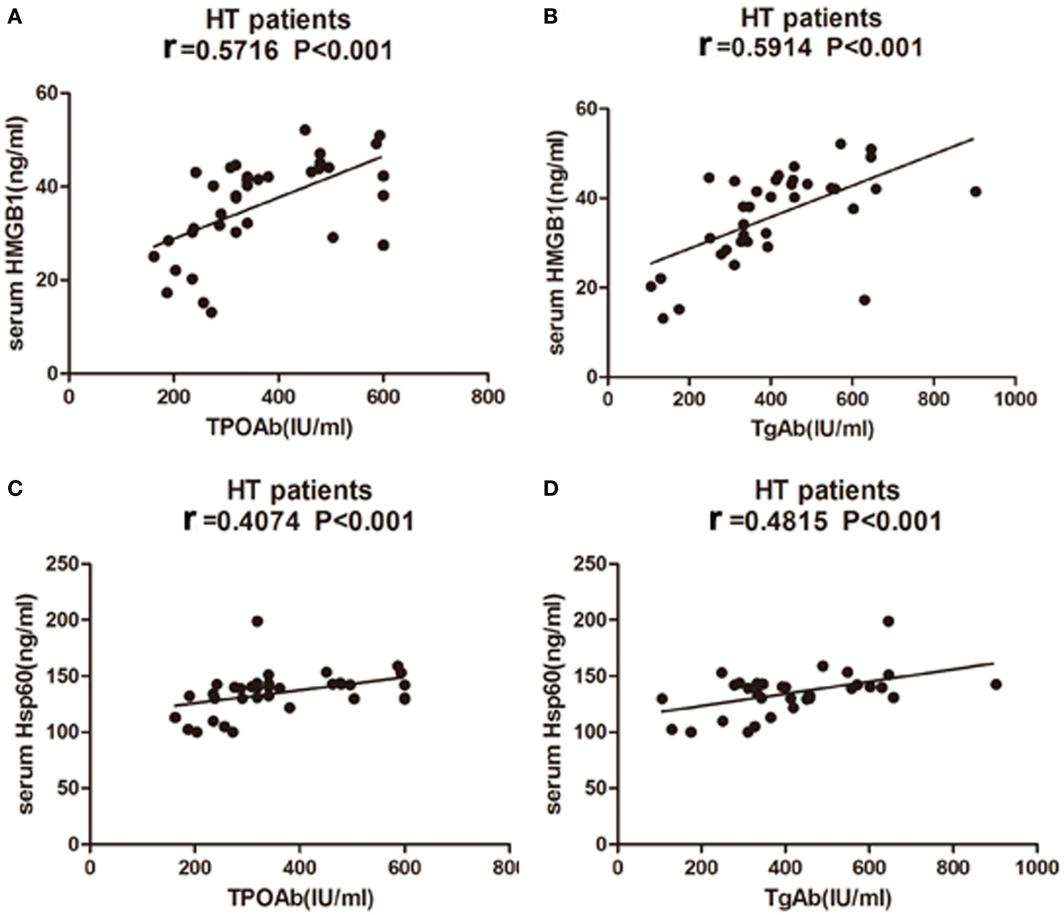

FIGURE 9 | Correlation analysis of laboratory parameters and serum levels of high mobility group box 1 (HMGB1) and heat shock protein 60 (HSP60) in HT patients. Relationship between serum expression of HMGB1 with TPOAb **(A)** and TgAb **(B)**, HSP60 with TPOAb **(C)** and TgAb **(D)** are presented in scatter plots. Pearson's and Spearman's correlation and *P*-values are given in all cases, *r* = Pearson correlation coefficient. HT, Hashimoto's thyroiditis.

HSP60 is an intracellular chaperone that plays an important role in protein folding and stability. Kotani et al. first demonstrated that HSP60 is more highly expressed in HT follicular cells than in normal thyrocytes (46), and it has been reported that the serum levels of HSP60 are significantly increased in HT patients compared to controls (47). HSP60 is expressed in the

thyroid glands of HT subjects and is localized to thyrocytes. This previous study also reported a correlation between HSP60 and both TgAb and TPOAb antibodies in the serum of HT patients. These studies suggest that HSP60 may actively participate in the development of HT through antibody recognition. This is the first finding regarding the elevated HSP60 expression in GD patients. Although no studies have addressed the levels of serum HSP60 in GD patients, HSP70, another member of the HSP family, is highly expressed in the thyroid tissue of patients with GD or HT (48, 49). The results of the current study show significantly decreased serum levels of sRAGE in HT and GD patients compared with controls. It has recently been reported that levels of sRAGE are significantly decreased in HT patients and that serum levels of AGEs are inversely correlated with those of sRAGE, a finding that is consistent with the results of the current study. Another study also showed increased expression of AGEs in HT patients, with positive correlations with TPOAb (50). A tendency toward elevated expression of AGEs in hyperthyroidism patients has also been reported, but this was significant only in patients with GD, which is consistent with our results (51). The serum levels of HSP60 and HMGB1 tend to correlate with TgAb and TPOAb in HT patients. These findings suggest that HSP60 and HMGB1 may play a vital role in the development of HT and are thus related to disease progression.

CONCLUSION

This is the first study reporting increased TLR expression, activation, and signaling adaptor molecules in PBMCs from AITD patients; a significant elevation in TLR endogenous ligands was also observed in the serum of AITD group. In a future study, the mechanism underlying the effects of viral and endogenous ligands on TLR activation and signaling pathways will be examined.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: WT, SP, and CL. Performed the experiments: SP, CL, XL, CH, TJ, SL, XZ, XH, HZ, XX, and CF. Analyzed the data: XW, SP, and CL. Contributed reagents/materials/analysis tools: WT, LS, ZS, XY, and CW. Contributed to the writing of the manuscript: SP and CL. All the authors reviewed and approved the final manuscript.

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The HMGB1–CXCL12 Complex Promotes Inflammatory Cell Infiltration in Uveitogenic T Cell-Induced Chronic Experimental Autoimmune Uveitis

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It is largely unknown how invading autoreactive T cells initiate the pathogenic process inside the diseased organ in organ-specific autoimmune diseases. In experimental autoimmune uveitis (EAU) induced by uveitogenic, interphotoreceptor retinoid-binding protein (IRBP)-specific T cells (tEAU) in mice, we have previously reported that high mobility group box 1 (HMGB1) released as a consequence of the direct interaction between IRBP-specific T cells and retinal parenchymal cells is an early and critical mediator in induction of intraocular inflammation. Our present study explored the roles of HMGB1 in intraocular inflammation, focusing on its role in recruiting inflammatory cells into the eye. Our results showed that supernatants from retinal explants either stimulated with HMGB1 or cocultured with IRBP-specific T cells attracted leukocytes. Notably, HMGB1 antagonists blocked supernatant-induced chemoattraction when present from the start of coculture, but not when added to the culture supernatants after coculture, indicating that molecules released by HMGB1-treated retinal cells are chemoattractive. Moreover, CXCL12 levels in the coculture supernatants were dependent on HMGB1, since they were increased in the cocultures and reduced when HMGB1 antagonists were added at the beginning of the coculture. When either anti-CXCL12 Ab was added to the supernatants after coculture or the responding lymphocytes were pretreated with Ab against CXCL12 specific receptor, CXCR4, chemoattraction by the coculture supernatants was decreased. Finally, induction of tEAU was significantly inhibited by a CXCR4 antagonist, AMD3100, at the time of autoreactive T cell transfer. Our study demonstrates that, at a very early stage of intraocular inflammation initiated by uveitogenic autoreactive T cells, synergism between HMGB1 and CXCL12 is crucial for the infiltration of inflammatory cells.

Keywords: autoimmune disease, uveitis, immune regulation, autoreactive T cells, damage-associated molecular patterns, HMGB1, CXCL12, CXCR4

Abbreviations: EAU, experimental autoimmune uveitis; HMGB1, high mobility group box 1; IRBP, interphotoreceptor retinoid-binding protein; RAGE, receptor for advanced glycation end products; tEAU, activated IRBP-specific T cell-induced EAU, TREM-1, triggering receptor expressed on myeloid cells-1.

INTRODUCTION

Autoimmune uveitis is a group of potentially visually disabling intraocular inflammatory diseases that arise without a known infectious trigger. Although the etiology remains unclear, it is generally believed that a T cell-mediated immune response to unique ocular proteins underlies the pathogenesis of the disease and this is supported by the observation that adoptive transfer of uveitogenic autoreactive T cells into susceptible, syngeneic rodents induces experimental autoimmune uveitis (tEAU) (1–4). Studies in rodent models of experimental autoimmune uveitis (EAU) induced by immunization of a well-characterized uveitogenic autoantigen, interphotoreceptor retinoid-binding protein (IRBP), have shown that activation of autoreactive T cells is a key pathogenic event linked to disease induction, progression, and recurrence (2, 4–7). While a great deal of information is available about the development and activation of autoimmune T cells in the periphery in EAU, the mechanism by which the low frequency of infiltrating uveitogenic T cells triggers and maintains the intraocular inflammatory cascade is unknown. The tEAU model, which, in contrast to the antigen-immunization EAU model, is induced without injecting microbial products and resembles human chronic uveitis, allows us to study the behavior of infiltrating effector T cells inside the eye.

Using this tEAU model, we have previously demonstrated that, once peripheral activated IRBP-specific T cells enter the eye, they interact with parenchymal cells, resulting in the production of high mobility group box 1 (HMGB1) by these cells (8). Released HMGB1 in the eye plays an early and critical role in IRBP-specific T cell-induced intraocular inflammation, because neutralization or blockade of HMGB1 using antagonists reduces ocular inflammation and suppresses uveitogenic T cell function, such as IRBP-specific T cell proliferation and cytokine production (8). We also found that HMGB1 is actively secreted within the eye within 24 h after IRBP-specific T cell transfer as a consequence of direct cell–cell contact between infiltrating IRBP-specific T cells and viable retinal cells (8), which is mediated by the Fas/FasL interaction (9). However, the mechanisms by which released HMGB1 induces intraocular inflammatory cascade are unknown. In the present study, we aimed to define the role of HMGB1 in inflammatory cell infiltration into the eye, one of the major pathogenic events initiated by the few IRBP-specific T cells that enter the eye.

High mobility group box 1 is one of the most important damage-associated molecular pattern molecules. Although itself is only a weak inflammatory mediator, its interaction with receptor for advanced glycation end products (RAGE) (10) or TLRs (11) leads to increased production and release of cytokines and other inflammatory molecules. HMGB1 also binds to nucleosomes, RNA and DNA, lipopolysaccharide, thrombospondin, triggering receptor expressed on myeloid cells-1, CD24, and CXCL12, promoting inflammatory responses (10). Three isoforms of HMGB1 have been discovered, with disulfide HMGB1 inducing cytokine production *via* TLR4, fully reduced HMGB1 promoting chemotaxis by binding CXCL12 for stimulation *via* CXCR4, and the fully oxidized HMGB1 being inactive (12).

We therefore investigated whether the interaction of autoreactive T cells and retinal cells leads to cooperation of HMGB1 and CXCL12 in promoting leukocyte migration *in vitro* and *in vivo*, and whether blocking HMGB1/CXCL12 complex inhibits its chemoattractive function, resulting in the reduction of intraocular inflammation.

MATERIALS AND METHODS

Animals and Reagents

Eight- to ten-week-old female C57BL/6J (B6) mice, purchased from the Jackson Laboratory (Bar Harbor, ME, USA), were housed and maintained in the animal facilities of the University of Louisville (KY, USA). All animal studies conformed to the Association for Research in Vision and Ophthalmology statement about the use of animals in ophthalmic and vision research. The protocol (#14052) was approved by the Institutional Animal Care and Use Committee of the University of Louisville.

All T cells were cultured in complete medium [RPMI 1640 medium (Mediatech, Manassas, VA, USA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), 5×10^{-5} M 2-mercapatoethanol, and 100 μg/ml of penicillin/streptomycin]. The human IRBP peptide 1–20 (GPTHLFQPSLVLDMAKVLLD) was synthesized by Sigma-Aldrich (St. Louis, MO, USA). The fully reduced HMGB1 (Cat#HM-115) was purchased from HMGBiotech (Milano, Italy) and chicken anti-HMGB1 polyclonal antibody (Cat#326052233) from Shino-Test Corporation (Kanagawa, Japan).

Induction of tEAU

The method used to induce tEAU has been reported previously (4). Briefly, T cells from mice immunized 12 days previously with peptide $IRBP_{1–20}$ were purified from draining lymph nodes and spleen cells by passage through a nylon wool column (Polysciences, Warrington, PA, USA), then 1×10^7 cells in 2 ml of RPMI 1640 medium were added to each well of a six-well plate (Costar, Corning, NY, USA) and stimulated with 20 μg/ml of $IRBP_{1–20}$ in the presence of 1×10^7-irradiated syngeneic spleen cells as antigen-presenting cells (APCs). After 2 days, activated lymph blasts were isolated by gradient centrifugation on Lymphoprep (Sigma-Aldrich) and injected intraperitoneally (i.p.) in 0.2 ml of PBS into naive B6 recipients (5×10^6 cells/mouse). The clinical course of the disease was assessed by indirect fundoscopy once or twice a week and graded as described previously (13).

Pathological Examination

Inflammation of the eye was confirmed by histopathology. Whole eyes were collected, immersed for 1 h in 4% phosphate-buffered glutaraldehyde, and transferred to 10% phosphate-buffered formaldehyde until processed. The fixed and dehydrated tissue was embedded in methacrylate, and 5 μm sections were cut through the pupillary-optic nerve plane and stained with hematoxylin and eosin. Presence or absence of disease was evaluated blind by examining six sections cut at different levels for each eye. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments based on the

presence of inflammatory cell infiltration of the iris, ciliary body, anterior chamber (AC), and retina (4).

Isolation of Retinal Explants and Coculture with Activated $IRBP_{1–20}$-Specific T Cells or HMGB1

Eyes were collected from B6 mice and neural retinas isolated and used as retinal explants as described previously (8, 9). Retinal explants were placed with the inner membrane facing up in a 24-well plate and cultured in 500 µl of DMEM/F12 medium (Mediatech Inc., Manassas, VA, USA) containing 0.1% fetal calf serum, then either HMGB1 (0.1 or 1 µg/ml) or 5×10^4-activated T cells, prepared from $IRBP_{1–20}$-immunized mice at day 11–14 postinjection as described above, was added and the cells incubated at 37°C with 5% CO_2 for 6 h, then CXCL12 and HMGB1 levels in the supernatants were measured by ELISA, as described below. In inhibitor studies, retinal explants were cocultured with activated $IRBP_{1–20}$-specific T cells in the presence or absence of anti-HMGB1 mAb (IBL international GmbH, Toronto, ON, Canada), anti-RAGE mAb (R&D, Minneapolis, MN, USA), or glycyrrhizin (Calbiochem) for 6 h, then the supernatants were collected for ELISA or chemotactic assays.

Intraocular Inoculations

B6 mice were anesthetized by i.p. injection of ketamine (80 mg/kg, JHP Pharmaceuticals, Rochester, MI, USA) and xylazine (10 mg/kg, Akorn, Decatur, IL, USA). One drop of 0.5% tropicamide and 1.25% phenylephrine hydrochloride ophthalmic solutions was applied topically on the eye before injection. Under a dissecting microscope, 1 µl of 1 µg anti-HMGB1 Ab was injected into the AC, one eye with a microliter syringe and a 33-gauge needle (Hamilton, Reno, NV, USA).

Intraocular Fluid Collection

The removed eyeball was immersed in 200 µl of PBS and cut in two, then the cornea, sclera, and lens were discarded and the rest of the tissue cut into small pieces and the suspension containing the aqueous humor, vitreous fluid, and fine pieces of tissue centrifuged at 500 *g* for 5 min at 4°C, then the supernatant (intraocular fluid) was immediately stored in a −80°C freezer until use. Half (about 100 µl) of each collection from one eyeball was used for CXCL12 measurement by ELISA.

Isolation of Eye-Infiltrating Cells

Eyes were collected after PBS perfusion, and a cell suspension was prepared by digestion for 10 min at 37°C with collagenase (1 mg/ml) and DNase (100 µg/ml) in RPMI 1640 containing 10% FCS. The cells were washed, re-suspended in staining buffer (PBS containing 3% FCS and 0.1% sodium azide), and stained with fluorescent mAbs to identify inflammatory cells by flow cytometry.

ELISA for HMGB1 and CXCL12

Culture supernatants from retina explants or intraocular fluid, prepared as described above, were added to wells pre-coated with HMGB1 (Abcam, Cambridge, MA, USA) or CXCL12 capture Abs (R&D System, Minneapolis, MN, USA) and levels of HMGB1 or CXCL12 measured following the manufacturer's instruction.

Immunohistochemistry for CXCL12 and CXCR4

To detect expression of CXCL12 and CXCR4 on the retina, paraffin-embedded tissue slides were deparaffinized and rehydrated with xylene and 100, 95, and 80% ethanol. After antigen retrieval in a citrate-buffered solution in a boiling water bath, the tissue was blocked by incubation with 3% BSA for 1 h at room temperature, then the slides were double-stained by overnight incubation at 4°C with phycoerythrin (PE)-labeled anti-CXCL12 Ab (R&D) or anti-CXCR4 Ab (R&D) and fluorescein isothiocyanate (FITC)-labeled anti-glutamine synthetase (GS) Ab (Sigma, St. Louis, MO, USA) or anti-Iba-1 Ab (Abcam, Cambridge, MA, USA), then the nuclei were counterstained with DAPI (Sigma) and the slides examined by fluorescence microscopy.

In Vivo Treatment with AMD3100, a Specific Inhibitor of CXCR4

We followed the protocol for AMD3100 treatment previously described by Matthys et al. (14). The mice were anesthetized by i.p. injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), and a number 2002 Alzet osmotic minipump (Alza, Palo Alto, CA, USA) was implanted dorsolaterally under the skin. The pumps were filled with 5 mg of AMD3100 in 90 µl of PBS, which was delivered at a rate of 0.25 µl/h (357 µg/day) for 14 days. Groups of mice implanted with pumps containing only PBS were also included. Other untreated mice were anesthetized like the treated ones but were not implanted with pumps.

Assays for IRBP-Specific T Cell Proliferation and Cytokine Production

Nylon wool-enriched T cells prepared at 15 days after transfer of $IRBP_{1–20}$-specific T cells into B6 mice were seeded at 4×10^5 cells/well in 96-well plates and cultured at 37°C for 60 h in 200 µl of complete medium with or without the indicated concentration of $IRBP_{1–20}$ in the presence of irradiated syngeneic spleen APCs (1×10^5), and [^{3}H]thymidine incorporation during the last 8 h assessed using a microplate scintillation counter (Packard Instruments). The proliferative response was expressed as the mean cpm ± SD for triplicate samples or the proliferative stimulus index, calculated as the mean cpm for antigen-stimulated cultures/mean cpm for unstimulated controls ratio, for triplicate samples. To measure cytokine production by responder T cells, supernatants were collected 48 h after T cell stimulation and assayed for IFN-γ, IL-17, and IL-10 using ELISA kits (R&D).

Chemotaxis Assay

Splenocytes (3×10^5 cells/well) from naïve B6 mice that had either been left untreated or been incubated for 30 min with 50 µg/ml of anti-CXCR4 Ab, then washed, were added to the upper wells of 24-well Transwell micro-chemotaxis devices (5 µm pore size; Costar) and either medium with or without HMGB1 or CCL2 or supernatants from cell cultures or cocultures was added to the lower wells. In inhibitor studies, the inhibitor was added either at

the beginning of cocultures or to the coculture supernatant at the end of coculture. After 2 h, cells that had migrated to the lower wells were collected, counted, stained with antibodies against CD3 for T cells, CD11b for macrophages, or Gr-1 for granulocytes, and examined by flow cytometry. The chemotactic index was calculated as the ratio of the number of migrated cells in chemoattractant-containing wells divided by the number of migrated cells in medium-containing wells. All assays were performed in triplicate.

Statistical Analysis

Experiments were repeated at least three times. An unpaired Student's *t*-test for two sets of data, one-way or two-way ANOVA for three or more means, or the Mann–Whitney *U* test for the pathological score of uveitis was used for statistical analysis. A p value < 0.05 was considered significant.

RESULTS

HMGB1, Released as a Consequence of the Interaction between Uveitogenic T Cells and Retinal Cells, Is Critical for the Production of Chemotactic Molecules by Retinal Cells

To examine the chemotactic ability of HMGB1 released after the interaction between uveitogenic T cells and retinal cells, we collected the supernatants from cocultures of activated IRBP-specific T cells and retinal explants for an *in vitro* chemoattractant migration assay. In this assay, splenocytes from naïve B6 mice were added to the top well of a chemotaxis chamber and coculture supernatants alone or together with 8 μg/ml of anti-HMGB1 mAb, 200 μg/ml of glycyrrhizin (an HMGB1 inhibitor), or 30 μg/ml of anti-RAGE mAb was added to the lower well, then the number of cells that migrated to the lower well after 2 h was counted by flow cytometry. As shown in **Figure 1A**, HMGB1 was released into the medium of the cocultures, as shown previously in Ref. (8). These supernatants were chemoattractive for splenocytes, both alone and after addition of anti-HMGB1 Ab, glycyrrhizin, or anti-RAGE Ab (**Figure 1B**). However, when the same concentration of anti-HMGB1 Ab, glycyrrhizin, or anti-RAGE Ab was added at the beginning of the coculture of retinal explants and activated IRBP-specific T cells, the supernatants from these cocultures no longer attracted splenocytes (**Figure 1C**), showing that HMGB1 and RAGE are involved in the release of a chemotactic molecule produced during the interaction between T cells and retinal cells.

HMGB1 Alone Cannot Recruit Inflammatory Cells, Whereas Supernatants from Retinal Explants Stimulated with HMGB1 Do Recruit Inflammatory Cells

High mobility group box 1 alone is reported to have no chemotactic activity to leukocytes (15, 16), and we confirmed this in **Figure 2A**, in which no migration of splenocytes from naïve

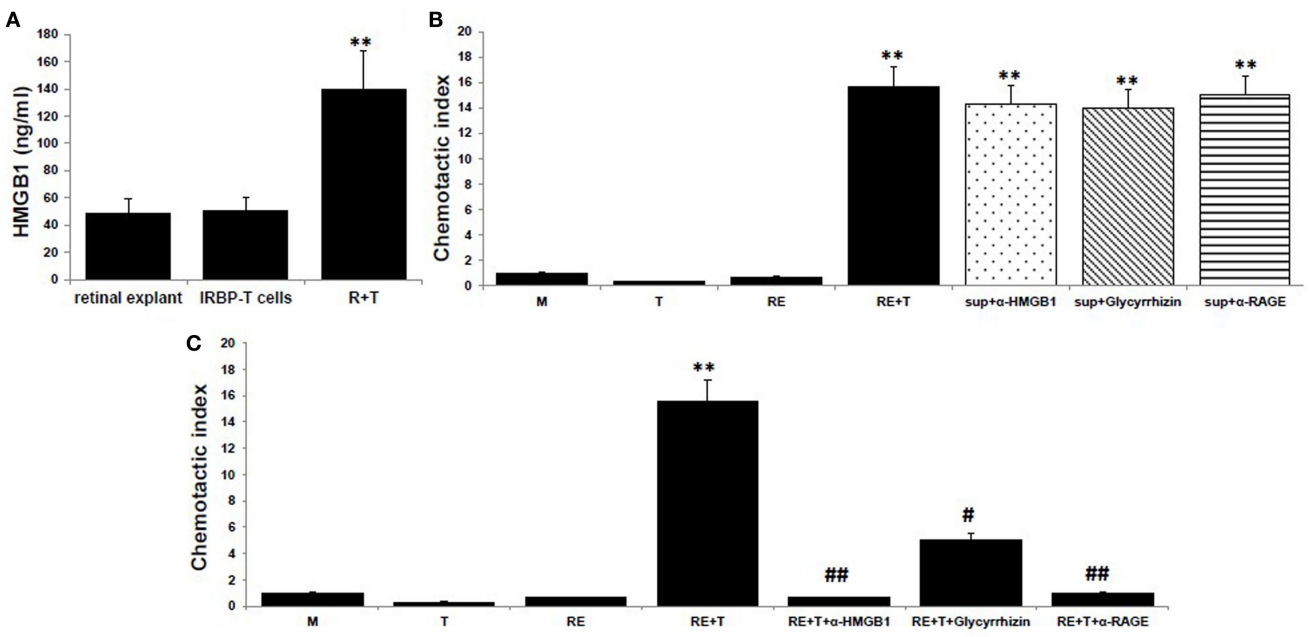

FIGURE 1 | High mobility group box 1 (HMGB1) release as a consequence of the interaction of uveitogenic T cells and retinal cells is critical for the production of chemotactic molecules by retinal cells. **(A)** Retinal explants from naïve B6 mice or activated interphotoreceptor retinoid-binding protein (IRBP)-specific T cells were cultured alone or together for 6 h, then culture supernatants were assayed for HMGB1 by ELISA. **(B,C)** Chemotactic index of medium or the supernatant from the T cells or retinal explants or the supernatant from the cocultures either alone or with anti-HMGB1 Ab (8 μg/ml), glycyrrhizin (200 μg/ml), or anti-receptor for advanced glycation end products (RAGE) Ab (30 μg/ml) added at the end of the coculture **(B)**, or at the beginning of the coculture **(C)**. The results are representative of those for three experiments. $^{**}p < 0.01$ compared to medium alone, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the coculture of retinal explants and IRBP-specific T cells in one-way ANOVA.

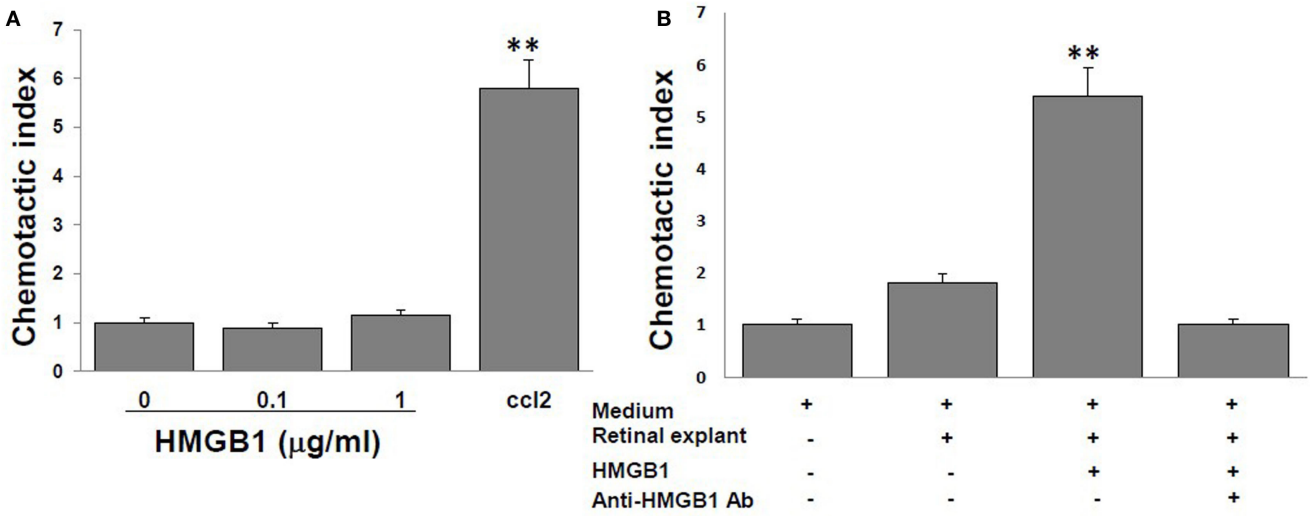

FIGURE 2 | High mobility group box 1 (HMGB1) alone cannot induce splenocyte migration, whereas supernatants collected from retinal explants stimulated with HMGB1 can. Splenocytes from naïve B6 mice were added to the top well of the chemotaxis chamber. **(A)** The chemotactic index of different concentrations of HMGB1 or 10 nM CCL21. **(B)** The chemotactic index of the medium only or supernatant from cultures of retinal explants incubated for 6 h with medium or 1 μg/ml of HMGB1 in the presence or absence of 1 μg/ml of anti-HMGB1Ab. $^{**}p < 0.01$ compared to medium alone in one-way ANOVA.

B6 mice was seen in the medium containing 0, 0.1, or 1 μg/ml of reduced form of HMGB1, contrast to that of the medium containing 10 nM CCL12, a chemoattractant for lymphocytes. However, as shown in **Figure 2B**, supernatants collected from retinal explants stimulated with 1 μg/ml of HMGB1 did attract splenocytes, while supernatants from the same cells incubated with HMGB1 and 1 μg/ml of anti-HMGB1 Ab did not. The results show that molecules released from HMGB1-stimulated retinal cells attract immune cells.

CXCL12 Is the Molecule Induced by HMGB1 That Attracts Immune Cells

The HMGB1-induced recruitment of inflammatory cells depends on CXCL12, and HMGB1 and CXCL12 form a heterocomplex that binds exclusively to CXCR4 (15–17). We therefore speculated that the chemotactic molecule released by retinal cells in the presence of HMGB1 was CXCL12. To test this, we first measured CXCL12 levels in the supernatants of retinal cells cultured alone or cocultured with IRBP-specific T cells in the presence or absence of anti-HMGB1 Ab, glycyrrhizin, or anti-RAGE Ab. As shown in **Figure 3A**, high levels of CXCL12 were found in supernatants from cocultures of IRBP-specific T cells and retinal explants, whereas only low amounts were detected in cultures of retinal explants or IRBP-specific T cells alone or in cocultures of retinal explants and IRBP-specific T cells in the presence of anti-HMGB1 Ab, glycyrrhizin, or anti-RAGE Ab, showing that both cell types were required and that these HMGB1 inhibitors either inhibited or reduced CXCL12 production by cocultured retinal cells. The *in vitro* increase in CXCL12 levels in cocultures of retinal explants and activated IRBP-specific T cells was confirmed *in vivo* in a study in which the transfer of IRBP-specific T cells to naïve mice was accompanied by AC injection of either anti-HMGB1 Ab or control Ig into each eye, then CXCL12 levels in the intraocular fluid were measured the next day. As shown in **Figure 3B**, high CXCL12 level was seen in mice that received IRBP-specific T cells and control Ig, but not anti-HMGB1 Ab.

Moreover, neutralization of CXCL12 in the coculture supernatants by anti-CXCL12 Ab (**Figure 4A**) or prior blockade of CXCR4, the CXCL12 receptor, on the responder splenocytes using anti-CXCR4 Ab (**Figure 4B**) inhibited induction of splenocyte migration by coculture supernatants.

As shown by flow cytometry in **Figures 4C–E**, coculture supernatants from IRBP-specific T cells and retinal explants were effective in attracting both $CD3^+$ T cells (**Figure 4C**) and $CD11b^+$ macrophages (**Figure 4E**), and this effect was blocked by the presence of anti-HMGB1 Ab (anti-H) during the coculture of CXCL12 release or by addition of anti-CXCL12 Ab (anti-C) to the coculture supernatants. Coculture supernatants from IRBP-specific T cells and retinal explants were not effective in attracting Gr-1-positive granulocytes (**Figure 4D**).

tEAU Was Attenuated by Blockade of CXCR4 Using AMD3100

We then examined the role of HMGB1/CXCL12 *in vivo* in tEAU by comparing severity in mice with and without treatment with the CXCR4 inhibitor, AMD3100. B6 mice were implanted with osmotic minipumps releasing AMD3100 at a rate of 357 μg/day for 14 days (see Materials and Methods), a treatment regime used in a study in which AMD3100 successfully inhibited induction of collagen-induced arthritis in mice (14). Two groups of control mice were included, one implanted with pumps containing PBS (PBS pump) and one with no implant (Ctrl). One day later, all mice were injected with IRBP-specific

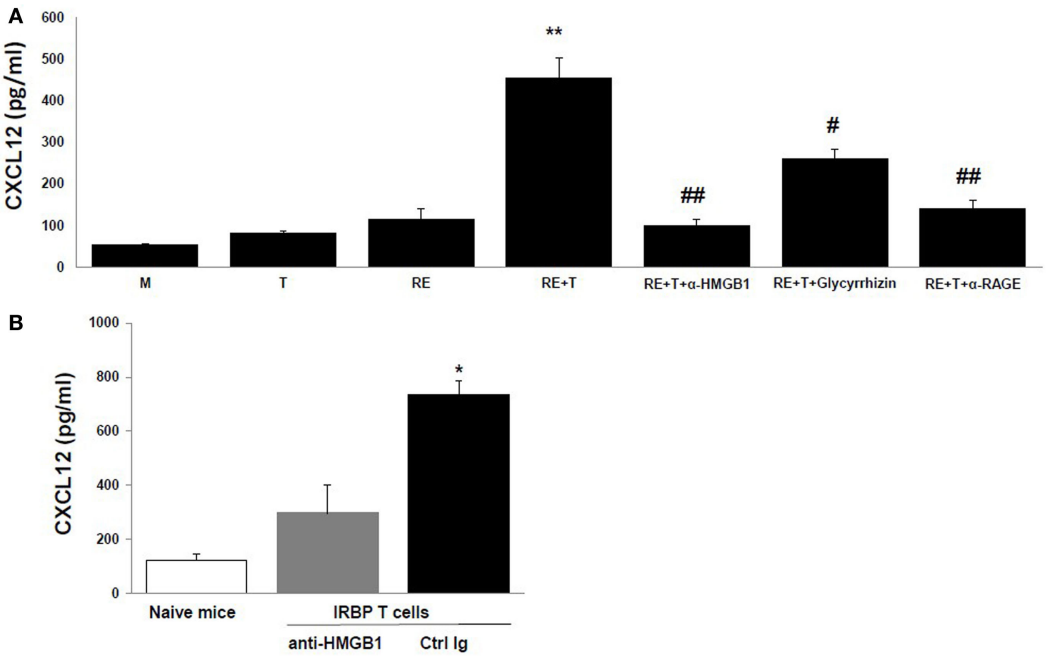

FIGURE 3 | High mobility group box 1 (HMGB1) induces retinal cells to secrete CXCL12. **(A)** Medium or the supernatant from the T cells or retinal explants or the supernatant from the cocultures alone or with addition of anti-HMGB1 Ab, glycyrrhizin, or anti-receptor for advanced glycation end products (RAGE) Ab at the same doses as **Figure 1B** at the beginning of the coculture was assayed for CXCL12 by ELISA. $^{**}p < 0.01$ compared to medium alone, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the coculture of retinal explants and IRBP-specific T cells in one-way ANOVA. **(B)** Naïve B6 mice were left untreated or were adoptively transferred with activated $IRBP_{1-20}$-specific T cells (5×10^6 cells/mouse) and 1 μg of either anti-HMGB1 Ab or control Ig was injected into the anterior chamber of each eye. Next day, the intraocular fluid was collected and CXCL12 levels measured. The data are the mean ± SD for eight eyes per group. $^{*}p < 0.05$ compared to the anti-HMGB1 Ab-treated eye in one-way ANOVA.

T cells to induce tEAU, then were examined for clinical signs of intraocular inflammation. Compared with either set of controls, the AMD3100-treated mice developed much milder ocular inflammation, as determined by the clinical score (**Figure 5A**). Histological evaluation on day 15 post-transfer of the control group and the AMD3100 pump group showed a well preserved retinal structure and minimal vitreous infiltrate in the AMD3100-treated mice (**Figure 5B**).

To determine which leukocyte subsets were dominant in tEAU and blocked by AMD3100, we analyzed eye-infiltrating cells in control- and AMD3100-treated mice on day 8 after disease induction by staining pooled single eye cells from each group with a panel of antibodies specific for different leukocytes and flow cytometric analysis. As shown in **Figures 5C,D**, AMD3100-treated mice showed a profound reduction in infiltrating cells. The percentage of αβ T cells dropped from 23.7% in the disease controls to 8.4% in mice treated with AMD3100, and the percentage of $CD11b^{+}Gr\text{-}1^{-}$ monocytes/macrophages decreased from 8.4 to 2%. In addition, the percentage of $CD11b^{+}$ $Gr\text{-}1^{+}$ neutrophils was dramatically reduced (11 versus 1.3%) (**Figure 5C**). Moreover, the absolute number of infiltrating cells in each eye of AMD3100-treated mice was significantly reduced compared to the untreated control eye (**Figure 5D**). At the day 8 post-T cell transfer, there were not many infiltrating $CD11C^{+}$ dendritic cells and $CD19^{+}$ B cells detected in both control and treated eyes.

Furthermore, IRBP-specific responses of T cells were significantly reduced after treatment with AMD3100. Significant lower IRBP-specific T cell proliferation (**Figure 6A**) and IFN-γ and IL-17 release (**Figure 6B**) in response to stimulation with $IRBP_{1-20}$ were seen with T cells from mice treated with AMD3100. However, no significant difference in IL-10 release was seen between AMD3100-treated and control mice, as we previously reported using anti-HMGB1Ab injection (8). To examine the reduced proliferation of lymphocytes a direct effect of CXCR4 inhibition or an indirect effect on antigen-presenting cells, we performed cross tests in which T cell proliferation was measured using all four combinations of responder T cells and APCs from $IRBP_{1-20}$-specific T cell transferred and AMD3100-treated or disease control mice. T cells from AMD3100-treated mice did not respond to increasing doses of $IRBP_{1-20}$ Ag in the presence of APCs from either AMD3100-treated or disease control mice (**Figure 6C**), whereas T cells from control mice reacted well in the presence of APCs from control mice, but not AMD3100-treated mice (**Figure 6D**), indicating that dysfunction of both T cells and APCs contributed to the T cell hyporesponsiveness in AMD3100-treated mice.

AMD3100 Inhibits the Increase in CXCL12 and CXCR4 Expression in the Eye in tEAU

Having demonstrated CXCL12 release by retinal cells after stimulation with HMGB1 or activated IRBP-specific T cells

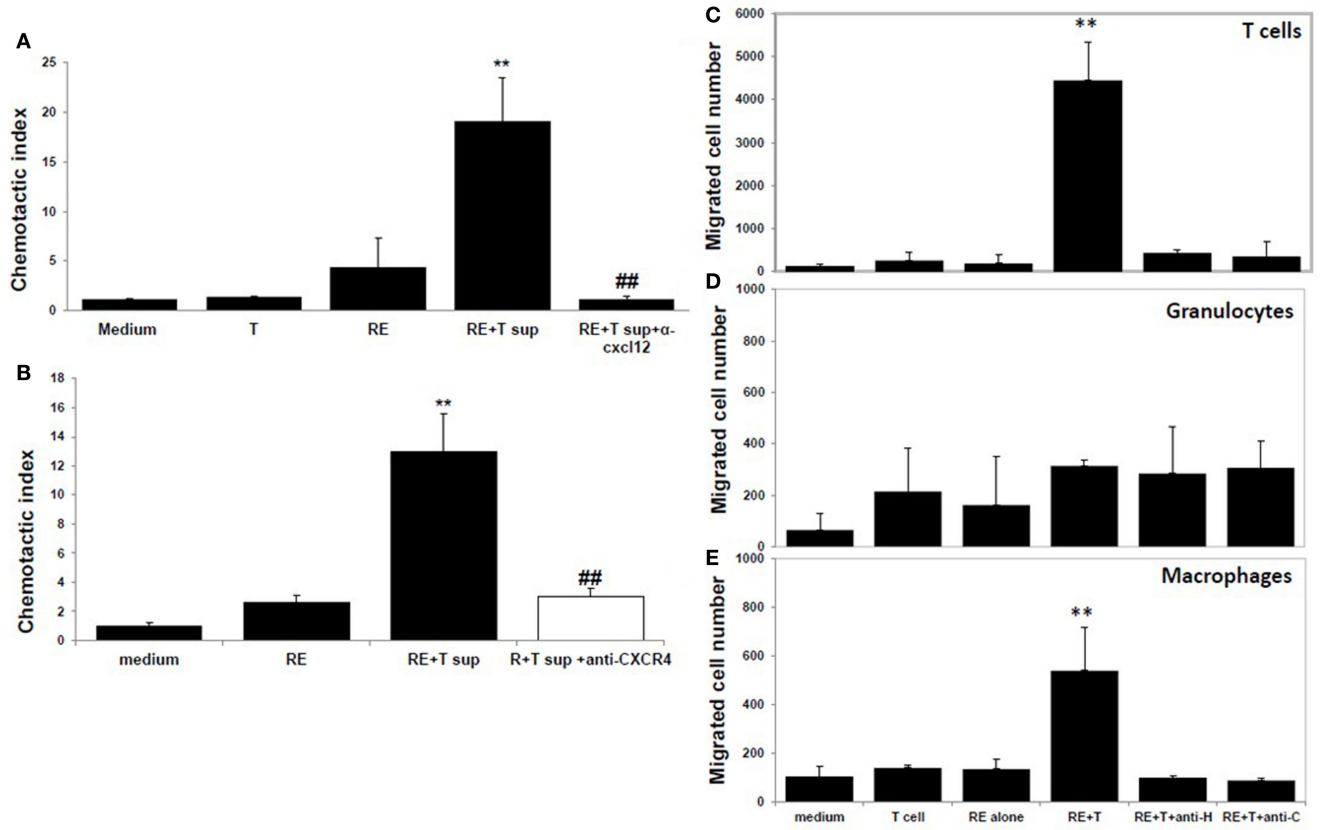

FIGURE 4 | **CXCL12 is the high mobility group box 1 (HMGB1)-induced molecule that attracts T cells**. **(A)** Chemotactic index of medium or the supernatant from the T cells or retinal explants or the supernatant from the cocultures either alone or with addition of anti-CXCL12 Ab (5 μg/ml) at the end of the coculture. **(B)** Splenocytes were incubated with or without anti-CXCR4 Ab (50 μg/ml) for 30 min, then were washed and used in the migration assay as in panel **(A)**. $^{**}p < 0.01$ compared to medium alone; and $^{\#\#}p < 0.01$ compared to the coculture supernatants of retinal explants and interphotoreceptor retinoid-binding protein-specific T cells, in one-way ANOVA. **(C–E)** Medium or the supernatant from the T cells or retinal explants or the supernatant from cocultures in the presence or absence of 1 μg/ml of anti-HMGB1 (anti-H) for 6 h or supernatant from cocultures to which anti-CXCL12 Ab (anti-C; 5 μg/ml) was added after coculture was used in the migration assay and the cells that migrated into the lower chamber were stained with anti-CD3 Ab **(C)**, Ly6G Ab **(D)**, or CD11b Ab **(E)** and analyzed by flow cytometry. $^{**}p < 0.01$ compared to medium alone in one-way ANOVA.

in vitro (**Figure 3**), we examined *in vivo* CXCL12 expression in naïve mice and in mice on day 15 after transfer of IRBP-specific T cells with or without AMD3100 treatment. As shown in **Figure 7A** (left), immunochemical staining for CXCL12 (red stain, first row, center panel) showed that CXCL12 was constitutively expressed on the retina, especially in the ganglion cell layer (GCL), inner plexiform layer (IPL), and outer plexiform layer (OPL). At the peak of disease (d15 post-T cell transfer; middle row), the retina became thinner and CXCL12 expression was increased in the neuroretina in all regions from the inner nuclear layer to the outer boundary of the outer nuclear layer. However, tEAU mice treated with AMD3100 (bottom row) showed similar CXCL12 expression to naïve mice (first row). Staining for astroglial cells using the marker GS (green stain) showed that not many GS cells expressed CXCL12 in naïve mice (first row, right panel), while during inflammation, more GS cells expressed CXCL12 (indicated by arrows, middle row, right panel), which was markedly reduced by AMD3100 treatment (bottom row, right panel). Our results suggest that CXCL12 may play a role in the maintenance of retinal cells in specific layers in the adult retina.

We also examined expression of the specific CXCL12 receptor, CXCR4, in the eye before, or after, 15 days of IRBP-specific T cell transfer with or without AMD3100 treatment. As seen in **Figure 7B**, no CXCR4 signal (red stain) was seen before tEAU induction (first row, center panel) indicating no endogenous expression of CXCR4 in the eye of naïve mice. However, CXCR4 was detected in the infiltrating cells at the peak of disease (middle row, center panel) and significantly reduced by AMD3100 treatment (bottom row, center panel). Staining for ionized calcium-binding molecule 1 (IBa1-1), a marker for macrophages/microglia, was markedly increased at the peak of tEAU (green, middle row, left panel) and some Iba-1-expressing cells also expressed CXCR4 (indicated by arrows, middle row, right panel), and both effects were abolished in the AMD3100-treated mice (bottom row).

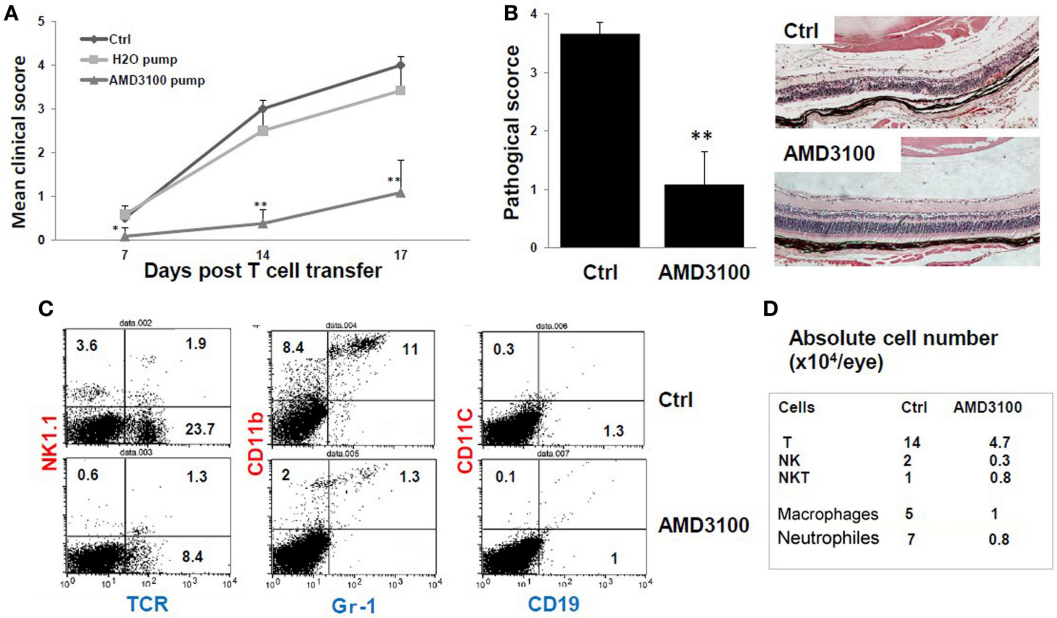

FIGURE 5 | **The CXCR4 inhibitor AMD3100 significantly inhibits tEAU**. Groups of mice were implanted with pumps filled with 5 mg of AMD3100 in 90 µl of PBS (AMD3100 pump) or PBS alone (PBS pump) 1 day before interphotoreceptor retinoid-binding protein (IRBP)-specific T cell transfer as described in Section "Materials and Methods" and examined weekly for clinical score **(A)** or on day 15 for **(B)**. A group of mice was left untreated as positive controls (Ctrl). **(A)** The mean clinical score for all three groups and **(B)** the pathological score for the indicated groups ($n = 12$ mice) presented as the mean ± SE. Hematoxylin and eosin; original magnification, ×100. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the Ctrl group using Mann–Whitney U test. **(C)** Identification of infiltrating leukocytes in the eye of day 8 post-$IRBP_{1-20}$-specific T cell-injected mice treated with pumps containing AMD3100 or PBS (Ctrl group). A suspension of single ocular cells for each group was pooled (total five mice in control group, and four in treated group), stained with the indicated fluorescein isothiocyanate- and phycoerythrin-conjugated antibodies and analyzed by flow cytometry. The percentage of positive cells is indicated. **(D)** Summary of infiltrative leukocyte subsets, from each eye is shown. Cells recovered from eyes of each group were counted after trypan blue staining. The number of cells was calculated based on the percentage of subsets as determined by flow cytometry.

DISCUSSION

We have previously reported that HMGB1 is detected in culture supernatants as early as 2 h of coculture of retinal explants and activated IRBP-specific T cells and at 1 day after adoptive transfer of activated IRBP-specific T cells, and blockade of HMGB1 using antagonists reduces ocular inflammation and suppresses uveitogenic T cell function (8, 9). However, how HMGB1 is involved in the pathogenesis of effector phase of uveitis is not clear.

It was recently demonstrated that HMGB1 is an enhancer of the activity of CXCL12 in stimulating the migration of mouse embryonic fibroblasts (15, 16). Our study further supports this and explores the synergistic roles of HMGB1 and CXCL12 in the infiltration of inflammatory cells at a very early stage of intraocular inflammation induced by uveitogenic autoreactive T cells. Our results in **Figures 1** and **3** show that interaction of uveitogenic T cells with retinal cells initiated release of HMGB1 and CXCL12 by retinal cells. CXCL12 release was dependent on HMGB1, since CXCL12 was not released from retinal cells in the presence of an anti-HMGB1 Ab (**Figure 3**). This process is very important for the subsequent role of inflammatory migration. Once the complex forms, the migration-stimulating activity of the supernatant was not affected by neutralization of HMGB1 by anti-HMGB1 Ab or glycyrrhizin (**Figure 1B**), a glycoconjugated triterpene produced by the licorice plant *Glycyrrhiza glabra*, which has been reported to inhibit the chemoattractant and mitogenic activities of HMGB1 on 3T3 fibroblasts and binds to both HMG box domains (BoxA and BoxB) (18). We previously showed that injection of mice with HMGB1 antagonists on the same day as IRBP-specific T cell transfer inhibits uveitogenic T cell-induced intraocular inflammation, but not when given later, indicating that HMGB1-induced CXCL12 release might be an early event initiated by infiltrating effector T cells (8).

It is not known how these two molecules are structurally associated. Nuclear magnetic resonance chemical-shift mapping indicates contacts between CXCL12 and full-length HMGB1 and its individual HMG boxes (17). HMGB1 binds to CXCL12 monomers or promote binding of CXCL12 to CXCR4 by fixing the N-terminal domain of CXCL12 in the best conformation for triggering CXCR4 signaling (16). Fluorescence resonance energy transfer studies with tagged CXCR4 have indicated that the conformation of CXCR4 dimers interacting with the HMGB1/CXCL12 heterocomplex is different from the conformation of those interacting with CXCL12 alone (17).

Receptor for advanced glycation end products has been shown to be the receptor responsible for HMGB1-induced migration (19). Our study showed that RAGE was required

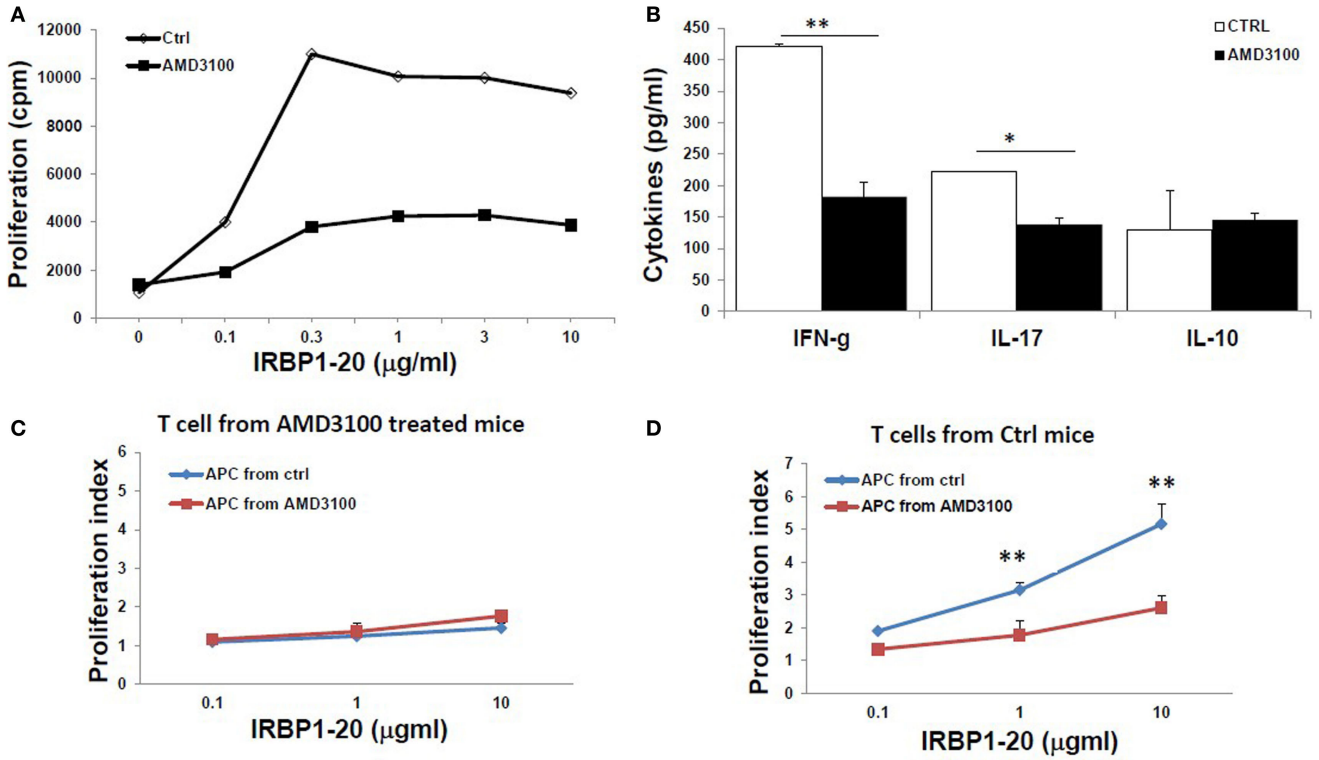

FIGURE 6 | Reduced responses of interphotoreceptor retinoid-binding protein (IRBP)-specific T cells in AMD3100-treated mice. **(A,B)** T cells from tEAU mice treated with AMD3100 or the Ctrl group were collected on day 15 after T cell transfer and cultured with irradiated antigen-presenting cells (APCs) and increasing doses of $IRBP_{1-20}$, then proliferation of responding T cells **(A)**, and levels of IFN-γ, IL-17, and IL-10 **(B)** released into the culture supernatants were measured. **(C,D)** Responder T cells prepared at day 8 post-$IRBP_{1-20}$-specific T cell-injected mice treated with AMD3100 [**(C)**, five mice] or control groups [**(D)**, four mice] were incubated with increasing doses of $IRBP_{1-20}$ in the presence of irradiated splenic APCs from either AMD3100- or control-treated mice and their proliferation measured. *$p < 0.05$ and **$p < 0.01$ compared to the Crtl group in Student's *t*-test.

for HMGB1-induced CXCL12 release from retinal cells and probably for the formation of the HMGB1/CXCL12 complex, but not for inflammatory cell migration *per se*, since HMGB1-induced CXCL12 release was inhibited by anti-RAGE mAb (**Figure 1C**), whereas HMGB1/CXCL12-induced migration of splenocytes was not (**Figure 1B**). The reason why a previous study found that HMGB1-induced chemotaxis of rat smooth muscle cells (SMCs) was inhibited not only by anti-HMGB1 Abs but also by anti-RAGE Abs (20), might be that anti-RAGE Abs inhibit HMGB1-induced CXCL12 release from SMCs. Our results are also compatible with those reported by Schiraldi et al. (17), who showed that, *in vitro*, CXCL12 expression and release were increased in HMGB1-stimulated mouse fibroblasts from wild-type B6 mice, but not from RAGE-deficient ($RAGE^{-/-}$) mice, whereas migration of bone marrow cells induced by a combination of HMGB1 and CXCL12 was similar in both sets of mice, while, *in vivo*, absence of RAGE in C57BL/6 mice did not prevent, but actually promoted, recruitment of monocytes into the injured muscle, excluding the possibility that inflammatory cells were recruited through RAGE signaling. They argued that the role of RAGE in cell migration is to trigger CXCL12 transcription (21) so that CXCL12 production is increased and sustained over time and that RAGE is no longer needed when sufficient CXCL12 is produced or present.

We found that CXCL12 was constitutively expressed in the GCL, IPL, and OPL of the mouse retina (**Figure 7A**). However, its role in the normal retina is not known. Since it is chemotactic for CXCR4 positive immune cells such as T cells and monocytes, it might be actively involved in intraocular inflammation. CXCR4 has been implicated in ocular leukocyte trafficking in an ovalbumin-induced acute uveitis model in DO11.10 mice with CD4+ T cells genetically engineered to react with ovalbumin (22). In addition, it might promote endothelial cell migration and enhance angiogenesis, the latter effect being supported by reports that CXCL12 promotes angiogenesis in the diabetic retina in rats (23) and oxygen-induced retinopathy in mice (24).

Most adoptively transferred disease-inducing T cells require "licensing for pathogenicity" in the lung and other organs in order to induce disease in target organs (23, 25)—"a hub-and-spoke pattern" (24). Both "licensing for pathogenicity" and HMGB1 production might take place during the induction of the effector phase of EAU. Release of intraocular HMGB1 and CXCL12 on day 1 after cell transfer shown in our previous

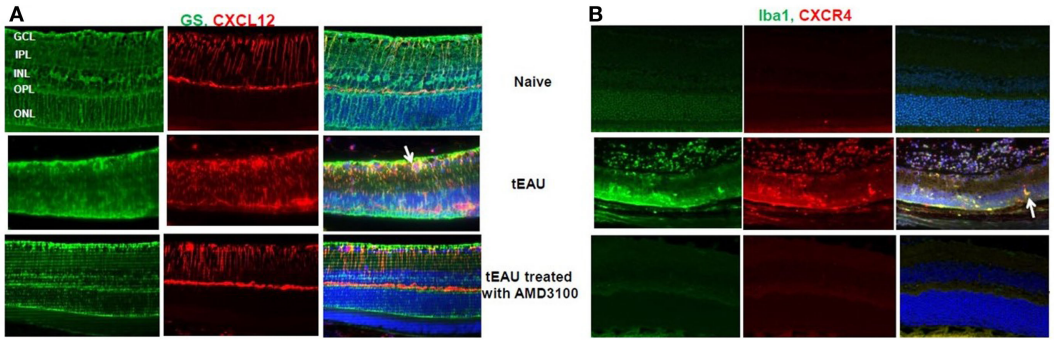

FIGURE 7 | **CXCL12 and CXCR4 expression in retinas**. Expression of CXCL12 **(A)** and CXCR4 **(B)** was examined on paraffin-embedded sections of retina from naïve mice and from mice on day 15 after interphotoreceptor retinoid-binding protein-specific T cell transfer treated with or without AMD3100 by staining with phycoerythrin-conjugated anti-CXCL12 Ab [red in panel **(A)**] or anti-CXCR4 Ab [red in panel **(B)**] and fluorescein isothiocyanate-conjugated anti-glutamine synthetase (GS) Ab [green in panel **(A)**] or anti-Iba-1 Ab [green in panel **(B)**]. The right columns show the fused image. Cell nuclei are stained blue with DAPI.

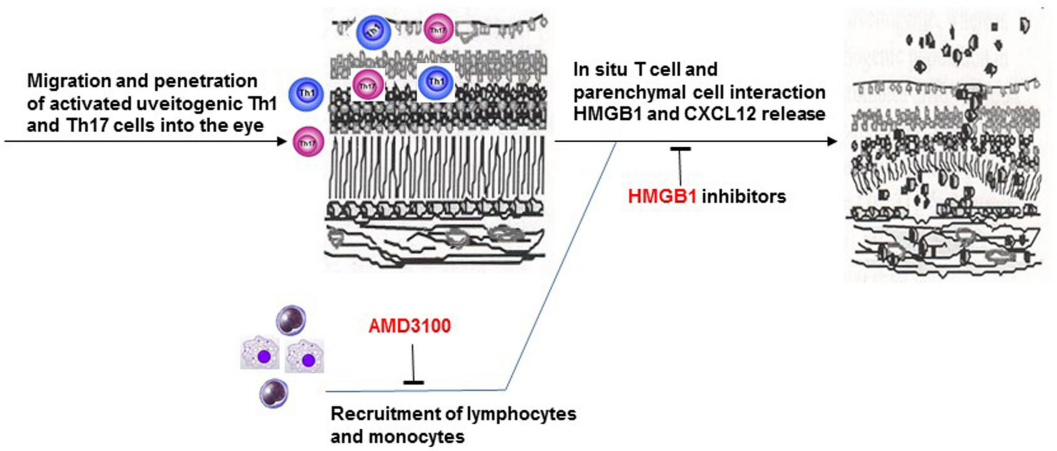

FIGURE 8 | **Brief summary of the mechanism of HMGB1-CXCL12 in the pathogenesis of effector phase of EAU**.

study (8) might attract "licensed" pathogenic T cells to the eye, and the later HMGB1 increase might be related to the involvement of these T cells and the damage they invoke in the retina. AMD3100 treatment might block the migration of licensed pathogenic T cells to the eye, leading to T cell apoptosis due to lack of reactivation in the target organ. Moreover, as shown in this study, AMD3100 treatment reduced the subsequent intraocular cascading and retina damage (**Figures 5** and **7**), resulting in a reduced frequency of naïve T cells activated by uveitogenic antigens released from the damaged retina, as demonstrated by low proliferative and cytokine production responses *in vitro* by IRBP-specific T cells from AMD3100-treated mice (**Figure 6**). Results in **Figures 6C,D** showed that AMD3100-treated APCs could not present antigens to activate T cells from controlled mice, indicating that low responsiveness of specific T cells is partially due to the reduced antigen-presenting functions of APCs such as monocytes/macrophages. Blockade of CXCR4 on APCs probably reduces the expression of positive costimulatory molecules, which is an interesting observation and needs to be further investigated.

In summary, our study demonstrates synergistic roles of HMGB1 and CXCL12 in the infiltration of inflammatory cells at a very early stage of intraocular inflammation induced by uveitogenic autoreactive T cells. In our animal model, early blockade of released HMGB1 decreases CXCL12-induced leukocyte migration into the eye and subsequent intraocular inflammation and photoreceptor cell damage (**Figure 8**).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of University of Louisville. Institutional approval was obtained and institutional guidelines regarding animal experimentation were followed.

AUTHOR CONTRIBUTIONS

GJ and JY performed the experiments and generated and analyzed the data; YW and TX assisted with the experiments and the data analysis; YZ, DS, and HK helped with the design of the experiments and edited the manuscript; and HS directed the study, planned experiments, interpreted the data, and wrote the manuscript. All authors read and approved the final manuscript.

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Systemic HMGB1 Neutralization Prevents Postoperative Neurocognitive Dysfunction in Aged Rats

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Postoperative neurocognitive disorders are common complications in elderly patients following surgery or critical illness. High mobility group box 1 protein (HMGB1) is rapidly released after tissue trauma and critically involved in response to sterile injury. Herein, we assessed the role of HMGB1 after liver surgery in aged rats and explored the therapeutic potential of a neutralizing anti-HMGB1 monoclonal antibody in a clinically relevant model of postoperative neurocognitive disorders. Nineteen to twenty-two months Sprague-Dawley rats were randomly assigned as: (1) control with saline; (2) surgery, a partial hepatolobectomy under sevoflurane anesthesia and analgesia, + immunoglobulin G as control antibody; (3) surgery + anti-HMGB1. A separate cohort of animals was used to detect His-tagged HMGB1 in the brain. Systemic anti-HMGB1 antibody treatment exerted neuroprotective effects preventing postoperative memory deficits and anxiety in aged rats by preventing surgery-induced reduction of phosphorylated cyclic AMP response element-binding protein in the hippocampus. Although no evident changes in the intracellular distribution of HMGB1 in hippocampal cells were noted after surgery, HMGB1 levels were elevated on day 3 in rat plasma samples. Experiments with tagged HMGB1 further revealed a critical role of systemic HMGB1 to enable an access to the brain and causing microglial activation. Overall, these data demonstrate a pivotal role for systemic HMGB1 in mediating postoperative neuroinflammation. This may have direct implications for common postoperative complications like delirium and postoperative cognitive dysfunction.

Keywords: aging, HMGB1, inflammation, memory, microglia, surgery

INTRODUCTION

Postoperative neurocognitive disorders, encompassing postoperative delirium (POD) and postoperative cognitive dysfunction (POCD), are common complications in elderly patients over 65 years of age following surgery or critical illness (1). Up to 47% of elderly patients experience cognitive dysfunction at hospital discharge and about 10% retain persistent cognitive deficits 3 months after major

surgery (2). Occurrence of these postoperative complications significantly associate with increased morbidity and mortality, greater length of hospital stay, increased costs, and decreased life independence (3). These outcomes, combined with the constant increase in the geriatric population, render postoperative neurocognitive disorders a significant complication that currently lacks defined mechanisms and treatments.

It is well appreciated that sterile injury, including surgical trauma, triggers a milieu of factors that ultimately contribute to the inflammatory process (4). Damage-associated molecular pattern molecules (DAMPs), in particular high mobility group box 1 (HMGB1), are rapidly released after tissue trauma and mediate immune cell recruitment and activation, cytokine release, and cell death (5). This molecule is highly preserved through evolution and is 99% identical between mammals (6). HMGB1 release has been described in preclinical models of cognitive decline (7–9). However, its contribution to postoperative neuroinflammation remains unclear. Increased levels of HMGB1 after surgery have been related to compromising the blood–brain barrier (BBB) and subsequent neuroinflammation (10, 11). Recently, changes in serum HMGB1 and other pro-inflammatory cytokines were positively associated with human POCD after gastrointestinal surgery in elderly patients (12).

Based on the above, we assessed the role of HMGB1 after liver surgery in aged rats and the therapeutic potential of a neutralizing anti-HMGB1 monoclonal antibody (mAb) as a mean to prevent postoperative neurocognitive disorders. Herein, we show a prominent role for systemic HMGB1 signaling to mediate neuroinflammation and neurotoxicity. Anti-HMGB1 mAb treatment increased phosphorylation of cyclic AMP response element binding (p-CREB) in the hippocampus after surgery, suggesting that anti-HMGB1 exerts neuroprotective effects in aged rats. These effects on neuroinflammation and synaptic plasticity may inform on pathophysiological mechanisms of delirium and other postoperative neurocognitive disorders appearing after surgery.

MATERIALS AND METHODS

Animals

Experiments were performed in accordance with the guidelines for experimental animal use of the Central South University. The protocol [LLSC(LA)2015-003] was approved by the ethics committee of the third Xiangya Hospital of Central South University. Aged female Sprague-Dawley (SD) rats (19–22 months, 450–600 g) were purchased from Central South University (China). Rats had surgery in the diestrus phase when estrogen levels are at their minimum. All rats were housed in standard cages with free access to food and water.

Drug Administration

Aged rats were randomly divided into 3 groups: (1) control with intravenous (i.v.) saline only; (2) surgery + immunoglobulin (IgG) (S + IgG) (1 mg/kg i.v.); (3) surgery + anti-HMGB1 (S + anti-HMGB1) (1 mg/kg i.v.). Treatments were given *via* tail vein immediately before surgical incision and 6 h after surgery; dosage and timing was based on Okuma et al. (13). Anti-HMGB1 antibody (2G7, mouse IgG2b) was supplied by Dr. H.E. Harris's laboratory, Stockholm, Sweden. This antibody has been extensively characterized previously with respect to its HMGB1 neutralizing activity in *in vitro* and *in vivo* studies (9, 14–18). The 2G7 anti-HMGB1 mAb neutralizes both HMGB1-induced cytokine/chemokine release and chemotactic activities. Mouse IgG2b (Sigma, M1395-5MG) was used as an isotype control. A separate cohort of rats was divided into 4 groups: (1) control saline alone; (2) surgery with saline; (3) surgery with Histidine (His)-tagged HMGB1 (1 mg/kg); (4) His-tagged HMGB1 only (1 mg/kg). His-tagged HMGB1 (Sigma, cat. Number 4652) or saline was given *via* tail vein right before surgical incision.

Partial Hepatolobectomy

Rats were rapidly induced with 5% sevoflurane anesthesia (Maruishi Pharmaceutical Co., Ltd., Japan) with high flow of oxygen (6 L/min). As each rat achieved loss of righting reflex, it was intubated with a 14G catheter and anesthesia maintained with continuous delivery of 3.5–4.5% sevoflurane mixed with oxygen (80–85%). The gas was monitored and analyzed by a multi-function monitor (Datex-Ohmeda, Helsinki, Finland); respiratory rate (R), $PetCO_2$, FiO_2, and Fi_{Sev} were continuously recorded. The depth of anesthesia was modulated according to the R (30–50/min) and the body movement of the rats. The partial hepatolobectomy was performed as previously described with some modifications (19). Briefly, an incision about 2 cm long was made below the xyphoid; the left lobe of liver was carefully isolated, ligated, and then removed. Finally, muscles and skin were closed with sterile sutures and subcutaneous tissue infiltration with 0.2 mL of 0.25% bupivacaine was administered for the purpose of local postoperative analgesia. Animals were then allowed to recover for further testing.

Behavioral Tests

Barnes Maze

Rats were tested with a protocol previously described (20). Briefly, rats were trained to locate the escaping hole on a Barnes maze four times/day on postoperative days 1–4 (3 min/trial and 15 min between each trial). The number of incorrect hole investigation (termed error) during each trial were recorded. The platform surface was cleaned with 75% ethanol before each trial in order to remove odor cues.

Open Field

Open field test was used to evaluate the anxiety level of the animal. A rat was placed directly into the center of the open field (100 cm × 100 cm × 48 cm, length × width × height). Movement of the rat in the open field was recorded by a digital camera during the 5-min testing session. The total square crossings, time spent in the central area and the percentage of square crossings in the central area to the total square crossings, were counted.

Immunostaining

Rats were terminated with chloral hydrate (10%) and perfused transcardially with ice cold 0.01M phosphate-buffered saline

(PBS). The brain was rapidly dissected; one hemisphere was used for immunostaining and the other for western blot. The hemisphere used for immunostaining was postfixed in 4% paraformaldehyde overnight at 4°C. After dehydrating with 15% and 30% sucrose, the brains were embedded in OCT (SAKURA Tissue-Tek, USA), and cryostat transverse sections of the brain (20 μm) were obtained. Four sections of hippocampus were randomly picked from 4 sets of serial sections from each rat at −3.6 to −4.16 mm anteroposterior to the bregma for immunostaining. Sections were washed three times in 0.01M PBS and incubated in 3% H_2O_2 for 10 min. After three washes, sections were blocked with 5% BSA in 0.01M PBS plus 0.1% Triton X-100 for 1 h at room temperature and then incubated with primary antibodies (1:1000, Iba-1, HMGB1, p-CREB) at 4°C overnight. Full details on the antibodies used are presented in **Table 1**. After washing three times in 0.01M PBS, the sections were incubated with secondary antibodies (Biotinylated Goat Anti-Rabbit IgG, 1:200, Vector, USA) for 1 h at room temperature, subsequently washed three times in 0.01M PBS and finally covered with permount containing DAPI (Vector, USA, H-1200). For each staining, pictures of Cornu Ammonis (CA)1 and Dentate Gyrus (DG) areas were taken under the same magnification (40× objective lens) and constant light intensity with a microscope (DS-Ri1, Nikon, Japan) by an author blinded to treatments.

Western Blot

Western blotting was used to assess the expression of *N*-methyl-D-aspartate receptor (NMDAR) subunits NR2A and NR2B, HMGB1, and GAPDH in the hippocampus. Briefly, frozen hippocampus was homogenized in lysis buffer containing protease inhibitors cocktails (Roche, Germany, cat num P8340) and phenylmethanesulfonylfluoride (PMSF, Sigma, USA, cat num p7626). The quantity of protein of samples was determined using a BCA protein assay kit (CWBio, China) according to the manufacturer's instructions. Equal amounts of protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 10% skim milk in TBST buffer for 1 h and then incubated with primary antibodies, HMGB1 (1:1000 Abcam, USA), NR2A (1:2000 Epitomics, USA), NR2B (1:1000 Abcam, USA), GAPDH (1:2000 Proteintech, China), and GAPDH (1:2000 Proteintech, China), anti-His antibody (1:5000 Sigma) overnight at 4°C (**Table 1**). After three washes, membranes were incubated with the secondary antibodies (Goat Anti-Rabbit IgG, HRP Conjugated, 1:2000, CWBIO, China, catalog number CW0103, Goat Anti-Mouse IgG, HRP Conjugated, 1:2000, CWBIO, China, catalog number CW0102) at room temperature for 2 h. Finally, visualization of the proteins was accomplished by enhanced chemiluminescence detection kit (Pierce; Thermo Scientific, Shanghai, China), and the intensity of each band was quantified by densitometry. Relative expression levels of protein were normalized by the ratio of target protein (HMGB1, NR2A, and NR2B) to GAPDH.

Enzyme-Linked Immunosorbent Assay

Under anesthesia, blood was collected from the right auricle in EDTA coated tubes and then centrifuged for 10 min at 3000 rpm. Plasma was collected and frozen at −80°C for further analyses. Human plasma was collected under an approved protocol by the Ethics Committee of the Third Xiangya Hospital of Central South University (protocol number: 2015-S165). Six patients identified with POD by The Confusion Assessment Method (CAM) (21) daily testing were compared to age-matched surgical controls (non-delirious). Blood was centrifuged at 3000 rpm for 10 min at room temperature, and the plasma samples were immediately frozen at −80°C. Concentration of HMGB1 in plasma was measured using ELISA (IBL International, Germany, catalog number ST51011) according to the manufacturer's protocol.

Statistical Analysis

Data are shown as mean ± SEM. Two-way repeated measures ANOVA was used to analyze the data from Barnes Maze test. ANOVA was used to analyze the data from open field test, Western blot, and immunostaining. Bonferroni Multiple Comparison Test was performed to compare selected groups when ANOVA showed significance. Statistical analysis was performed using Prism 5 (Graph Pad Software Inc., La Jolla, CA, USA). Significance was set at $p < 0.05$.

RESULTS

Blocking HMGB1 Improves Postoperative Cognitive Decline and Anxiety in Aged Rats

All rats were included in the study protocol, and no mortality was reported after the surgical procedure. We evaluated learning

TABLE 1 | List of antibodies used in the study.

Antibody	Host	Antigen	Supplier/cat. num.	Concentration
HMGB1	Rabbit/monoclonal	Synthetic peptide corresponding to Human HMGB1 aa 150 to the C-terminus	Abcam, ab79823	1:1000
p-CREB	Rabbit/monoclonal	Synthetic phoshopeptide corresponding to residues surrounding Serine 133 of human CREB	Abcam, ab32096	1:1000
Iba1	Rabbit/polyclonal	Synthetic peptide corresponding to C-terminus of Iba1	Wako, 019-19741	1:1000
NR2A	Rabbit/monoclonal	Synthetic peptide corresponding to Human NMDAR2A	Epitomics, 3916-1	1:2000
NR2B	Rabbit/polyclonal	Synthetic peptide conjugated to KLH derived from within residues 1450 to the C-terminus of Rat NMDAR2B	Abcam, ab65783	1:1000
His	Mouse/monoclonal		Sigma, H1029	1:5000
GAPDH	Rabbit/polyclonal	full-length GAPDH of human origin	Proteintech, 10494-1-AP	1:2000

and memory by error number in the Barnes maze and detected levels of anxiety in the open field. In this test, repeated measures ANOVA showed that the error number was significantly affected by treatment [$F_{(2,69)} = 5.73$, $p = 0.01$], measure time [$F_{(3,69)} = 36.79$, $p < 0.001$], and the interaction of treatment and measure time [$F_{(6,69)} = 3.41$, $p = 0.005$]. Compared to the control group, the errors of the surgery group treated with control antibodies (S + IgG group) were significantly increased on days 2 and 3 after surgery, indicating an impairment in learning and memory of aged rats after surgery ($p = 0.001$, respectively) (**Figure 1A**). Compared to the S + IgG group, errors in S + anti-HMGB1 group were significantly decreased on days 2 ($p = 0.035$) and 3 after surgery ($p = 0.023$), denoting a protective effect by perioperative anti-HMGB1 treatment (**Figure 1A**).

In addition, in the open field test, the percentage of square crossing in the central area in the S + IgG group was significantly lower than that of control group on day 3 after surgery (7.88 ± 1.74 vs. 16.68 ± 0.79%, $p < 0.05$) (**Figure 1B**). Percentage square crossing in the central area in S + anti-HMGB1group significantly increased relative to the S + IgG group (16.79 ± 4.53 vs. 7.88 ± 1.74%, $p < 0.05$) (**Figure 1B**).

Effects of Anti-HMGB1 on Memory Marker p-CREB

To further investigate the mechanisms of anti-HMGB1 in preventing surgery-induced memory decline, we assessed the transcription factor CREB, which is closely involved in synaptic plasticity and memory function (22). Compared to the control group, the level of p-CREB in the DG area of the S + IgG group was distinctly decreased on day 3 (13.19 ± 1.05 vs. 2.85 ± 0.43, $p < 0.05$) (**Figures 2A,B**). Rats treated with anti-HMGB1 mAb had significantly higher p-CREB in the DG and CA1 areas than the S + IgG group (14.04 ± 0.80 vs. 2.85 ± 0.43 and 9.66 ± 0.47 vs. 4.68 ± 0.50, $p < 0.05$, respectively), which was similar to control rats (**Figures 2B,C**).

We also assessed NMDAR expression in hippocampal lysate. Compared to control, expression of NMDAR subunits NR2A and NR2B was increased at day 3 after surgery (1.00 ± 0.09 vs. 2.04 ± 0.21 and 1.00 ± 0.04 vs. 1.54 ± 0.08, $p < 0.05$, respectively) (**Figures 3A,B**). Treatment with anti-HMGB1 mAb significantly reduced the upregulation, with levels returning to baseline at day 3 as compared to the S + IgG group (2.04 ± 0.21 vs. 1.24 ± 0.07 and 1.54 ± 0.08 vs. 1.09 ± 0.15, $p < 0.05$, respectively) (**Figure 3**).

Microglia Activation Is Attenuated by Anti-HMGB1 Treatment

Neuroinflammation and microglia activation have been implicated in the pathophysiology of cognitive decline (23). Compared to control, the percentage of activated microglia, defined by pleomorphic and bigger cell body with de-ramification and shortening of cell processes, was significantly higher in the CA1 and DG hippocampal areas after surgery (59.64 ± 2.54 vs. 85.30 ± 0.90 and 57.23 ± 3.25 vs. 82.25 ± 1.36, $p < 0.05$, respectively) (**Figure 4**). Notably, treatment with anti-HMGB1 mAb decreased microglia activation in CA1 and DG at day 3 after surgery (71.10 ± 3.30 vs. 85.30 ± 0.90 and 65.92 ± 0.95 vs. 82.25 ± 1.36, $p < 0.05$, respectively) (**Figure 4**).

Peripheral HMGB1 Contributes to Postoperative Neuroinflammation

To characterize the role of HMGB1 in neuroinflammation after surgery, we measured the cellular distribution and expression of HMGB1 in the hippocampus. Nuclear translocation of HMGB1 to the cytoplasm has been reported as a critical pathological mechanism in a number of different conditions (24, 25). Notably, we found no evident change in cellular localization of HMGB1 in the hippocampus after surgery (**Figure 5A**).

This prompted us to evaluate if peripheral HMGB1 was responsible for this neuroinflammatory response. We used His-tagged HMGB1 injected into the tail vein of aged rats to

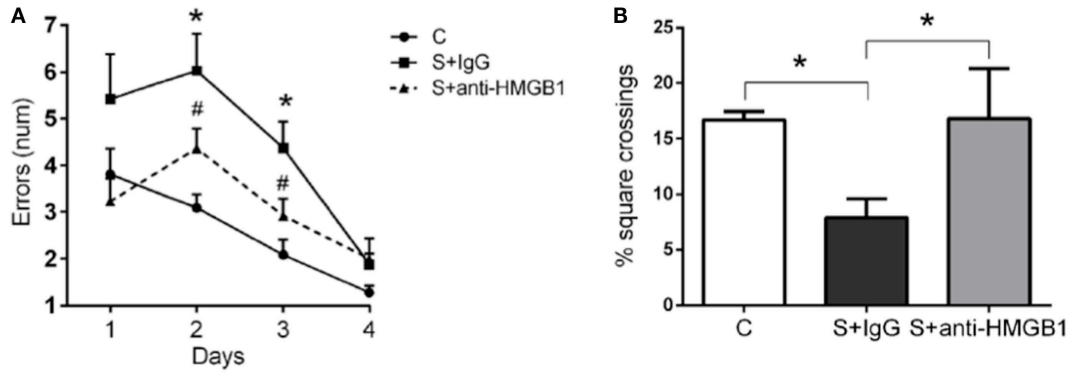

FIGURE 1 | Anti-HMGB1 improves memory dysfunction and anxiety in aged rats. Spatial memory was evaluated in the Barnes maze. Rats that had undergone liver surgery had significant memory impairments as detected by error numbers in reaching the correct target box **(A)**. Treatment with anti-HMGB1 mAb significantly improved postoperative memory dysfunction on both days 2 and 3 after surgery. **(B)** Anxiety was measured in an open field on postoperative day 3. Treatment with anti-HMGB1 mAb prevented postoperative anxiety as compared to the S + IgG group. Results are expressed as mean ± SEM ($n = 12$). *$p < 0.05$ vs. C; #$p < 0.05$ vs. S + IgG group by two-way repeated measures ANOVA for Barnes Maze and one-way ANOVA for open field. Abbreviations: C, control; S, surgery.

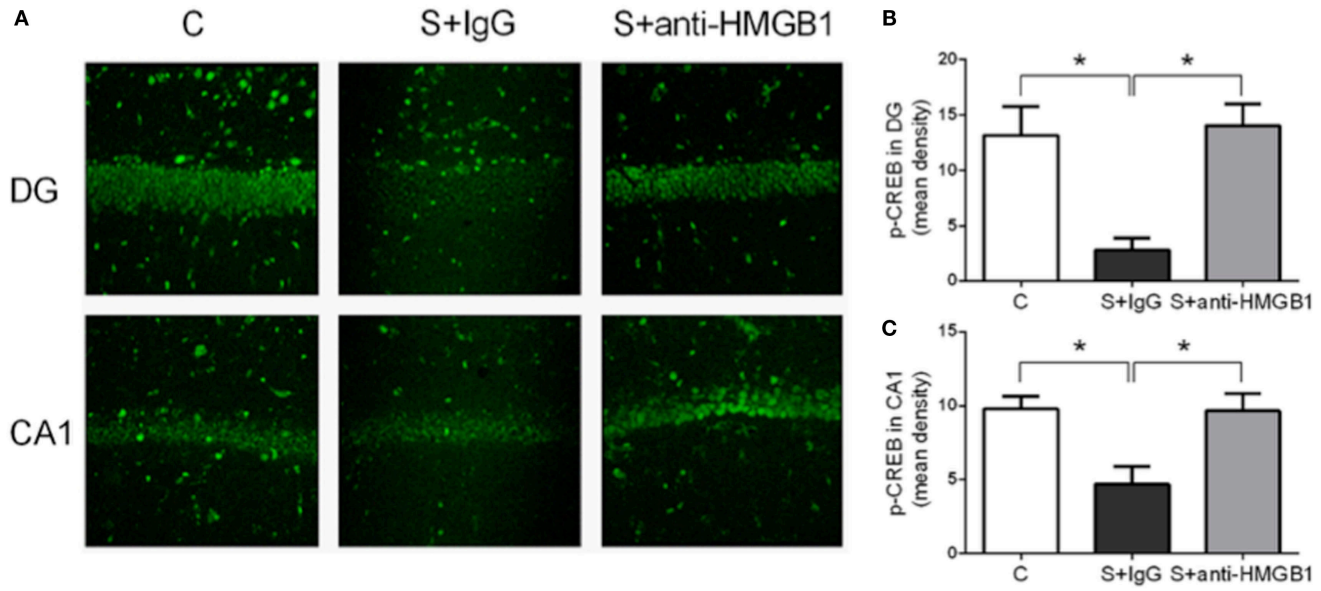

FIGURE 2 | **Modulation of p-CREB by anti-HMGB1**. Representative p-CREB immunofluorescence in the hippocampus, DG and CA1 areas, on postoperative day 3 **(A)**. Expression of p-CREB was reduced after surgery (S + IgG), and anti-HMGB1 treatment was effective in rescuing the expression back to levels similar to the levels of control animals. **(B,C)** show relative mean optical density for p-CREB staining in DG and CA1. Results are expressed as mean ± SEM ($n = 5$). Abbreviations: C, control; S, surgery. Scale bar = 50 μm.

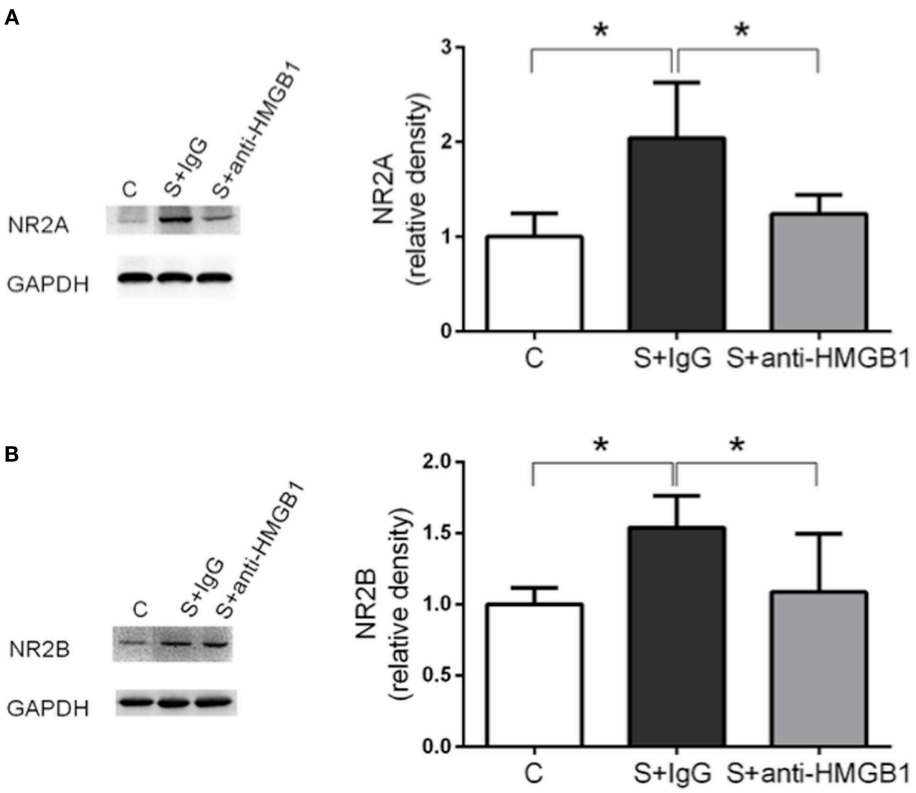

FIGURE 3 | **Effects of anti-HMGB1 on NMDAR subunits**. Expression of NR2A **(A)** and NR2B **(B)** was assessed in the hippocampus on day 3. Immunoblots and quantifications are shown describing an increase in NR2A and NR2B levels after surgery that was restored almost to baseline by anti-HMGB1 mAb treatment. Results are expressed as mean ± SEM ($n = 5$). *$p < 0.05$ by one-way ANOVA. Abbreviations: C, control; S, surgery. NR2A/B: *N*-methyl-D-aspartate receptor subunit 2A/B.

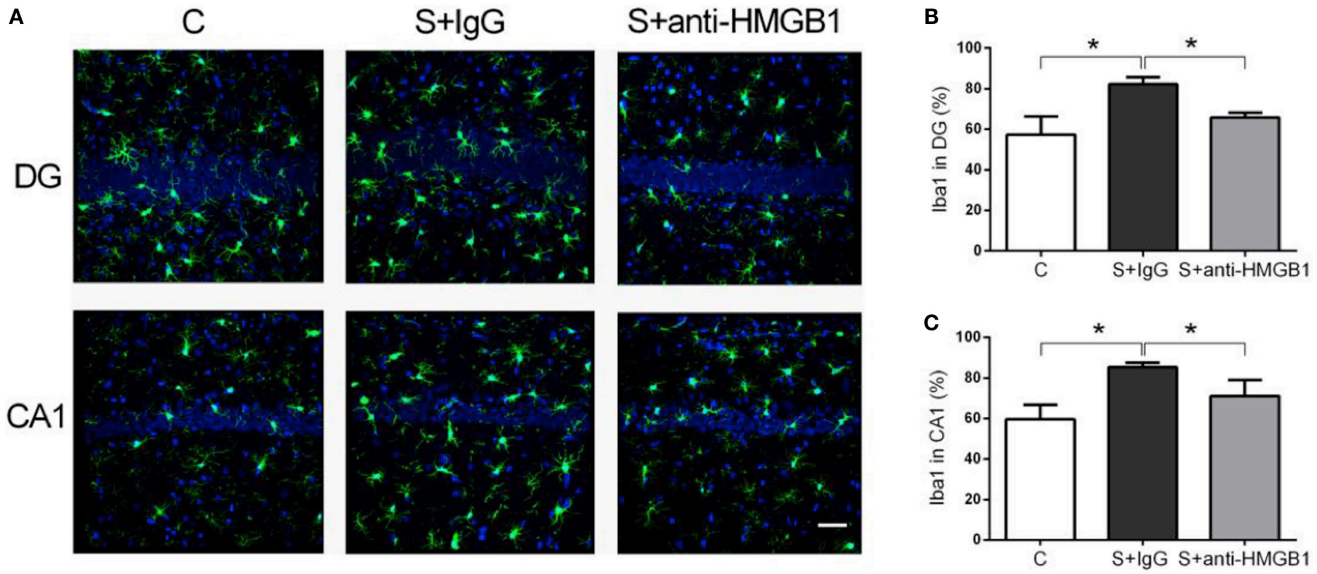

FIGURE 4 | **Microglia activation after surgery and effects of anti-HMGB1**. Neuroinflammation was assessed by Iba-1 immunofluorescence on postoperative day 3. Photomicrographs from DG and CA1 areas of the hippocampus are shown **(A)**. Surgery activated microglia as noted by morphological changes on day 3, including enlargement of cell bodies, which was attenuated by anti-HMGB1 mAb treatment. **(B,C)** show the quantification of Iba-1 immunofluorescence in DG and CA1, respectively. Results are expressed as mean ± SEM ($n = 5$). *$p < 0.05$ by one-way ANOVA. Abbreviations: C, control; S, surgery. Scale bar = 50 μm.

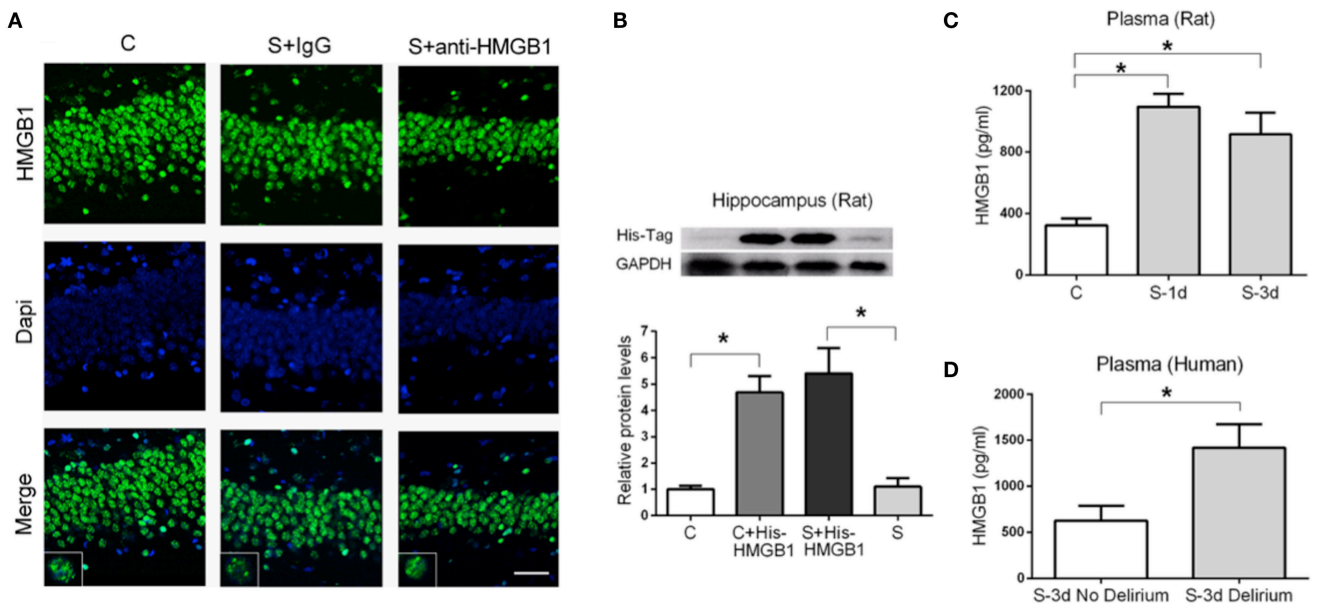

FIGURE 5 | **Expression of HMGB1 in the hippocampus and plasma after surgery**. HMGB1 localization in the hippocampus was assessed on postoperative day 3 **(A)**. No evident changes regarding nuclear translocation or extracellular HMGB1 were detected after surgery (S + IgG) or in S + anti-HMGB1 groups. Images and insets are representative of the hippocampal DG area. We used His-tagged HMGB1 to detect its presence in the CNS after tail vein administration **(B)**. Representative immunoblot and quantification show the presence of His-tagged HMGB1 in the hippocampus of both control (C + His-tagged HMGB1) and operated rats (S + His-tagged HMGB1). Systemic levels of HMGB1 in rats **(C)** and human **(D)** plasma were measured by ELISA on days 1 and 3 after surgery. Results are expressed as mean ± SEM. Scale bar = 50 μm.

detect blood-derived HMGB1 and found a significant increase in His-tagged HMGB1 in the hippocampus of both control and surgery groups (1.00 ± 0.13 vs. 4.69 ± 0.61 and 5.41 ± 0.95 vs. 1.10 ± 0.32, $p < 0.05$, respectively) (**Figure 5B**) suggesting that peripheral HMGB1 could directly enter into the CNS. Plasma levels of HMGB1 were significantly increased in aged rats at

days 1 and 3 after surgery (322.20 ± 45.25 vs. 1098.00 ± 82.64 and 322.20 ± 45.25 vs. 918.30 ± 140.80, $p < 0.05$, respectively) (**Figure 5C**). We also evaluated human plasma levels of HMGB1 from patients diagnosed with POD on day 3 and found a significant elevation compared to age-matched surgical subjects without delirium (627.1 ± 160.2 vs. 1418 ± 258.4, $p < 0.05$) (**Figure 5D**).

DISCUSSION

The current study aimed at investigating the role of HMGB1 signaling after surgery and the therapeutic potential of neutralizing anti-HMGB1 mAbs to prevent surgery-induced memory dysfunction. Our data indicate a key role for peripherally released HMGB1 causing neuroinflammation and memory dysfunction after abdominal surgery. Treatment with a neutralizing anti-HMGB1 mAb reduced microglial activation and prevented synaptic plasticity dysfunction. Herein, we uncovered a novel mechanism for HMGB1 in regulating p-CREB and synaptic plasticity after surgery.

HMGB1 is a multifunctional molecule with a critical role in sterile injury, including surgical manipulations (26). Passive and active release of HMGB1 may occur after injury: passive release occurs instantaneously as a result of disruption of cellular integrity and tissue necrosis; active secretion is a slower process due to the interaction with other cellular products (5, 27, 28). Release of HMGB1 as well as other DAMPs has been reported after surgery, with levels rapidly increasing within 30 min after orthopedic injury (10), suggesting that the release of HMGB1 may be critical in initiating an inflammatory cascade leading to CNS dysfunction. Furthermore, increased systemic levels of HMGB1 have been reported in elderly patients after gastrointestinal surgery, and these levels correlated with the development of POCD (12). Vacas et al. (29) showed a single dose of HMGB1 mAb effectively reduce circulating levels of HMGB1 and IL-6 after orthopedic surgery in younger mice. In our model, we found a more prolonged upregulation of systemic HMGB1. This may be related to a direct surgical insult to the liver, which contains a large pool of HMGB1, and greater cellular senescence promoting age-associated inflammation (30, 31). Thus, we treated animals twice with mAb to prevent both the initial, passive, release of HMGB1 from the direct tissue trauma, and the secondary increase due to the ensuing inflammatory response.

The mechanism whereby peripheral inflammation contributes to neuroinflammation, neurodegeneration, and cognitive dysfunction remains unclear. Intracellular translocation of HMGB1 from the nucleus to the cytoplasm is critical for extracellular release by a non-classical secretory mechanism (24, 32). In our model, we did not observe evident translocation of HMGB1 from the nucleus to the cytoplasm in the CNS. The subcellular localization of HMGB1 in the studied hippocampal areas after surgery was similar to normal expression in the rat brain (33). It is possible that the protective effects in our model are mediated by an overall dampening of systemic DAMPs and cytokines, which in turn prevent endothelial dysfunction and neuroinflammation, and also a direct CNS effect or vagal regulation (34). Neuroinflammation has been reported in several models of POCD (10, 11, 35), and here we could confirm microglia activation in the hippocampal formation after surgery. The important discovery of the present study is that neuroinflammation was reduced by treatment with anti-HMGB1 mAb. Notably, HMGB1 is necessary and sufficient to trigger memory dysfunction (29), microglia activation (36), and prime the immune system (37) as demonstrated by administering recombinant HMGB1 in otherwise healthy animals. Thus, in POCD models preventing systemic HMGB1 increase may be critical in limiting BBB opening (11, 38, 39), subsequent monocyte chemoattractant protein (MCP-1) expression in the hippocampus (29), microglial activation, and memory deficits. The role of the systemic milieu in triggering CNS complications after surgery is further demonstrated by the experiments with His-tagged HMGB1, suggesting that peripheral HMGB1 can directly access the brain parenchyma of aged rats.

In this study, we measured CREB expression in the hippocampus, which is critical in memory and synaptic plasticity (22). Phosphorylated CREB was significantly decreased 3 days after surgery and treatment with anti-HMGB1 mAb reversed this impairment in the hippocampus. Since CREB is implicated in hippocampal-dependent memory function and synaptic plasticity, the rescuing effect of the anti-HMGB1 mAb treatment is consistent with the improved behavioral outcomes demonstrated in this study. Although we can describe a novel effect of anti-HMGB1 mAb to modulate neuroplasticity, the effects of peripheral surgery on neuronal function and p-CREB regulation require further elucidation. POCD has been associated with changes in synaptic plasticity, including long-term potentiation (LTP), and this may be dependent on the neuroinflammatory response after trauma (40). In this study, we found changes in NMDAR expression after surgery, which was restored by anti-HMGB1 mAb treatment. NR2A and NR2B are critical for sustaining LTP and are prominently expressed in hippocampal pyramidal excitatory cells (41). The increase in NR2A and NR2B after surgery may relate to acute neurotoxicity as higher expression of NR2A in the hippocampus has been associated with poorer cognitive outcomes (42). Furthermore, HMGB1 has been demonstrated as an excitatory and neurotoxic signaling molecule in the CNS (43) and exerts a prominent role in conditions like epilepsy (44).

The anti-inflammatory effects of HMGB1 neutralization have been described in several models, including POCD (7–9, 29). Here, we provide additional evidence for HMGB1 modulation of neuronal molecules of relevance to synaptic plasticity and memory function.

In the present study, we have not determined the redox states of HMGB1. This is critical to a better understating of the role of HMGB1 signaling after trauma as posttranslational modifications regulate receptor usage and thus functionality (45). The HMGB1-receptors TLR4 and RAGE have been demonstrated to be upregulated in POCD models (11, 35). No HMGB1 redox isoform restriction is known for RAGE signaling, while disulfide-HMGB1 is required for interaction with TLR4 and TLR4-mediated neuroinflammation and disruption of memory-related signaling in the hippocampus. Moreover, TLR4 activation modulates NMDAR subunits through HMGB1 signaling (46),

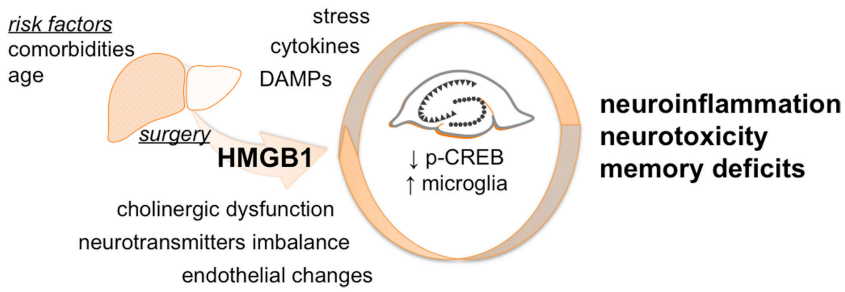

FIGURE 6 | Proposed working model. Surgery triggers peripheral HMGB1 release that can gain access to the CNS contributing to neuroinflammation. The effects of HMGB1 in the CNS are not limited to microglia activation but also involve modulation of p-CREB expression in the hippocampus. Overall, this acute neurotoxicity leads to behavioral deficits that are effectively attenuated by selective anti-HMGB1 mAb treatment.

suggesting that neuroinflammation may be critical in mediating the cognitive deficits.

Postoperative neurocognitive disorders are multifactorial conditions with several factors contributing to overall changes in mental status (**Figure 6**). Recent evidence suggests women are at greater risk for cognitive decline after surgery than men (47, 48). Although we did not include age-matched males or younger females for comparison in this study, several studies have focused on POCD using males and younger rodent models. Herein, we attempted to bridge a critical gap in translation by applying a clinically relevant model to study the therapeutic effects of HMGB1 mAb in aged females. Given HMGB1 is a protein highly conserved across species (6), these findings may have implications beyond specific sex, age, and strain differences.

In conclusion, blocking HMGB1 during the perioperative period attenuated postoperative neuroinflammation and memory dysfunction. Based on these findings, we propose a key role for HMGB1 in mediating POD and provide new evidence for a protective role of neutralizing HMGB1 treatment in the perioperative setting.

AUTHOR CONTRIBUTIONS

NT, TY, WO, and JT conceived of the study, analyzed the data, and wrote the manuscript. XW, JF, and MC performed the research. UA and HEH participated in the design of the study and contributed key reagents. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Inflammatory Regulation by Driving Microglial M2 Polarization: Neuroprotective Effects of Cannabinoid Receptor-2 Activation in Intracerebral Hemorrhage

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The cannabinoid receptor-2 (CB2R) was initially thought to be the "peripheral cannabinoid receptor." Recent studies, however, have documented CB2R expression in the brain in both glial and neuronal cells, and increasing evidence suggests an important role for CB2R in the central nervous system inflammatory response. Intracerebral hemorrhage (ICH), which occurs when a diseased cerebral vessel ruptures, accounts for 10–15% of all strokes. Although surgical techniques have significantly advanced in the past two decades, ICH continues to have a high mortality rate. The aim of this study was to investigate the therapeutic effects of CB2R stimulation in acute phase after experimental ICH in rats and its related mechanisms. Data showed that stimulation of CB2R using a selective agonist, JWH133, ameliorated brain edema, brain damage, and neuron death and improved neurobehavioral outcomes in acute phase after ICH. The neuroprotective effects were prevented by SR144528, a selective CB2R inhibitor. Additionally, JWH133 suppressed neuroinflammation and upregulated the expression of microglial M2-associated marker in both gene and protein level. Furthermore, the expression of phosphorylated cAMP-dependent protein kinase (pPKA) and its downstream effector, cAMP-response element binding protein (CREB), were facilitated. Knockdown of CREB significantly inversed the increase of M2 polarization in microglia, indicating that the JWH133-mediated anti-inflammatory effects are closely associated with PKA/CREB signaling pathway. These findings demonstrated that CB2R stimulation significantly protected the brain damage and suppressed neuroinflammation by promoting the acquisition of microglial M2 phenotype in acute stage after ICH. Taken together, this study provided mechanism insight into neuroprotective effects by CB2R stimulation after ICH.

Keywords: intracerebral hemorrhage, cannabinoid receptor-2, neuroinflammation, microglial polarization, CREB

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INTRODUCTION

Intracerebral hemorrhage (ICH) is a subtype of stroke with high morbidity and mortality, accounting for about 15% of all deaths from strokes (1). Pronounced inflammatory reactions play an important role in secondary brain injury following ICH (2, 3); various stimuli, including thrombin or glutamate, activate microglia and initiate an inflammatory response, and subsequently release pro-inflammatory cytokines or chemokines to enhance neuroinflammation (3, 4).

Microglia are the resident macrophages of the brain and the first responders of the immune system (5). They are highly plastic cells that can assume diverse phenotypes and engage different functional programs in response to specific microenvironmental signals. In particular, stimulation with interferon-γ promotes classically activated microglia/macrophage (M1 phenotype) that release destructive pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), causing damage to healthy cells and tissues (6). In contrast, cytokines such as interleukin-4 and interleukin-10 (IL-10) induce an alternative activated microglia (M2 phenotype) that generates anti-inflammatory cytokines, such as transforming growth factor beta (TGF-β) and IL-10, which possess neuroprotective properties (7, 8). Microglia activation and M1/M2 polarization have been reported in several types of acute central nervous system (CNS) injury, such as traumatic brain injury, spinal cord injury, and ischemic stroke (9, 10). Microglia activation and polarization also occur in hemorrhagic stroke (11, 12); however, their underlying mechanisms after ICH remain unclear.

The cannabinoid receptor-2 (CB2R) signaling pathway plays an important role in CNS injury via regulation of microglial activities (13, 14). In addition, the expression of CB2R on microglia mainly depends on the activation state of microglia (15). CB2R on activated microglia have been shown to modulate properties of microglial migration and infiltration into the CNS during active neuroinflammation and degeneration (16). In our previous study, we showed that CB2R stimulation attenuated microglial accumulation after germinal matrix hemorrhage (GMH) in neonatal rats (17). However, whether CB2R has effects on microglia polarization following ICH remains unknown.

In this study, we investigated the therapeutic effects of CB2R stimulation in acute phase following experimental ICH and its related mechanisms. We found that selective stimulation of CB2R significantly protected brain injury and suppressed neuroinflammation by promoting M2 polarization of microglia. In addition, we also demonstrated that PKA/CREB signaling pathway was involved in, at least partially, the suppression of neuroinflammation. These results indicated that CB2R may be a potential therapeutic target for ICH.

ANIMALS AND METHODS

Animals

Excluding 6 rats that died of an overdose of anesthetic before modeling, total 213 adult male Sprague-Dawley rats (250–300 g) were used for this study. Rats were housed under specific pathogen-free conditions and had free access to food and water. Animals were sacrificed at the endpoint under deep anesthesia using an overdose of intraperitoneal pentobarbital. All efforts were made to minimize suffering and animal numbers according to the *Guide for the Care and Use of Laboratory Animals*; the study was approved by the Animal Care and Use Committee at the Third Military Medical University.

Experimental Design

Experiment I

To determine the expression time course of CB2R after ICH, 18 rats were randomly assigned into three groups: ICH 0 h ($n = 6$), ICH 24 h ($n = 6$), and ICH 72 h ($n = 6$). Perihematomal tissue (shown as the white quadrangle in **Figure 1A**) was collected to detect the protein expression of CB2R.

Experiment II

A total of 168 rats were randomly divided into four groups for the mechanism study: sham-operated (Sham group, $n = 42$), ICH + Vehicle (ICH + Vehi group, $n = 42$), ICH + JWH133

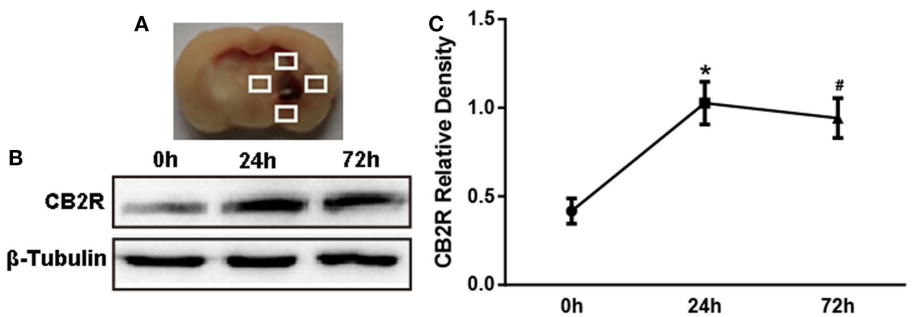

FIGURE 1 | The expression of cannabinoid receptor-2 (CB2R) after intracerebral hemorrhage (ICH). The coronal section of specimen **(A)**. Western blot test **(B)** of CB2R expression in total tissue around the hematoma (shown as the white quadrangles) at 0, 24, and 72 h after ICH, and the relative densitometric values analysis **(C)**. Values were expressed as the mean ± SD, $n = 6$ in per group. $^{*}P < 0.05$ vs. 0 h group; $^{\#}P > 0.05$ vs. 24 h group.

(ICH + JWH group, $n = 42$), and ICH + JWH133 + SR144528 (ICH + JWH + SR group, $n = 42$). Sham group received only a needle insertion. The ICH + Vehi group received an equal volume of vehicle, and the ICH + JWH group received an intraperitoneal injection of a selective CB2R agonist, JWH133 (1.5 mg/kg, Tocris Bioscience, Minneapolis, MN, USA) at 1 h after surgery. The ICH + JWH + SR group was treated with a selective CB2R antagonist, SR144528 (3 mg/kg, Santa Cruz Biotechnologies, Dallas, TX, USA) 3 min before JWH133 (1.5 mg/kg) intraperitoneally. Brain water content ($n = 6$ in each group) and neurological scores ($n = 6$ in each group), including the modified Neurological Severity Score (mNSS) and forelimb placing test, were tested at 24 and 72 h after ICH. Gene levels of microglial M1/M2 markers and inflammatory cytokines in perihematomal tissue were evaluated at 6, 12, 24, and 72 h after ICH ($n = 3$ in each group). Western blots ($n = 6$ per group) and immunofluorescence staining ($n = 6$ per group) for protein expression of the tissues around the hematoma were conducted at 24 h post-ictus. The use of JWH133 and SR144528 was according to the publication (18).

Experiment III

For further study of the mechanism, 27 rats were randomly assigned into three groups: ICH + JWH133 + Vehicle group (JWH + Vehi, $n = 9$), ICH + JWH133 + CREB-1 siRNA group (JWH + si-CREB, $n = 9$), and ICH + JWH133 + scrambled siRNA group (JWH + scr-siRNA, $n = 9$). siRNA dilution buffer (Santa Cruz Biotechnologies, Dallas, TX, USA), CREB-1 siRNA (Santa Cruz Biotechnologies, Dallas, TX, USA), or scrambled siRNA (Santa Cruz Biotechnologies, Dallas, TX, USA) was intracerebroventricularly injected at 24 h before ICH modeling, and JWH133 was injected 1 h after surgery. PCR for gene levels ($n = 3$ in each group) and western blots for protein levels ($n = 6$ in each group) were performed at 24 h after ICH in each group.

ICH Model

To induce ICH, rats were anesthetized with an intraperitoneal injection of 5% chloral hydrate (350 mg/kg). A feedback-controlled heating pad was used to maintain body temperature at 37.0°C. A cranial burr hole (about 1 mm) was drilled, and a 29-gauge needle was inserted stereotaxically into the right basal ganglia (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 4.0 mm lateral to the bregma) (19, 20). A total of 100 μl autologous arterial blood was infused at a rate of 10 μl/min using a microinfusion pump in each rat. The sham groups received only a needle injection into the right basal ganglia.

Brain Water Content Measurement

Brain water content was examined 24 and 72 h after surgery. As previously described (21), animals were anesthetized with an intraperitoneal injection of 5% chloral hydrate (350 mg/kg). Brains were removed, and the tissue was sliced coronally (4 mm thickness) around the hematoma. Samples were divided into four parts: ipsilateral basal ganglia, ipsilateral cortex, contralateral basal ganglia, and contralateral cortex. Cerebellum was regarded as the internal control. Sample weights were determined immediately after removal and after drying for 24 h in a 100°C oven using an electric analytical balance. Brain water content (%) was calculated as (wet weight − dry weight)/wet weight × 100%.

Assessment of Behavioral Outcome

Behavioral outcomes were assessed in a blinded fashion at 24 and 72 h after surgery. The neurological abnormalities were assessed by the mNSS method. The evaluation was performed by an investigator blinded to the experimental scheme. The mNSS is a composite test of motor, sensory, and balance functions. Neurological function was graded on a scale of 0–18 (normal score, 0; maximal deficit score, 18) (22). For the forelimb placing test, each rat was tested 10 times for each forelimb; the percentage of trials that the rat placed the appropriate forelimb at the edge of the countertop in response to the vibrissae stimulation was determined. Testers were experienced and blind to the condition of the animal. The mean neurological score was evaluated by two blinded observers (23).

Fluoro-Jade C and Terminal dUDP Nick End Labeling (TUNEL) Staining and Cell Counting

Fluoro-Jade C staining was used to assess neuronal degeneration. Briefly, sections were rinsed for 5 min in basic alcohol, followed by a 2-min rinse in 70% alcohol. Then, the sections were rinsed in distilled water and incubated in 0.06% $KMnO_4$ for 10 min. They were rinsed in distilled water to remove excess $KMnO_4$ and incubated in 0.0001% Fluoro-Jade C stain (Millipore, Boston, MA, USA) stain in 0.1% acetic acid for 10 min. Following Fluoro-Jade C labeling, the sections were rinsed three times in distilled water, air dried for 10 min, cleared in xylene, and covered with DPX. TUNEL staining was performed using an *in situ* cell death detection kit-POD (Roche, Switzerland) to reveal DNA damage according to the manufacturer's instruction (24). High-power images (×40 magnification) were taken around the hematoma using a digital camera. Fluoro-Jade C and TUNEL-positive cells were counted. Counts were performed on four areas in each brain section.

Real-Time PCR

PCR was performed and analyzed as previously described (25). Total RNA from brain tissue around the hematoma was extracted using Qiagen RNeasy mini kits. One microgram of RNA was reverse-transcripted and cDNA was synthesized using the PrimescriptTM RT kit (Takara, Dalian, China), substituting DNase and RNase-free water for no-RT controls. qPCR reactions were set up in 25 μl using SYBR Premix Ex TaqII kit (Takara, Dalian, China) and conducted on a CFX-96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The running procedure was 30 s at 95°C, 40 cycles of 5 s at 95°C, and 30 s at 60°C, following a melt curve. The qPCR primers were listed in Table S1 in Supplementary Material. Gene expression was quantified with standard samples and normalized with GAPDH. The data are expressed as normalized messenger RNA (mRNA) expression (fold mRNA increase).

Immunofluorescence Staining

Immunofluorescence staining was performed as previously described (21). Briefly, free-floating slices were incubated with primary goat anti-Iba1 (1:200, Abcam, Cambridge, United Kingdom) at 4°C overnight, followed by Alexa 555-labeled rabbit anti-goat IgG (H + L) (1:500; Beyotime, Wuhan, China) secondary antibody (1 h, 37°C). Sections were washed and blocked with 10% normal goat serum for 1 h, then incubated overnight with mouse anti-CD68 (1:200, 1:500, AbD Serotec, Oxford, UK) or rabbit anti-CD206 (1:400, Santa Cruz Biotechnologies, Dallas, TX, USA). Finally, sections were incubated with the appropriate secondary antibodies for 1 h at 37°C. Colocalization was examined using a fluorescent microscope (Zeiss, LSM780).

Western Blot Analysis

Western blot assays were performed as described previously (26). A total of 50 μg of prepared protein was loaded into each lane of SDS-PAGE gels. Gel electrophoresis was performed, and protein was transferred to a nitrocellulose membrane. The membrane was blocked in Carnation® non-fat milk and probed with primary and secondary antibodies. The following primary antibodies were used: rabbit anti-CB2R (1:500, Abcam, Cambridge, UK), mouse anti-CD68 (1:500, AbD Serotec, Oxford, UK), rabbit anti-CD206 (1:500, Santa Cruz Biotechnologies, Dallas, TX, USA), rabbit anti-phospho-CREB (Ser133; 1:1,000, Cell Signaling Technology, Boston, MA, USA), rabbit anti-phospho-PKAC (Thr197; 1:1,000, Cell Signaling Technology, Boston, MA, USA), rabbit anti-β-tubulin (1:1,000, Abcam, Cambridge, UK), and mouse anti-β-actin (1:1,000, Santa Cruz Biotechnologies, Dallas, TX, USA). Then, membranes were incubated in the appropriate HRP-conjugated secondary antibody (diluted 1:1,000 in secondary antibody dilution buffer) for 1 h at 37°C. Protein bands were visualized using a nickel-intensified DAB solution, and the densitometric values were analyzed using Image J software. The housekeeping proteins β-tubulin and β-actin were used as internal controls.

Intracerebroventricular Infusion

Intracerebroventricular infusion was performed as described previously (27). Rats were anesthetized with an intraperitoneal injection of 5% chloral hydrate (350 mg/kg). The needle of a 10-μl Hamilton syringe (Microliter No. 701; Hamilton Company) was inserted through a burr hole in the skull into the left lateral ventricle, according to the following coordinates: 1.5 mm posterior, 4.2 mm ventral, and 0.8 mm lateral to the bregma. CREB-1 siRNA or an irrelevant scrambled siRNA [500 pmol each in 1 μl siRNA dilution buffer (Santa Cruz Biotechnology)] was injected (0.5 μl/min) using a microinfusion pump at 24 h before ICH induction. The needle was removed 10 min later to prevent backflow. The burr hole was sealed with bone wax, and skin incisions were closed with sutures after the needle was removed. All rats received JWH133 (1.5 mg/kg) intraperitoneally 1 h after ICH. CREB-1 siRNA consists of three different siRNA duplexes to improve the knockdown efficiency. All CREB-1 siRNA sequences were provided in 5′–3′ orientation as shown in Table S2 in Supplementary Material.

Statistical Analysis

Data are reported as the mean ± SD. Data were analyzed using one-way analysis of variance tests followed by Student–Newman–Keuls tests. A non-parametric test (Kruskal–Wallis H) was used if the data were not normally distributed, followed by a Nemenyi test when a two-group comparison was necessary. Differences were considered statistically significant at $P < 0.05$.

RESULTS

CB2R Was Upregulated after ICH Injury

We first investigated whether CB2R alterations would respond to brain injury after ICH. Data showed that CB2R levels around hematoma (**Figure 1A**) were significantly increased at 24 h after ICH when compared with the ICH 0 h group ($P < 0.05$) and remained at a high level until 72 h (**Figures 1B,C**).

JWH133 Reduced Brain Water Content and Improved Neurobehavioral Outcomes both at 24 and 72 h after ICH

To assess brain edema and neurobehavioral outcomes after ICH, brain water content and behavioral testings, including mNSS test and forelimb placing test, were used. Data showed that both at 24 and 72 h after surgery, rats subjected to ICH had increased perihematomal edema in the ipsilateral basal ganglia ($P < 0.01$ vs. Sham; **Figures 2A,B**). The animals also had significantly worse mNSS test performance ($P < 0.01$ vs. Sham; **Figures 3A,B**) and forelimb placing scores ($P < 0.01$ vs. Sham; **Figures 3C,D**). In ICH + JWH groups, the perihematomal brain edemas were significantly reduced ($P < 0.05$ vs. ICH + Vehi; **Figures 2A,B**), and the neurobehavioral outcomes were significantly improved ($P < 0.05$ vs. ICH + vehi; **Figures 3A–D**). All of these JWH133 effects were reversed by SR144528.

JWH133 Attenuated Neuronal Death and DNA Damage after ICH

To investigate the neuronal degeneration after ICH, Fluoro-Jade C and TUNEL staining were used. At 24 h after ICH, the treatment of JWH133 significantly decreased the number of Fluoro-Jade C-positive cells (497 vs. 720/mm^2 in ICH + Vehi group, $P < 0.01$) (**Figures 4A,B**). Similarly, the number of TUNEL-positive cells were significantly decreased in the ICH + JWH group (524 vs. 325/mm^2 in ICH + Vehi group, $P < 0.01$) (**Figures 4A,C**). The result indicated that JWH133 could significantly attenuate neuronal death and DNA damage after ICH.

Microglia Primarily Polarized to Classic M1 Phenotype during the ICH Acute Phase, and JWH133 Promoted M2 Polarization

To investigate the characteristics of microglial activation during the acute phase following ICH induction, gene levels of M1/M2

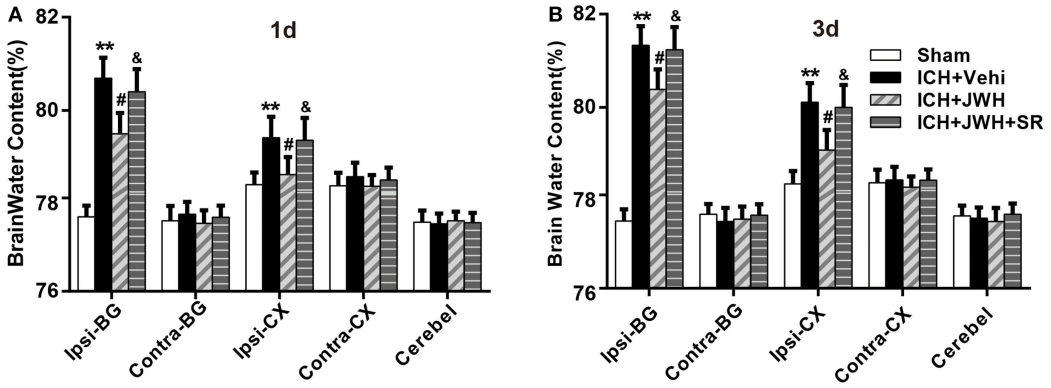

FIGURE 2 | Brain water content after intracerebral hemorrhage (ICH). Brain water content in ipsilateral basal ganglia at 24 h **(A)** and 72 h **(B)** after ICH. Ipsi-BG, ipsilateral basal ganglia; Ipsi-CX, ipsilateral cortex; Cont-BG, contralateral basal ganglia; and Cont-CX, contralateral cortex; Cerebel, Cerebellum; Vehi, Vehicle; JWH, JWH133; SR, SR144528. Values were expressed as the mean ± SD, $n = 6$ in each group. $^{**}P < 0.01$ compared with sham group; $^{\#}P < 0.05$ compared with ICH + Vehi group; $^{\&}P < 0.05$ compared with ICH + JWH group.

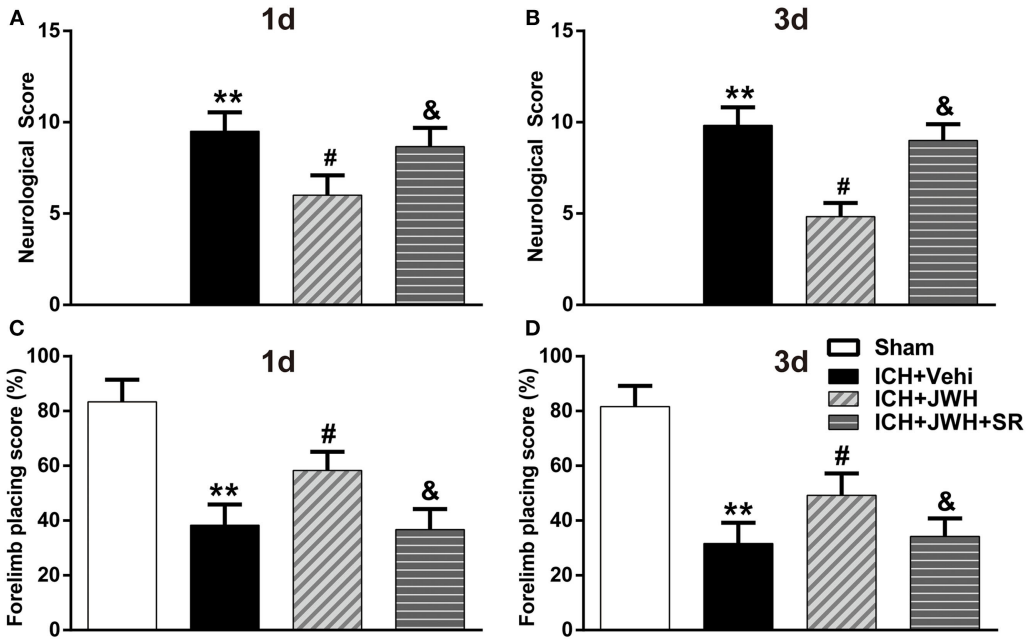

FIGURE 3 | Neurological deficit scores. Results of modified Neurological Severity Score test at 24 h **(A)** and 72 h **(B)**, and forelimb placing test at 24 h **(C)** and 72 h **(D)**. Each experiment was repeated three times and the average value was taken for statistics. Vehi, Vehicle; JWH, JWH133; SR, SR144528. Values were expressed as the mean ± SD, $n = 6$ per group. $^{**}P < 0.01$ compared with sham group; $^{\#}P < 0.05$ compared with ICH + Vehi group; $^{\&}P < 0.05$ compared with ICH + JWH group.

markers were investigated time dependently. After ICH, M1-associated markers CD32, CD68, and CD86 increased immediately and peaked at 6 h, and remained a high level to 72 h (**Figures 5A,C,E**). M2-associated markers Arg-1, Ym-1, and CCL-22 increased slowly and peaked at 24 h, and kept a low level from 6 to 72 h (**Figures 5B,D,F**). However, the use of JWH133 significantly inhibited the increase of M1-associated mRNA levels (**Figures 5A,C,E**) and promoted M2-associated mRNA levels (**Figures 5B,D,F**), with the most obvious differences at 24 h, and all the effects could be significantly reversed by SR144528 (**Figures 5A–E**). Consistent with the qPCR results, immunofluorescent double-labeled staining and western blot results showed that the M1-associated marker CD68 was also more significantly upregulated than M2-associated marker CD206 at 24 h after ICH (**Figures 6A,B**). Treatment with JWH133 promoted the protein expression of CD206 and inhibited CD68 expression

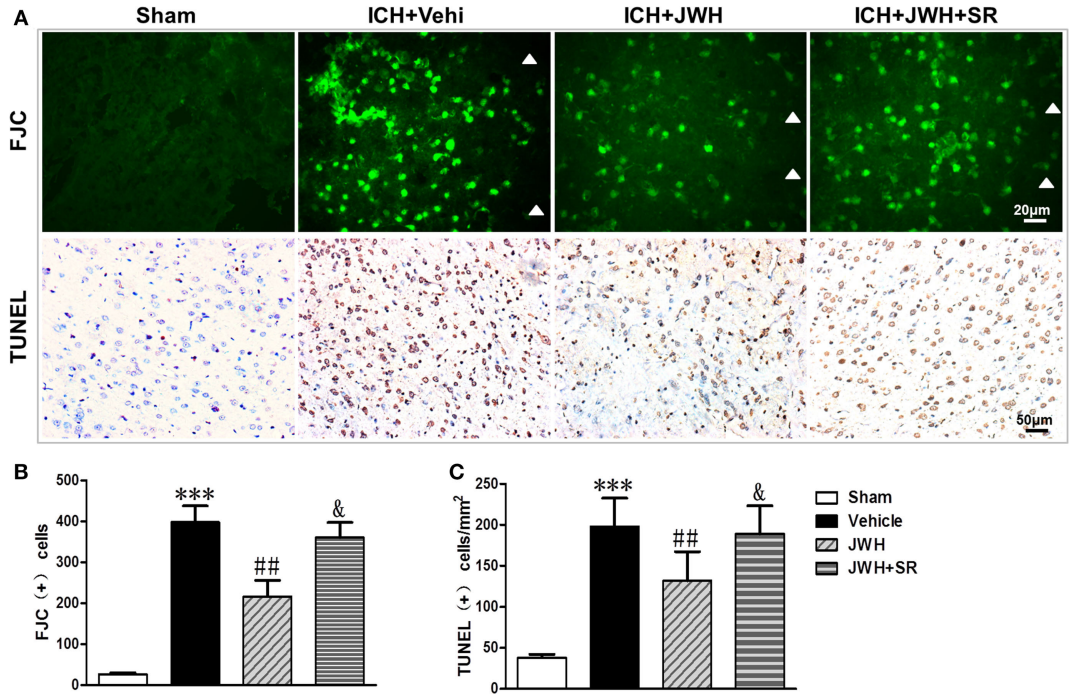

FIGURE 4 | Cell death detection following intracerebral hemorrhage (ICH). Fluoro-Jade C and terminal dUDP nick end labeling (TUNEL) staining for tissue around the hematoma in the ipsilateral basal ganglia at 24 h after ICH **(A)**, and analysis of positive cell counting of Fluoro-Jade C (green) **(B)** and TUNEL (brown) **(C)** Green represents for FJC-positive cells and brown represents for TUNEL-positive cells. FJC, Fluoro-Jade C; Vehi, Vehicle; JWH, JWH133; SR, SR144528. Values were expressed as mean ± SD, $n = 6$ in each group. $^{**}P < 0.01$ compared with sham group; $^{\#}P < 0.05$ compared with ICH + Vehi group; $^{\&}P < 0.05$ compared with ICH + JWH group.

(**Figures 6A–C**). These results indicated that CB2R stimulation could drive the acquisition of M2 polarization in microglia and reduce M1 phenotype.

Inflammatory Responses during ICH Were Inhibited by JWH133 Treatment

Inflammatory cytokine secretion plays an important role in brain injury after ICH. In this study, the mRNA expression levels of both pro-inflammatory (IL-1β, TNF-α, and iNOS) and anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) in peri-hematomal brain tissue were detected at different time points after ICH. Expression levels of pro-inflammatory cytokines increased immediately and IL-1β peaked at 6 h (**Figure 7A**), while TNF-α and iNOS (**Figures 7C,E**) peaked at 24 h post-ICH. The gene levels of IL-4, IL-10, and TGF-β were upregulated at 6 h after ICH and reached a peak at 24 h (**Figures 7B,D,F**). However, treatment with JWH133 prevented the increase of IL-1β, TNF-α, and iNOS at 6 h after ICH induction, and the effect persisted to 72 h, with the most significant changes occurring at 24 and 72 h (**Figures 7A,C,E**). In contrast, IL-4, IL-10, and TGF-β were significantly increased at 6 h, with the most significant changes occurring at 24 h (**Figures 7B,D,F**). All of the JWH133-mediated effects were prevented by treatment with SR144528.

pPKA–CREB Signaling Pathway Was Upregulated by JWH133 Treatment

To further explore the underlying mechanisms, we tested the expression of pPKA–CREB signaling pathway. Results of western blot showed that pPKA and pCREB expression was significantly lower in ICH + Vehi group when compared with Sham group ($P < 0.01$), and the ICH + JWH group had significantly higher expression of pPKA ($P < 0.01$) and pCREB ($P < 0.05$) when compared with the ICH + Vehi group (**Figures 8A,B**), indicating that pPKA–CREB signaling pathway was significantly upregulated by JWH133 treatment. The pretreatment of SR144528 prevented the upregulation effects.

pPKA–CREB Signaling Pathway Was Involved in the JWH133-Induced Increase of Microglial M2 Polarization

cAMP-response element binding protein (CREB) *in vivo* knockdown was performed to investigate the potential role of CREB in the effects of JWH133 induced the increase of microglial M2 polarization and the decrease of M1 phenotype. Pretreatment with CREB-1 siRNA sufficiently downregulated the expression of CREB both at gene and protein levels (Figures S1A,B in Supplementary Material). Data showed that CREB-1 siRNA abolished the JWH133-induced increase of M2-associated markers

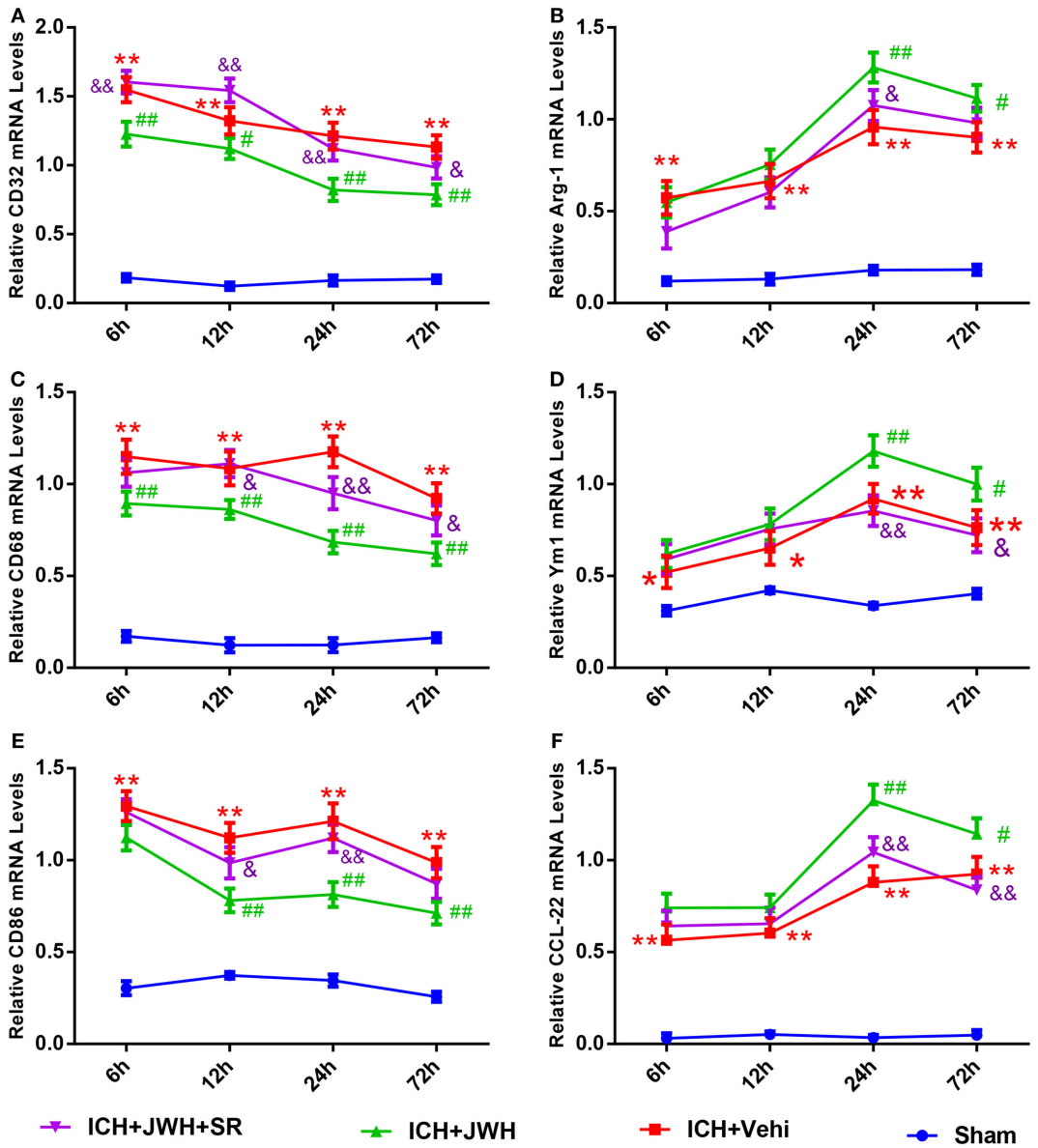

FIGURE 5 | M1/M2-associated markers of microglia at messenger RNA level. CD32 **(A)**, CD68 **(C)**, and CD86 **(E)** are M1-associated markers. Arg-1 **(B)**, Ym-1 **(D)**, and CCL-22 **(F)** are M2-associated markers. Values were expressed as mean ± SD, $n = 6$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. ICH + Vehi group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. ICH + JWH group.

CD206 and Ym-1 at protein or gene level (**Figures 9B,D,F**), as well as the decrease of M1-associated markers CD68 and CD32 in protein or gene levels (**Figures 9A,C,E**). These results demonstrated that PKA/CREB signaling pathway plays a crucial role in the JWH133-mediated acquisition of M2 phenotype of microglia.

DISCUSSION

To our knowledge, this is the first study to demonstrate that JWH133, a selective CB2R agonist, reduces brain water content, neurological deficits, DNA damage, and neuron death in an autologous blood infusion rat ICH model. We also demonstrated that JWH133 suppresses neuroinflammation by driving microglial M2 polarization through PKA/CREB pathway.

Increasing evidence indicates that inflammatory mechanisms are involved in stroke-induced brain injury, and microglia/macrophage activation is thought to play a pivotal pathophysiological role (1, 4). Overactivation of microglia/macrophages can exacerbate the inflammatory response after a stroke, resulting in blood–brain barrier disruption and neuronal damage, but also represents a promising target for stroke treatment. However,

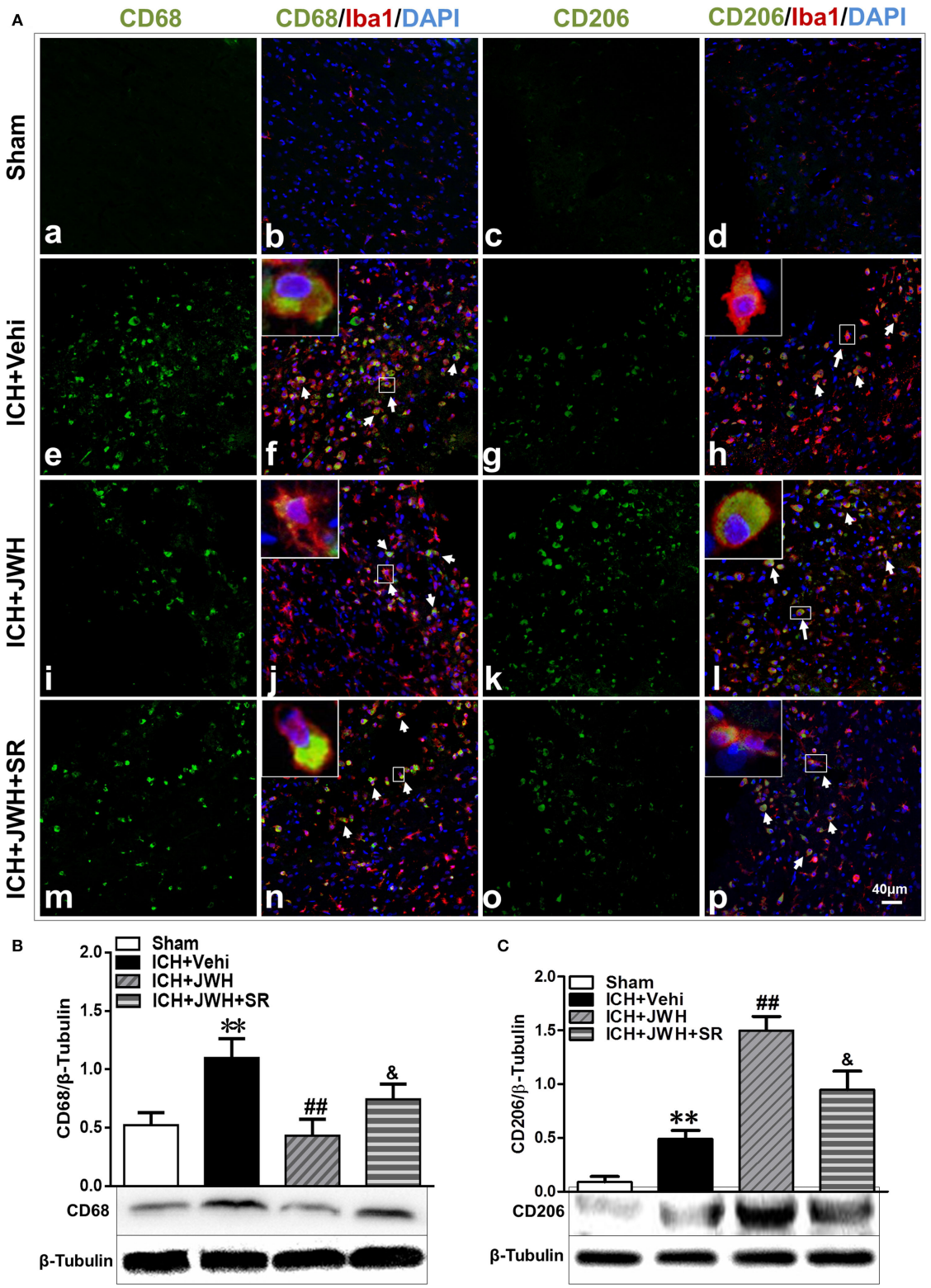

FIGURE 6 | M1/M2-associated markers of microglia at protein level. Perihematomal tissue was costained for CD68 (M1 marker) (green) and Iba1 (microglia marker) (red), or CD206 (M2 marker) (green) and Iba1 (red) at 24 h after intracerebral hemorrhage (ICH) **(A)**. Cell nucleus was stained with DAPI (blue). White arrows pointed to typical cells. $n = 6$ in per group. CD68 **(B)** and CD206 **(C)** were also tested using western blots. Values of the relative densitometric analysis were expressed as mean ± SD, $n = 6$ in each group. $^{**}P < 0.01$ compared with sham group; $^{\#\#}P < 0.01$ compared with ICH + Vehi group; $^{\&}P < 0.05$ compared with ICH + JWH group.

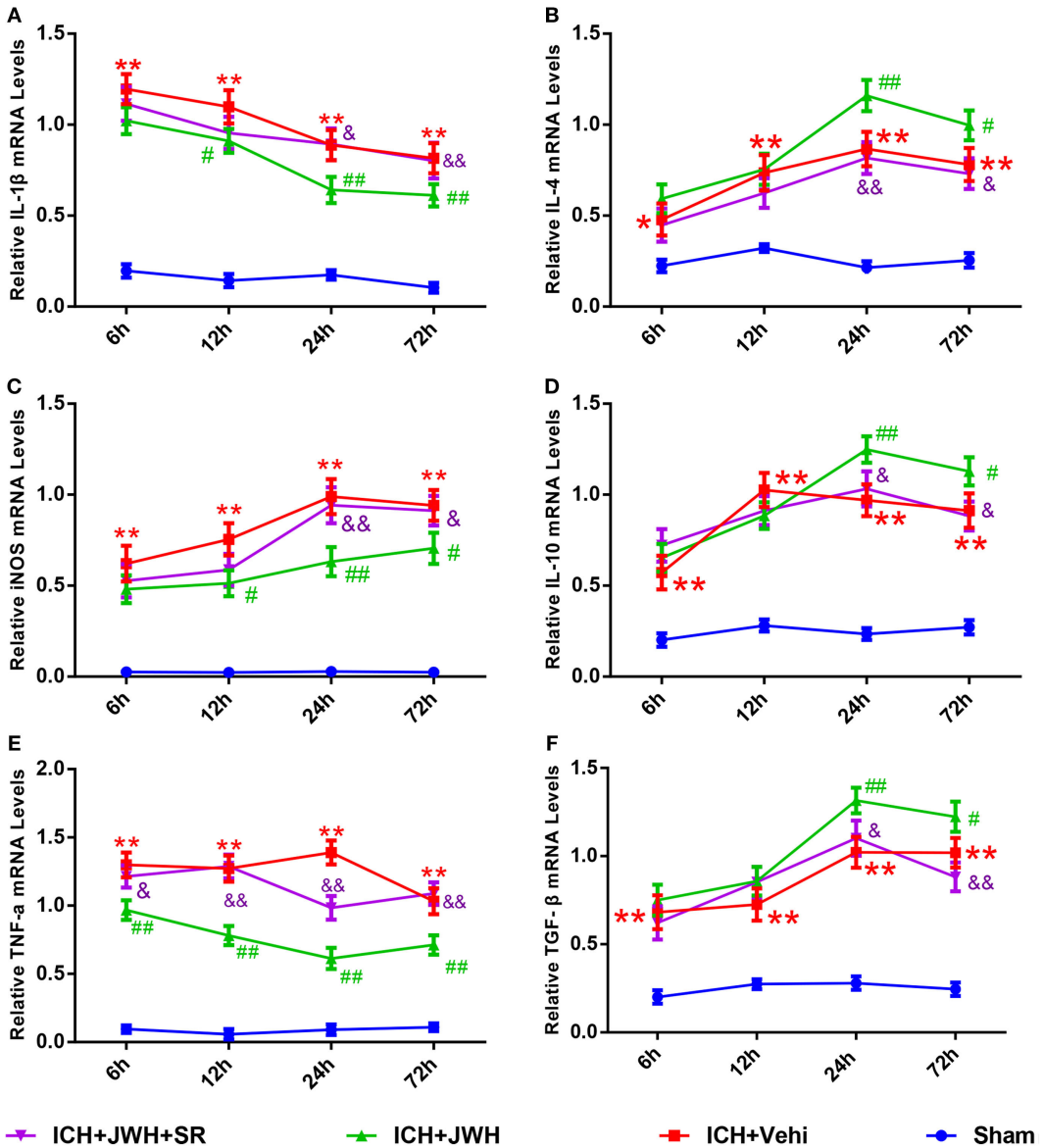

FIGURE 7 | Inflammation cytokines at messenger RNA level in acute phase after intracerebral hemorrhage (ICH). Interleukin-1β **(A)**, iNOS **(C)**, and tumor necrosis factor-α **(E)** are pro-inflammatory cytokines, and the IL-4 **(B)**, interleukin-10 **(D)**, and transforming growth factor beta **(F)** are anti-inflammatory cytokines. The values were mean ± SD, $n = 6$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with sham group, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with ICH + Vehi group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared with ICH + JWH group.

broad suppression of microglia/macrophages may deprive the normal physiological defense mechanism of the CNS and lead to unintended side effects. Therefore, an improved understanding of the dual beneficial and detrimental roles of microglia in CNS injury and recovery is critical for improving stroke treatment (11).

Once activated, microglia/macrophages serve as a double-edged sword during the battle between neurological damage and protection. Microglia can be activated to two polarization states: the M1, classically activated phenotype, and the M2, alternatively activated phenotype (28). Upon stimulation with lipopolysaccharide or interferon gamma, microglia can be activated to the M1 phenotype and produce pro-inflammatory mediators, chemokines, redox molecules, costimulatory proteins, and major histocompatibility complex II (29, 30), exacerbating inflammatory damage. Alternatively, microglia can also be activated to the M2 polarization state, which produces anti-inflammatory mediators that promote brain recovery by scavenging cell debris, resolving local inflammation, and are involved in tissue remodeling (31, 32). There is no doubt that it is very important to understand the status and function of polarized microglia activation for assessing inflammation progression and optimizing treatment.

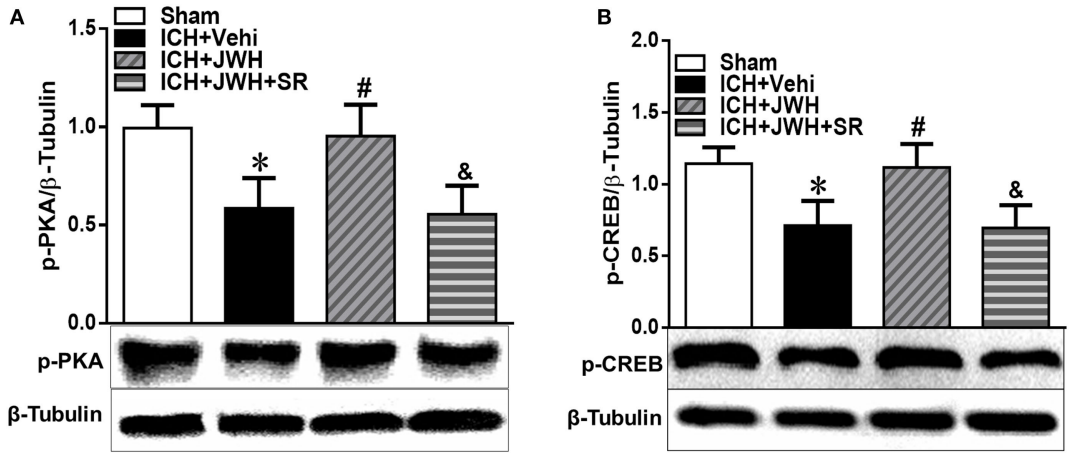

FIGURE 8 | The expression of pPKA–CREB signaling pathway at 24 h after intracerebral hemorrhage (ICH). Total protein around the hematoma was extracted for investigation. Western blot analysis was used to detect the protein expression of **(A)** pPKA and **(B)** pCREB. Values of the relative densitometric analysis were expressed as mean ± SD, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with ICH + Vehi group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared with ICH + JWH group.

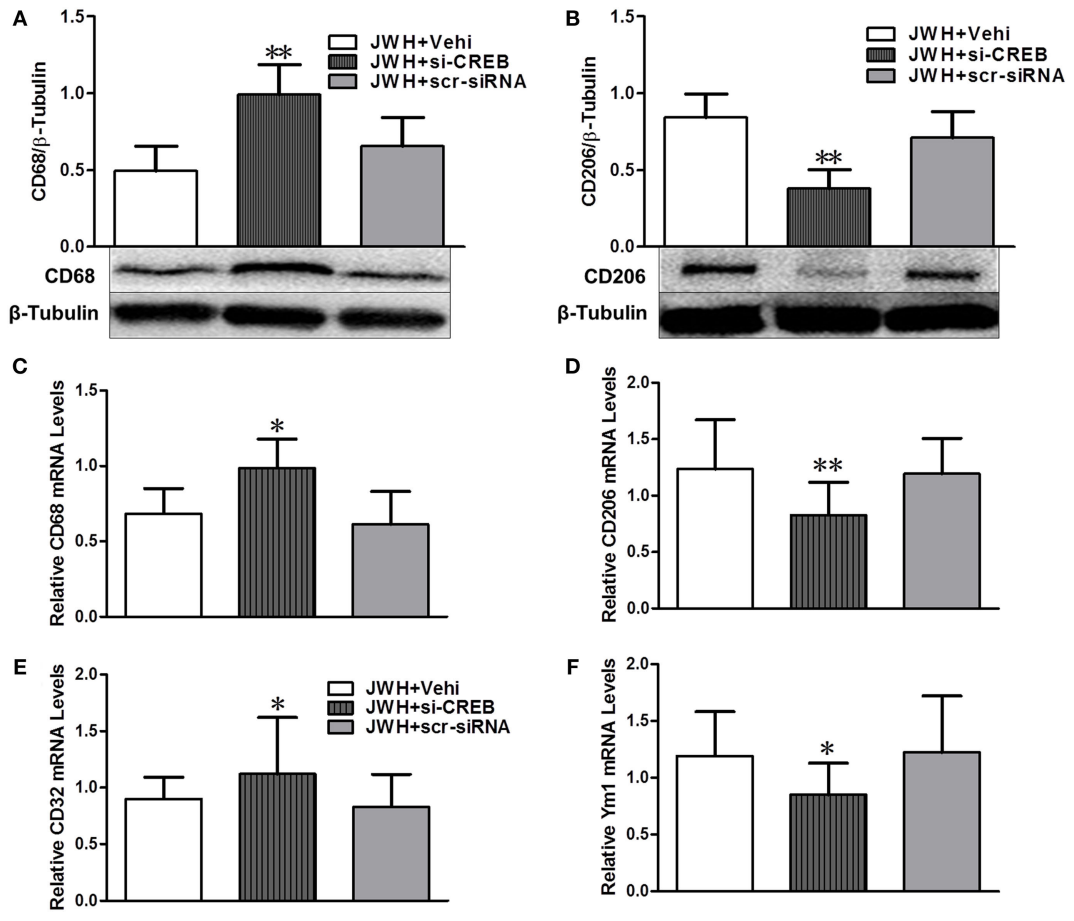

FIGURE 9 | The relationship between pPKA–CREB signaling pathway and microglial polarization. Western blot test and relative densitometric analysis ($n = 6$ in each group) of CD68 **(A)** and CD206 **(B)**. Relative messenger RNA level of M1-associated markers CD68 **(C)** and CD32 **(E)**, and M2-associated markers CD206 **(D)** and Ym-1 **(F)** ($n = 3$ in each group). The values were mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with JWH + Vehi group.

TBI was reported to create a severe cortical lesion, which led to chronic and persistent M1-primed activation that lasted for months to years. On the other hand, ICH often occurs after TBI. However, characteristic of microglial polarization and inflammatory response after ICH remains unclear (33, 34). Xi et al. (11) found that ICH induces microglia activation and polarization in mice. They reported that M1 phenotypic markers were increased and reached a peak as early as 4 h, remained high at 3 days, and decreased 7 days after ICH, whereas M2 phenotypic markers were upregulated later than M1 markers, reaching a peak at day 1 and declined on day 7 after ICH, which is largely consistent with the findings of this study. The results confirmed our hypothesis that in the ICH acute phase, microglia/macrophage were largely activated toward the classical M1 phenotype, and JWH133 influenced the polarization process by promoting the anti-inflammatory M2 phenotype.

We further investigated the intracellular molecular switches that regulate microglia/macrophage polarization. A recent breakthrough in research on microglia/macrophages has revealed that several transcriptional regulators may serve crucial roles in M2-associated marker expression (5, 35). Interferon regulatory factor 4 serves as a key transcriptional factor modulating M2 polarization, whereas the interferon regulatory factor 5 and interferon regulatory factor 8 control macrophages toward M1 polarization. The nuclear hormone receptor peroxisome proliferator-activated receptor γ is an important transcriptional factor that mediates macrophages primed toward M2 polarization (36, 37). CB2R can couple to G_i proteins, and CB2R activation has been shown to activate cAMP/PKA (38). Additionally, it has been demonstrated that CB2R stimulation enhances CREB activation after cerebral ischemia through phosphorylation of AMPK (39). JWH133 was also found to attenuate apoptosis by activation of phosphorylated CREB-Bcl-2 pathway after subarachnoid hemorrhage in rats (27). cAMP signaling is spatially and temporally regulated, allowing for the selective activation of a subset of targets. A-kinase anchoring proteins provided the platform for the assembly of signalosomes, which consist of cAMP effectors and their substrates. Among them, the role that cAMP plays in maintaining both microglia and monocyte homeostasis to prevent M1 activation has been reported. PKA was considered to be the cAMP receptor and seem to be closely associated with inflammation (40). Although CREB could be activated by various signaling pathways, including cAMP/PKA, ERK1/2, and PI3K/Akt (41), in this study, we selectively detected PKA/CREB signaling pathway. CB2R agonist JWH133 significantly enhanced the phosphorylated expression of PKA and CREB and knockdown of CREB significantly inversed the JWH133-induced increase of M2 polarization in microglia, indicating that PKA/CREB pathway participates in the effects of CB2R stimulation on microglia polarization during ICH. The results are in agreement with our previous study that the promotion of microglial M2 polarization through the cAMP/PKA pathway participates in the CB2R-mediated anti-inflammatory effects during GMH (26).

Until now, most studies on microglial polarization have focused on TBI and cerebral ischemia, but relatively little was known about the polarization of microglia/macrophages following ICH (31). In this study, we demonstrated the neuroprotective effects of a CB2R agonist via microglial polarization and the underlying molecular mechanisms that involved in microglial polarization, which may represent a new target for ICH therapy.

In summary, our results reveal microglia/macrophage activation and polarization after ICH in rats. Our data showed that the CB2R agonist JWH133 lessened the brain water content, improved neurobehavioral outcomes, and ameliorated DNA damage and neuron death, which resulted in alleviating brain injury after ICH. We also found that JWH133 inhibited the pro-inflammatory cytokine release and promoted microglia M2 polarization, leading to a beneficial anti-inflammatory cytokine release. JWH133 also facilitated the phosphorylation of PKA and its downstream effector, CREB. Moreover, knockdown of CREB *in vivo* abolished the effects of JWH133 on microglial polarization, indicating that the PKA/CREB signaling pathway plays a critical role in the JWH133-mediated effects. Therefore, CB2R may provide a new target to modulate microglia/macrophage-mediated inflammatory injury and recovery after ICH.

ETHICS STATMENT

All institutional and national guidelines for the care and use of laboratory animals were followed.

AUTHOR CONTRIBUTIONS

LL, TY, FZ, NY, TQ, ZH, CQ, TJ, and ZY contribute to the implementation of the experiment. ZG, FH, YY, and CZ contribute to the design and paper writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00112/full#supplementary-material.

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REVIEW
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Mitochondria-Derived Damage-Associated Molecular Patterns in Neurodegeneration

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Inflammation is increasingly implicated in neurodegenerative disease pathology. As no acquired pathogen appears to drive this inflammation, the question of what does remains. Recent advances indicate damage-associated molecular pattern (DAMP) molecules, which are released by injured and dying cells, can cause specific inflammatory cascades. Inflammation, therefore, can be endogenously induced. Mitochondrial components induce inflammatory responses in several pathological conditions. Due to evidence such as this, a number of mitochondrial components, including mitochondrial DNA, have been labeled as DAMP molecules. In this review, we consider the contributions of mitochondrial-derived DAMPs to inflammation observed in neurodegenerative diseases.

Keywords: damage-associated molecular pattern, mitochondria, neuroinflammation, neurodegeneration, sterile inflammation

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INTRODUCTION

Inflammatory pathways are activated through either pathogen-initiated or damage-initiated events. Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) activate similar inflammatory cascades and are therefore difficult to distinguish. Inflammation stimulated by DAMPs is an area of study that has recently gained notice. In particular,

Abbreviations: 3NP, 3-nitropropionic acid; ACS, apoptosis-associated speck-like protein containing a CARD; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APOE, apolipoprotein E; APP, amyloid precursor protein; ATP, adenosine triphosphate; BBB, blood–brain barrier; CD33, Siglec-3; CD38, cluster of differentiation 38; CLU, clusterin; CNS, central nervous system; COX, cytochrome oxidase; CR1, complement rector 1; CSF, cerebral spinal fluid; CSF1R, colony-stimulating factor 1 receptor; CXCR31, CX3C chemokine receptor 1 or fractalkine receptor; cybrid, cytoplasmic hybrid; DAMP, damage-associated molecular pattern; EPHA1, ephrin type A receptor 1; ER, endoplasmic reticulum; FCγRII, Fc receptor or CD32; FPR or FPRL1, formyl peptide receptors; FTLD, frontotemporal lobar degeneration; GFAP, glial fibrillary acidic protein; GWAS, genome-wide association study; HLA-DRB5/DRB1, major histocompatibility complex, class II, DR beta 5; HMGB1, high mobility group box 1; IFN-γ, interferon-γ; IgG, immunoglobulin G; IL-1, interleukin-1; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; INPPD5, inositol polyphosphate-5-phosphatase; IP, intraperitoneal; LPS, lipopolysaccharide; LRRK2, leucine-rich repeat kinase 2; MCI, mild-cognitive impairment; MCP-1, monocyte chemotactic protein 1; M-CSF, macrophage colony-stimulating factor; MHC, major histocompatibility complex; MIP-1α, macrophage inflammatory protein-1α; MMP-8, matrix metalloproteinase 8; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mtDNA, mitochondrial DNA; NLRP3, NLR family pyrin domain containing 3; NO, nitric oxide; NOD, nucleotide-binding oligomerization domain-like receptors; NSAID, non-steroidal anti-inflammatory drug; PAMP, pathogen-associated molecular pattern; PD, Parkinson's disease; PET, positron emission tomography; PRR, pattern-recognition receptor; RAGE, receptor for advanced glycation end products; RANTES, regulated on activation, normal T cell expressed and secreted; ROS, reactive oxygen species; SNP, single-nucleotide polymorphism; SOD1, superoxide dismutase 1; SRA, microglial scavenger receptor A; TBI, traumatic brain injury; TFAM, transcription factor A; TGF-β, transforming growth factor β; TNFα, tumor necrosis factor α; TREM2, triggering receptor expressed on myeloid cells 2.

DAMPs derived from mitochondrial components are interesting due to the prokaryotic origin of this organelle. Furthermore, mitochondrial-derived DAMP molecules may play a role in heart disease, arthritis, liver disease, trauma, and sepsis (1–5).

Neuroinflammation and mitochondrial dysfunction are observed across numerous neurodegenerative diseases (6–8). Mitochondrial dysfunction can induce inflammation and *vice versa*. Mitochondrial dysfunction may modulate the release of mitochondria-derived DAMP molecules (9, 10). Here, we discuss the relationship between neuroinflammation, mitochondrial dysfunction, and mitochondria-derived DAMP molecules in the context of neurodegenerative diseases.

NEUROINFLAMMATION IN NEURODEGENERATIVE DISEASES

Neuroinflammation is classically defined as proliferation and activation of microglia (microgliosis), and/or astrocytes (astrogliosis). Microglia are macrophage cells of mesenchymal origin, which monitor the central nervous system (CNS) for pathogens. Astrocytes are derived from the ectoderm and have numerous functions including metabolic support for neurons, regulating synapses, brain structure, and repair. Further evidence of neuroinflammation can encompass activation of inflammatory pathways, increased expression of cytokines or chemokines, and in some cases disruption of the blood–brain barrier (BBB) accompanied by infiltration of peripheral immune cells (such as T cells). Neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) all have evidence of neuroinflammation pathology.

Alzheimer's Disease

Initial interest in neuroinflammation as a causative factor in AD centered on the link between reduced risk for AD and long-term non-steroidal anti-inflammatory drug (NSAID) use. Early evidence from epidemiological studies suggested that long-term use of NSAIDs led to a decreased risk of developing AD (11, 12). Subsequent studies found a correlation between apolipoprotein E (APOE, a genetic risk factor for AD) genotype and the protective effects gained from NSAID use. The correlation between decreased AD risk and NSAID use is greatest in individuals who harbor an APOE ε4 allele (13, 14). Furthermore, the age of the individual taking NSAIDs and the type of NSAID administered affect the association of AD risk reduction (15).

Clinical trials investigating whether NSAIDs might benefit AD subjects, though, were disappointing. Early trials experienced high attrition rates among participants due to adverse effects and thus did not provide clear answers (16, 17). A larger trial of one NSAID, tarenflurbil, showed largely negative results (18). However, a recent publication that showed positive memory and brain inflammation outcomes for a different NSAID, fenamate, in an AD mouse model has led to renewed interest (19).

Genome-wide association studies (GWAS) have also renewed interest for neuroinflammation as a potential causative factor for AD. In particular, a rare variant of triggering receptor expressed on myeloid cells 2 (TREM2), R47H, is associated with an increased risk for late onset Alzheimer's disease (LOAD) (20). TREM2 is a membrane protein in myeloid cells (such as microglia), which modulates inflammatory pathways by inhibiting cytokine production. TREM2 is likely responsible for determining microglial phenotypes (i.e., M1 activation versus M2 activation). Furthermore, TREM2 is also important for lipid sensing, providing a further link between neuroinflammation and bioenergetic pathways (21). The R47H TREM2 variant leads to a reduction in microglial phagocytosis (22). Soluble TREM2 levels are increased in cerebral spinal fluid (CSF) of AD subjects, a parameter that positively correlates with gray matter volume but negatively correlates with diffusivity (sometimes considered a function of cell integrity) (23). Other single-nucleotide polymorphisms (SNPs) associated with AD risk also affect inflammatory pathways. These include SNPs in complement receptor 1, clusterin (CLU), major histocompatibility complex (MHC), class II, DR beta 5, ephrin type A receptor 1, inositol polyphosphate-5-phosphatase, and or Siglec-3 (24–29). The relationship of each of these genes to inflammatory signaling has been reviewed elsewhere (30).

Microglia and reactive astrocytes are closely associated with amyloid plaques in AD brain (31, 32). The quantity of interleukin-1 (IL-1) reactive microglia is increased sixfold in AD brain (33). Furthermore, microglial IL-1 expression correlates with plaque distribution (34). Levels of macrophage colony-stimulating factor, an activator of macrophages, was found to be increased in plasma and CSF from AD subjects compared to those with mild-cognitive impairment (35). A number of cytokines and chemokines, which can be released by macrophages or other immune cells, are increased in AD, including interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor α (TNFα), interleukin-8 (IL-8), transforming growth factor β, and macrophage inflammatory protein-1α (36).

Parkinson's Disease

The discovery of reactive microglia in the substantia nigra of PD brain tissue provided early evidence for the role of neuroinflammation in PD (37). Further evidence for microglial activation in PD brain came from positron emission tomography (PET) imaging studies. PET studies showed increased microglial activation as evidenced by increased levels of peripheral benzodiazepine sites, a selective marker of activated microglia (38). Increased levels of glial cells expressing TNFα, IL-1β, and interferon-γ (IFN-γ) have also been observed in the substantia nigra of deceased PD subjects (39). Furthermore, microglial activation is observed in animal models of PD (36, 40).

Studies on astrogliosis in the PD brain have generated conflicting results. Early studies suggested increased astrogliosis in the PD brain. More recent studies have shown minimal levels of nigral astrogliosis, although astrocytic accumulation of α-synuclein was observed. The authors speculated that α-synuclein accumulation may cause astrocytes to be less reactive (41). A subsequent report showed minimal astrogliosis and an inverse correlation between levels of α-synuclein and astrogliosis in the PD brain, suggesting α-synuclein may suppress astrogliosis (42).

Similar to AD, PD risk is reduced with regular or chronic NSAID use. Long-term aspirin use is associated with less PD risk; however, non-aspirin-based NSAID use afforded better risk reduction. Furthermore, women who used aspirin in a chronic

(greater than 24 months) or regular manner had a lower risk for PD than men (43).

Genome-wide association studies have identified the gene that encodes leucine-rich repeat kinase 2 (LRRK2) as a risk factor for sporadic PD. LRRK2 mutations are also associated with autosomal dominant forms of PD (44). Similar to TREM2 in AD, LRRK2 mediates microglial function (45, 46). LRRK2 facilitates vesicle trafficking and cytoskeletal remodeling and may skew microglia toward a pro-inflammatory phenotype (45–47).

Changes in peripheral inflammation may contribute to PD. Studies of peripheral inflammation showed an increased ratio of CD8+ T cells to CD4+ T cells in the blood of PD patients (48). Peripheral inflammation may affect the brain through disruption of the BBB, an event that is observed in PD subjects (49). Furthermore, peripheral inflammatory changes correlate with PD risk. Increased plasma IL-6 levels correlated with disease risk, although with a small sample size (50). CD8+ and CD4+ T cell reactivity is increased in the substantia nigra of PD patients. Furthermore, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin used to generate a commonly utilized PD animal model, was found to increase T cell infiltration into the substantia nigra (51).

Amyotrophic Lateral Sclerosis

Inflammatory changes have been observed in the CNS of ALS patients. Early studies in ALS spinal cord and motor cortex discovered accumulation of immunoglobulin G and complement (52). Subsequent studies found T cell infiltration and increased levels of MHC I and II antigens on macrophages and dendritic cells (53, 54). Immunohistochemical studies in postmortem brain tissue showed increased levels of phagocytic and leukocyte surface proteins on microglia, as well as evidence to support the infiltration of activated lymphocytes into the precentral gyrus (55). PET studies using PK11195, a ligand that binds microglia, have provided further evidence of microglial activation in ALS (56).

Astrogliosis is observed within the spinal cord (ventral and dorsal horns) and brain (cortical gray matter and subcortical white matter) in postmortem ALS subjects (57). Microgliosis is evident in the spinal cord ventral horn, the corticospinal tract, and the motor cortex (55). The role of microglia and astrocytes in ALS disease progression is further supported in transgenic mutant superoxide dismutase 1 (SOD1) ALS mouse models. Selective deletion of mutant SOD1 from microglia (which also removes expression from peripheral myeloid cells) slowed disease progression (58). Similar results were observed when mutant SOD1 expression was deleted from astrocytes (59). Conversely, deleting mutant SOD1 expression from motor neurons had no effect on disease progression or survival, but did delay disease onset (58). These studies emphasize the potential role of neuroinflammation in ALS disease progression.

Genome-wide association studies in ALS have linked inflammatory genes with disease outcome. A SNP in CX3C chemokine receptor 1 (CXCR31, fractalkine receptor) is associated with reduced survival in sporadic ALS subjects; however, this SNP fails to associate with ALS risk (60). CXCR31 is important for the migration of leukocytes and may play a role in microglial migration (61). RNA seq and ingenuity pathway analysis of postmortem ALS spinal cord samples found upregulation of inflammatory pathways, with TNFα being a predicted upstream regulator of inflammation in these ALS samples (62).

Neuroinflammation is evident in AD, PD, and ALS. However, whether or not neuroinflammation contributes to disease onset, progression, and risk requires further study. To this end, a growing appreciation of a relationship between mitochondrial dysfunction and inflammatory pathways may provide insight into this important question. Below, we discuss evidence for mitochondrial dysfunction in AD, PD, and ALS.

Primary Mitochondrial Diseases

Primary mitochondrial diseases are caused by mutations in mitochondrial DNA (mtDNA) or nuclear DNA genes that encode mitochondrial proteins. Diseases caused by mtDNA mutations include Leber's hereditary optic neuropathy, myoclonic epilepsy and ragged red fiber, neuropathy ataxia and retinitis pigmentosa, Kearns–Sayre syndrome, and Leigh's syndrome. Diseases caused by a nuclear DNA mutation of which affects a mitochondrial localized protein include Friedreich's ataxia, Wilson's disease, and Mohr–Tranebjaerg syndrome. Commonly, these diseases manifest in neurological symptoms. A few of these diseases are associated with neuroinflammation pathology.

Friedreich's ataxia is caused by autosomal recessive inheritance of a mutant *Frataxin* gene. The product of the *Frataxin* gene is responsible for iron homeostasis within mitochondria, and loss of this gene in Schwann cells leads to reduced mitochondrial respiration, inflammation, increased mitochondrial iron concentrations, and cell death (63–65). COX2 expression is elevated in both animal models and Friedreich's ataxia patient lymphocytes, an indicator of increased inflammation (66).

For subjects with Leigh's syndrome, mtDNA mutations occur in several genes including ATPase 6, ND 1–6 (NADH dehydrogenase), and CO3 [cytochrome oxidase (COX)] (67, 68). These subjects often have deficient ETC enzyme activities (67). In a mouse model of Leigh's syndrome, evidence of neuroinflammation is abundant (69). However, inflammatory markers have not been measured from human subject tissues.

Wilson's disease is caused by a mutation in the *ATP7B* (ATPase copper transporting β polypeptide) gene and is characterized by liver disease, ataxia, parkinsonism, seizures, and reduced cognition (70, 71). This gene encodes a copper transporting ATPase that localizes to mitochondria and affects mitochondrial copper levels (70, 72). Subjects with this mutation have reduced ETC function (73, 74). Pentraxin 3, a marker of inflammation, is elevated in the serum of Wilson's disease subjects (75).

Despite the association of mitochondrial dysfunction and neuroinflammation or inflammation (discussed below), these processes have not been extensively studied in primary mitochondrial diseases. Future research endeavors into this area would likely benefit our understanding of these diseases.

MITOCHONDRIAL DYSFUNCTION IN NEURODEGENERATIVE DISEASES

The Krebs cycle and oxidative phosphorylation occur in the matrix and inner mitochondrial membrane, respectively. Oxidative

phosphorylation requires the mitochondrial respiratory chain. These bioenergetic pathways generate the high energy compound adenosine triphosphate (ATP) (76). Mitochondria and bioenergetic intermediates generated within mitochondria regulate cell signaling pathways (including pro-inflammatory responses, as discussed below).

The brain comprises approximately 2% of the body's weight yet consumes about 20% of its oxygen uptake. The brain requires high amounts of energy for numerous processes, including neurotransmitter production and synaptic activity. Therefore, the brain is highly susceptible to mitochondrial dysfunction, which has been observed in several neurodegenerative diseases including (but not limited to) AD, ALS, and PD (77–80). Furthermore, mitochondrial dysfunction declines with age, and age is the greatest risk factor for these neurodegenerative diseases (78, 80). Mitochondrial dysfunction can lead to increased reactive oxygen species (ROS) production, decreased ATP production, alterations in mitochondrial membrane potential, damage to mtDNA, and activation of cell death pathways (81).

Alzheimer's Disease

In postmortem AD brains, decreased COX function, reduced intact mitochondrial number, and increased mitochondrial autophagy have been reported (78, 82–86). Mitochondrial dysfunction appears to be systemic in AD, as deficits in COX activity are apparent in AD patient fibroblasts and platelets (83, 87–89). Changes in mtDNA may drive cell signaling changes, bioenergetic pathway deficits, and histopathological hallmarks of AD. Cytoplasmic hybrid (cybrid) studies in which mtDNA from human AD subjects is transferred into a donor cell line that lacks its own mtDNA provides a system in which mtDNA-derived biochemical and molecular consequences can be assessed. The cybrid model system controls for nuclear DNA alterations, as patient mtDNA is transferred into the context of a consistent nuclear DNA background (90). Cybrid cells generated using AD patient mtDNA have reduced COX activity, increased ROS production, and increased Aβ deposition (90, 91).

Evidence of mtDNA mutations, deletions, and oxidative modifications are present in AD subjects (92–97). mtDNA is inherited from the mother, and interestingly a maternal inheritance pattern for AD has been noted. This maternal inheritance pattern is associated with early changes in brain atrophy and mitochondrial biomarkers (98–103). Finally, mitochondrial haplotypes are associated with increased AD risk (104–106). These studies suggest changes in mitochondrial function, possibly at the level of mDNA maintenance and inheritance, are important in AD pathology.

Parkinson's Disease

The most studied respiratory chain aberration in PD is a deficit in complex I activity. Initial insight into this deficit stems from cases of recreational drug users exposed to MPTP. After MPTP exposure, individuals developed parkinsonian symptoms and at autopsy were found to have degeneration in the substantia nigra, similar to that observed with PD. This degeneration occurred in the absence of Lewy bodies (or aggregated α-synuclein). Following its accidental discovery, MPTP was adapted to produce monkey and rodent models of PD (107–109). MPTP is oxidized to MPP+, which accumulates in neurons and is a potent complex I inhibitor (107, 110).

Complex I deficits are observed in postmortem brain, platelets, and fibroblasts from PD subjects (111, 112). The observed deficits in complex I activity could be driven by oxidative damage to its catalytic subunits or altered mtDNA (90, 113, 114). Cybrid cell lines generated from PD subject mtDNA recapitulate the complex I deficit. Sporadic PD cybrid cell lines also show reduced mtDNA copy number, reduced ATP, cell death pathway activation, and a relatively depolarized mitochondrial membrane potential (113–115).

Parkinson's disease risk is associated with mtDNA haplotype, similar to AD (116–118). Changes to mtDNA are also observed in PD, such as mutations and deletions (119–121). Polymorphisms of mtDNA polymerase γ influences PD risk as well (122). Overall, mitochondrial function and mtDNA inheritance and maintenance are important to the pathology of PD.

Amyotrophic Lateral Sclerosis

ALS subjects have evidence of mitochondrial dysfunction within the CNS and periphery. CNS mitochondrial abnormalities include altered mitochondrial morphology, mitochondrial inclusions/aggregates, reductions in COX activity, and lower mtDNA levels with increased levels of mtDNA point mutations and deletions (123–128). COX deficits, lowered mitochondrial number, altered mitochondrial calcium levels, and mtDNA deletions, are also observed in ALS subject muscle (129–131). Lymphocytes from ALS subjects have reduced mitochondrial maximum respiration, liver mitochondria appear swollen, and platelet mitochondria manifest a depolarized mitochondrial membrane potential with increased apoptosis (132–135). Cybrid cells generated from ALS patient mtDNA have alterations in antioxidant enzyme activity and reduced complex I activity (136). The association of ALS with distinct mtDNA haplotypes is under investigation (137).

MITOCHONDRIAL DYSFUNCTION AND INFLAMMATION

Mitochondria are important modulators of innate immunity pathways. Mitochondria-derived ROS, calcium, and ATP are signaling molecules that activate inflammatory responses. Under conditions in which mitochondria are damaged, such as accumulated mtDNA mutations or mitochondrial dysfunction (possibly through aberrant ROS production), a sustained inflammatory response and downstream pathological inflammation could ensue. A relationship between mitochondrial dysfunction and inflammatory signaling is discussed below.

Mitochondria can directly activate inflammasome signaling. Mitochondria-derived ROS activate the NLR family pyrin domain containing 3 (NLRP3) inflammasome pathway. NLRP3 is normally associated with the endoplasmic reticulum membrane, but upon activation is redistributed to nuclear and mitochondrial membranes, where it oligomerizes with apoptosis-associated speck-like protein containing a CARD (ACS) and pro-caspase 1 to form the NLRP3 inflammasome (138–140). Mitochondria

also mediate inflammatory pathway activation through redox sensitive proteins (140).

Mitochondrial dysfunction initiates inflammation across various models. *In vitro* and *in vivo* experiments demonstrate complex II inhibition using 3-nitropropionic acid (3NP) induces microglial activation and reduces the ability of microglia to undergo alternate activation (141). Human microglial cells treated with 3NP became activated and showed increased ROS production and cell death rates. Similar results were observed following intrastriatal 3NP injection in adult rats, where microglial activation, ROS production, and cell death were increased (142). Chronic subcutaneous injections of rotenone in rats (a complex I inhibitor that is used to model PD in rodents) increases IL-1β within the hypothalamus. These rats also displayed a decreased number of tyrosine hydroxylase positive neurons in the substantia nigra, perturbed locomotion, and sleep abnormalities (143).

Conversely, pro-inflammatory cytokines also modulate mitochondrial function. For example, TNFα can decrease complex I activity, reduce ATP production, depolarize the mitochondrial membrane potential, increase ROS, and lower activities of complexes II and IV (depending on the cell type). In hepatocytes, TNFα uncouples mitochondria and increases ROS production. This ROS production is generated from complex I and III of the respiratory chain and initiates NFκB activation (144, 145). Similar effects have been reported in L929 cells (a mouse fibroblast cell line), a leukemia cell line, and 3T3-L1 adipocytes (146–148). In a mouse hippocampal cell line (HT22), TNFα induces mitochondrial respiration deficits and a loss of mitochondrial membrane potential (149). IL-1β also yields similar effects on mitochondrial function (150). Nitric oxide (NO), another inflammatory signaling molecule, disrupts mitochondrial membrane potential and inhibits COX activity. In human retinal pigment cells, TNFα, IL-1β, and IFN-γ increase the production of both mitochondrial- and NADPH oxidase-derived ROS (151).

In vivo studies also suggest pro-inflammatory molecules influence mitochondrial function. Intraperitoneal injection of lipopolysaccharide in rodents increases brain cytokine and inflammatory receptor expression [including IL-1β, toll like receptor 2 and 4, microglial scavenger receptor A (SRA), and Fc receptor (FCγRII)] in a region-specific manner. The authors also reported increased microglial number and mitochondrial functional alterations, including decreased glutathione (an antioxidant) and increased complex II/III activity (152).

A clear relationship between mitochondrial dysfunction and inflammatory cascades exists. Both of these pathological hallmarks are present across multiple neurodegenerative diseases. Mitochondria-derived DAMP molecules could provide a further link between these pathologies. Below, we discuss mitochondria-derived DAMP molecules, potential routes of release, and evidence for their role in neuroinflammation.

MITOCHONDRIA-DERIVED DAMPs: INDUCERS OF NEUROINFLAMMATION?

Previously identified mitochondria-derived DAMP molecules include mtDNA, cardiolipin, ATP, mitochondrial transcription factor A (TFAM), cytochrome *c*, and formyl-methionine-labeled peptides (30). Formyl-methionine-labeled peptides may also include mitochondrial protein-derived cryptides. Cryptides are endogenous, fragmented functional peptides. Pro-inflammatory cryptides from mtDNA and nuclear DNA-encoded mitochondrial proteins were recently described (153–155). As reviewed in **Table 1**, each of these molecules has been observed to initiate a pro-inflammatory phenotype under various disease and pathological states.

Damage-associated molecular pattern molecules activate inflammatory signaling in a manner similar to PAMPs. The danger molecule is recognized by a pattern-recognition receptor (PRR), and adaptor molecules initiate intracellular signaling cascades and cytokine production. Similar to PAMPs, mitochondria-derived DAMPs are recognized by various PRRs. These include TNF receptor, NLRP3, IL-1 receptor, nucleotide-binding oligomerization domain-like receptor, receptor for advanced glycation end products, formyl peptide receptors (FPR or FPRL1), and purogenic receptors. With the increasing recognition of mitochondria-derived DAMP molecules as pathogenic instigators in various diseases (**Table 1**), recent studies have begun to explore their contribution to neuroinflammation.

Mitochondria-Derived DAMP Molecules in Neuroinflammation

In the periphery, TFAM functions as a potent DAMP molecule (**Table 1**). Recently, it was found that TFAM, in combination with IFN-γ, can activate human microglial cells, human peripheral blood monocytes, and THP1 monocytic cells (173). Cotreatment of THP1 monocytic cells with IFN-γ and TFAM or TFAM alone lead to an increase in cytokine expression (including IL-1β, IL-6, and IL-8). While treatment of SH-SY5Y neuroblastoma cells with IFN-γ and TFAM was not toxic, exposure to conditioned medium from monocytic cells activated with IFN-γ and TFAM-induced SH-SY5Y cell death. Finally, mitochondrial proteins extracted from THP1 monocytic cells produced effects similar to TFAM.

Degraded and oxidized mtDNA can initiate pro-inflammatory pathways in astrocytes. Exposure of mtDNA to hydrogen peroxide can induce its degradation, and these mtDNA degradation products are found in human CSF and plasma (9). Furthermore, transfection of mouse primary astrocytes with oxidant-

TABLE 1 | Mitochondria-derived damage-associated molecular pattern molecules.

Molecule	Disease or pathologic context	Reference
Mitochondrial DNA	Trauma, heart failure, arthritis, Parkinson's disease, Alzheimer's disease, aging	(1, 3, 156–158)
Cardiolipin	Arthritis, bowl disease, myocardial infarct/heart disease	(159–166)
Adenosine triphosphate	Atherosclerosis, lung inflammation/fibrosis	(167, 168)
Formyl-methionine-labeled peptides	*In vitro* neuron degeneration, inflammatory activation of neutrophils and macrophages	(169–172)
Transcription factor A	*In vitro* microglial activation, hemorrhagic shock	(173, 174)
Cytochrome *c*	Arthritis, liver injury, myocardial infarct/heart disease, SIRS	(175, 176)

initiated, degraded mitochondrial polynucleotides caused a pro-inflammatory response. This inflammatory astrocyte phenotype was characterized by the upregulation of IL-6, monocyte chemotactic protein 1, TNFα, and IL-1β (9). It was also previously reported that mtDNA is degraded in response to hydrogen peroxide in HA-1 hamster ovarian cells, an effect that was not observed with nuclear DNA or cytoplasmic RNA. Degradation of the mitochondrial genome was apparent in both mtDNA and mitochondrial RNA species (10). These observations suggest a mechanism for mtDNA degradation, and also for the downstream activation of glial cell pro-inflammatory phenotypes.

Mitochondrial components induce inflammation in microglial (BV2) and neuronal (SH-SY5Y) cells (177). In the defining experiments, these cells were exposed to mitochondrial lysates prepared from SH-SY5Y cells containing mtDNA or alternatively SH-SY5Y cells lacking mtDNA. BV2 microglial cells exposed to mitochondrial lysates containing mtDNA had increased TNFα, IL-8, and matrix metalloproteinase 8 mRNA but decreased TREM2 mRNA. Furthermore, NFκB nuclear localization was increased. These effects were not observed when BV2 microglial cells were exposed to mitochondrial lysates prepared from cells that lacked mtDNA. In SH-SY5Y neuronal cells exposed to mitochondrial lysates containing mtDNA, TNFα mRNA and NFκB protein expression were elevated. In addition, mitochondrial lysate-exposed SH-SY5Y cells showed increased amyloid precursor protein (APP) mRNA and protein. Changes in APP expression or pro-inflammatory pathways did not occur when SH-SY5Y cells were exposed to mitochondrial lysates that lacked mtDNA.

Mitochondria-derived DAMP molecules induce neuroinflammation *in vivo*. We recently observed stereotactic injection of mitochondrial lysates or mtDNA into rodent hippocampi induced pro-inflammatory changes (178). Mitochondrial lysates increased hippocampal TNFα mRNA and decreased TREM2 mRNA expression. In addition, NFκB phosphorylation was elevated in the cortex, while glial fibrillary acidic protein (GFAP) protein levels were elevated within the hippocampus. Hippocampal mtDNA injection lead to increased hippocampal TNFα mRNA but reduced hippocampal TREM2 mRNA, increased GFAP hippocampal protein expression, elevated cortical NFκB phosphorylation, increased cortical colony-stimulating factor 1 receptor protein expression, and increased levels of phosphorylated AKT within the cortex. Beyond these inflammatory changes, whole mitochondria lysates increased protein and mRNA levels of endogenous rodent APP and $A\beta_{1-42}$. These effects on APP and $A\beta_{1-42}$ were not observed following injection of mtDNA. Overall, these studies provide evidence that mitochondria-derived DAMP molecules are capable of inducing neuroinflammation, as well as altering AD-related pathways.

While some studies suggest Aβ is pro-inflammatory and acts as a DAMP molecule, a recent study interestingly suggests Aβ has antimicrobial properties (179). In models that overproduce Aβ, including rodent, cell culture, and worm models, the severity of fungal and microbial infections was reduced. Injection of the bacterium *S. typhimurium* into the brain of an AD mouse model (5XFAD model) increased the propensity of Aβ to form plaques, and Aβ colocalized with the bacteria. In addition, the 5XFAD mice injected with *S. typhimurium* showed increased survival and reduced meningitis. This study further found that oligomerization of Aβ was required for this antimicrobial property to manifest. Earlier *in vitro* studies also showed Aβ initiated antimicrobial activity against several bacterial and fungal microorganisms. Aβ inhibited bacterial growth, and brain homogenates from AD subjects (containing Aβ) were also capable of inhibiting microbial growth (180). These studies suggest a possible connection between mitochondria-derived DAMP molecules, neuroinflammation, APP metabolism, and Aβ.

Mitochondria-Derived DAMP Molecules As Biomarkers of Brain Integrity

Cell-free circulating mitochondrial components (DAMPs) such as mtDNA are altered in trauma. For example, in children with traumatic brain injury (TBI), CSF mtDNA levels are elevated. CSF mtDNA levels also correlate with TBI outcome. In children who survived a TBI, CSF mtDNA levels were in the lower range, while in children whose outcome included either death or severe disability CSF mtDNA levels were in the upper range. CSF levels of another DAMP molecule, high mobility group box 1, correlated with mtDNA levels in these subjects. Overall, mtDNA appears to represent a potential CSF mitochondrial DAMP biomarker for TBI and to have the potential to correlate with patient outcomes (3).

Circulating mtDNA is also associated with aging. In a study of 831 Caucasian subjects spanning ages 1–104 years, plasma mtDNA levels began to increase after the fifth decade of life. Plasma mtDNA levels further correlated with cytokine levels, specifically TNFα, IL-6, and regulated on activation normal T cell expressed and secreted (RANTES). Subjects with the highest levels of plasma mtDNA also had the highest levels of these cytokines (TNFα, IL-6, and RANTES), while subjects with the lowest plasma mtDNA levels had the lowest amounts of measured cytokines (156).

Circulating mtDNA may also potentially have the ability to serve as an AD or PD biomarker. Cell-free CSF mtDNA levels are reduced in clinically asymptomatic subjects with a genetically defined increased AD risk and clinically symptomatic AD subjects (157). However, no differences were observed for subjects with frontotemporal lobar degeneration. In a separate study, PD subjects were found to have lower levels of cell-free CSF mtDNA (158). While some may argue lower cell-free CSF mtDNA negates the possibility of a mitochondria-derived DAMP-induced neuroinflammation, other evidence contradicts this idea. It is important to note levels of another AD biomarker, Aβ, are elevated in the brain but significantly reduced in CSF from AD subjects (181, 182). Therefore, it is difficult to draw conclusions regarding CSF biomarker data and causation without sufficient knowledge of the mechanisms that underlie those biomarker changes.

Specific Release of Mitochondria-Derived DAMP Molecules within the CNS

An important question relevant to the issue of mitochondria-derived DAMPs is how might these intracellular molecules access

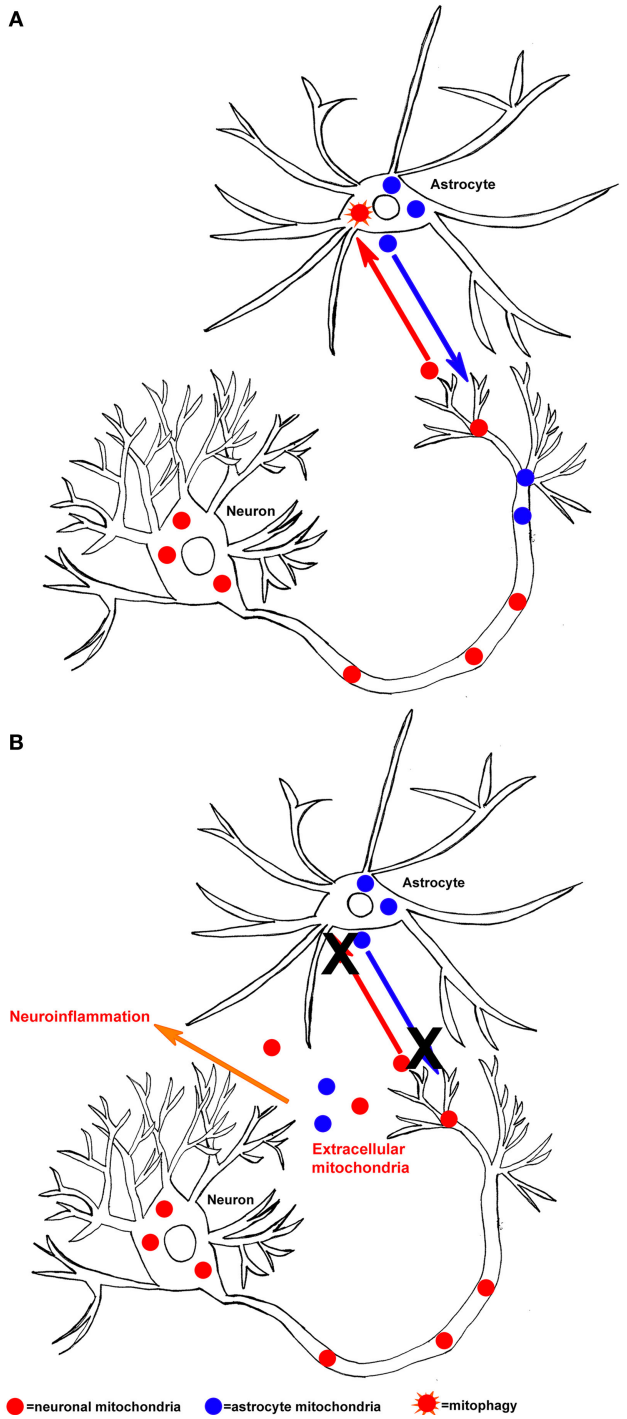

FIGURE 1 | Hypothesized mechanism of mitochondrial-derived damage-associated molecular pattern molecule released in the central nervous system. (A) Neurons can release mitochondria (neuronal mitochondria are shown in red) to astrocytes, where they then undergo mitophagy (orange star shape). In addition, astrocytes can release mitochondria (astrocyte mitochondria are shown in blue) to neurons, under conditions of bioenergetic stress. **(B)** If the process of mitochondrial exchange between neurons and astrocytes malfunctions (X), then mitochondria and their components could be released into the extracellular space and initiate neuroinflammation.

the extracellular space? While cellular components are released during necrotic cell death, more specific process through which mitochondria are released to the brain's extracellular compartment have recently been described. One of these processes has been termed transcellular mitophagy. Neurons are large cells and can span long distances. Mitochondria continuously move between the cell body and dendrites, and between the cell body and axons. When peripheral mitochondria cease to function properly, they were believed to return to the cell body for disposal through the process of mitochondrial autophagy or mitophagy. Data now indicate neurons can also export mitochondria to surrounding glial cells, where they then undergo mitophagy. This process was first described to occur within the optic nerve and the cortex (183). In a separate study, it was found that astrocytes also transfer mitochondria to neurons (184). The transfer of mitochondria from astrocytes to neurons enhanced neuronal survival following ischemia reperfusion injury and required signaling from cluster of differentiation 38, cyclic ADP ribose, and calcium.

CONCLUSION

In specific neurodegenerative diseases, if mitochondrial dysfunction overwhelmed the ability of neurons and astrocytes to adequately perform mitophagy, then mitochondria-derived DAMP molecules could predictably facilitate neuroinflammation (**Figure 1**). Clearly, a rational case can be made that mitochondria-derived DAMP molecules may contribute to neurodegenerative disease-associated neuroinflammation. However, important questions remain about how mitochondrial DAMPs contribute to neurodegenerative diseases and neurodegenerative disease-related pathologies. Future directions should focus on if and how specific mitochondrial-derived molecules initiate neuroinflammation. More specifically, how does mitochondrial dysfunction contribute to the release of DAMP molecules and is this process upstream or downstream of other disease pathologies? As this line of investigation moves forward, studies to address issues of cause versus consequence should help resolve these important knowledge gaps.

AUTHOR CONTRIBUTIONS

HW was responsible for writing the article. IW and YJ contributed to sections of the article. RS edited and provided direction for the article.

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IL-10-Producing CD1d^{hi}CD5^{+} Regulatory B Cells May Play a Critical Role in Modulating Immune Homeostasis in Silicosis Patients

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Silicosis is characterized by chronic lung inflammation and fibrosis, which are extremely harmful to human health. The pathogenesis of silicosis involves uncontrolled immune processes. Evidence supports that regulatory B cells (Bregs) produce negative regulatory cytokines, such as IL-10, which can negatively regulate immune responses in inflammation and autoimmune diseases. Our previous study found that IL-10-producing B cells were involved in the development of silica-induced lung inflammation and fibrosis of mice. However, little is known about the role of Bregs in silicosis patients (SP). In this study, we found that serum concentrations of IL-10 were significantly increased in SP by using protein array screening. We further determined that the frequency of IL-10-producing CD1d^{hi}CD5^{+} Bregs, not IL-10-producing non-B lymphocytes, was significantly higher in SP compared to subjects under surveillance (SS) and healthy workers (HW) by flow cytometry. We also found that regulatory T cells (Tregs) and Th2 cytokines (IL-4, IL-5, and IL-13) were significantly increased in SP. Th1 cytokines (IFN-γ, IL-2, and IL-12) and inflammatory cytokines (IL-1β, IL-6, and TNF-α) were not significantly different between SP, SS, and HW. Our study indicated that IL-10-producing CD1d^{hi}CD5^{+} Bregs might maintain Tregs and regulate Th1/Th2 polarization in SP, suggesting that IL-10-producing Bregs may play a critical role in modulating immune homeostasis in SP.

Keywords: IL-10, regulatory B cells, regulatory T cells, Th1/Th2 polarization, immune homeostasis, silicosis patients

INTRODUCTION

Silicosis is a potentially fatal, but preventable, occupational lung disease caused by inhaling respirable crystalline silica (1). It is characterized by chronic lung inflammation and irreversible fibrosis (2). Progressive fibrosis leads to disability and mortality in patients, which results in a heavy burden to society. In China, there were 11,471 cases of silicosis reported in 2014, accounting for 42.69% of 26,873 new pneumoconiosis cases (Ministry of Health of the People's Republic of China, 2015). Silicosis is reported to be a special concern in young

adults (aged 15–44 years) (3). Silicosis has become the most serious occupational disease in both developed and developing countries, yet little is known about the crucial cellular and molecular mechanisms that initiate and propagate the process of silicosis.

Previous evidence has shown that both innate and adaptive immune responses play a key role in the pathogenesis of silicosis (4, 5). Alveolar macrophages, the first defense against foreign substances, ingest inhaled silica and become activated to release a host of mediators, such as cytokines and chemokines, which initiate the influx of inflammatory cells and activation of immune cells (6, 7). The complex network of interactions between mediators and cells results in the onset of lung injury, inflammation, and potentially fibrosis. The inflammation mediators are involved in the causation of silicosis. Chemokines that primarily attract monocytes and macrophages induce inflammation mediators such as IL-1β, IL-6, and TNF-α, which initiate the activation of T and B immune cells and stimulate T-lymphocyte type switching (8, 9).

Previous studies have demonstrated that Th1 and Th2 cells participate in the pathogenesis of silicosis (10, 11). Regulatory T cells (Tregs) play a role of the maintenance of immune homeostasis, which limits inflammation and modulates Th1/Th2 balance in silica-induced lung fibrosis (12). However, other regulatory subsets in silicosis have not been investigated in detail, and the mechanism of immune homeostasis modulation needs further exploration. Recent studies have demonstrated that another regulatory lymphocyte, specific B cell subsets, so-called regulatory B cells (Bregs), are potent immune response regulators and play important roles in regulating immune homeostasis (13, 14). A number of Breg subsets have been reported, of which the IL-10-producing B cell (B10) subset is among the best characterized (15, 16). Subsequent studies have shown that $CD1d^{high}CD5^{+}$ B cells were associated with the suppression of inflammation and autoimmune disease through IL-10 secretion (17, 18). B10 was also found to participate in regulating Th immune response by affecting the secretion of inflammatory cytokines such as TNF-α (19–21). In addition, B10 influences the proliferation of Tregs (22). Recent findings have shown that frequencies of peripheral Bregs increased in patients with lung cancer and B10s were significantly elevated in non-small cell lung carcinoma patients (23, 24). $CD1d^{+}CD5^{+}$ B cell numbers were increased in patients with tuberculosis (25). Our previous study found that B10 was involved in the development of silica-induced lung inflammation and fibrosis of mice (26). However, little is known about the role of similar or equivalent regulatory B lymphocyte populations in silicosis patients (SP).

A protein microarray assay and Bio-Plex assay were used in the present study to find that the level of IL-10 was increased in the serum of SP. To explore whether the increased IL-10 was produced by B and/or T cell populations and to determine the role of Bregs in SP, we investigated peripheral B10 and $CD19^{+}$ $IL\text{-}10^{+}CD1d^{hi}CD5^{+}$ B cell subsets and Tregs and also determined the level of chemokines, inflammation mediators, and Th1 and Th2 cytokines in SP, subjects under surveillance (SS), and healthy workers (HW) that had prior exposure to silica dust. Our results may provide new insights into the role of Bregs in SP.

MATERIALS AND METHODS

Study Design

The study, conducted from May 2015 to March 2016, included 57 individuals. Nineteen first phase male SP were recruited from Shenyang No. 9 Hospital. Nineteen SS and nineteen HW with exposure to silica dust from Northern Heavy Industries Group Co., Ltd., matched by sex, age, year of exposure to silica dust, and ethnicity with SP were enrolled in this study. Criteria were based on the national diagnostic criteria of pneumoconiosis (GBZ70-2009). SS were workers with exposure to silica, who had no certain shadow changes visible in the chest X-ray films by health examination, and who needed periodic follow-ups within specified periods of time. The diagnosis of silicosis was based upon patient history of exposure to silica and available chest X-rays and was made by five qualified experts, who served as members of the Shenyang Municipal Pneumoconiosis Diagnosis Committee. Patients with chronic inflammatory diseases, respiratory infectious diseases, tuberculosis, asthma, diabetes, or cancer autoimmune disease were excluded.

Pulmonary function was measured from 2015 to 2016 by using spirometry. The data collected included forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1). Personal information, including sex, age, exposure period, and smoking status were obtain *via* questionnaire.

This study was carried out in accordance with the recommendations of the Ethics Committee of the China Medical University with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol (CMU2100012006) was approved by the Ethics Committee of the China Medical University.

Blood Sample

Five milliliters of venous blood samples were collected into no additive vacutainer tubes. Serum was obtained after centrifugation and stored at −80°C for protein microarray analysis and the Bio-Plex assay.

Also, 6 ml venous blood samples were collected into vacutainer tubes containing EDTA. Peripheral blood mononuclear cells (PBMCs) were immediately isolated by Ficoll density gradient centrifugation (TBD sciences, China) for Flow cytometric analysis.

Protein Microarray Analysis

RayBiotech Human Cytokine Antibody Array 440 (RayBiotech, Inc., Cat# QAH-CAA-440) was used to profile the variation of proteins in serum. The samples were detected by the Shanghai XY Biotech Company. The experimental procedure was carried out in accordance with the manufacturer's instructions. Briefly, pre-coated antibody array membranes were incubated with coating buffer for 30 min. After that, the blocking buffer was decanted and replaced with sample dilutions. Membranes

were incubated overnight at 4°C with shaking. The next day, the membranes were incubated with biotin-conjugated antibody for 2 h after washing. Mixture of biotin-conjugated antibodies was removed and Streptavidin-Fluor was added to each sub-array. The incubation chambers were covered with adhesive film and incubated for 2 h. After washing, signals were detected with a GenePix 4000B system (Axon Instruments, Foster City, CA, USA). GenePix Pro 6.0 software (Axon Instruments) was used for densitometric analysis of the spots. These values were normalized to the ratio of positive control values for each sample. Afterward, the total normalized fluorescence values of replicate spots were averaged and expressed as fold increase over the control samples.

Bio-Plex Assay

The levels of 27 cytokines in serum were measured by the Bio-Plex Pro™ human cytokine 27-plex assay (Bio-Rad Laboratories, Hercules, CA, USA; Cat# M500KCAF0Y). This multiplex assay detects: FGF basic, Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1)α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF. The experimental procedure was carried out according to the manufacturer's instructions. Briefly, beads coated with capture antibodies were incubated with premixed standards or sample supernatants for 30 min. Following incubation, premixed detection antibodies were added and incubated as before. After washing, streptavidin-PE was added and incubated for 10 min. After washing, the beads were resuspended in Bio-Plex cytokine assay buffer, and results were read on the Bio-Plex 200 system using Low PMT setting. Data were analyzed with Bio-Plex Manager™ software version 2.0.

Cell Preparation and Flow Cytometry Analysis

Peripheral blood mononuclear cells were isolated from blood samples by Ficoll density gradient centrifugation. After washing and addition of RPMI1640 medium, PBMCs were stimulated with Leukocyte Activation Cocktail (BD Pharmingen, San Jose, CA, USA) for 5 h, followed by blocking with Human BD Fc Block™ (BD Pharmingen) for 10 min at room temperature. PE-Cy7-conjugated CD4 (BD Pharmingen) and PerCP-Cy5.5-conjugated CD19 (BD Pharmingen) antibodies were used for cell surface staining. Cells were fixed and permeabilized using a fixation/permeabilization kit (eBioscience, San Diego, CA, USA) or BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution kit (BD Pharmingen) according to the manufacturer's instructions. The cells were then stained at 4°C with APC-conjugated anti-Foxp3 (BD Pharmingen), APC-conjugated anti-IL-10 (BD Pharmingen), FITC-conjugated anti-CD5 (BD Pharmingen), and PE-conjugated anti-CD1d (BD Pharmingen). After washing, stained cells were resuspended in 1% paraformaldehyde-PBS. Analysis of cell marker expression was performed using a FACSCanto II system (BD, Franklin Lakes, NJ, USA). Dead cells were gated out depending on forward scattering and side scattering. Cells were analyzed with FlowJo X software (Tree Star, OR, USA).

Statistical Analyses

Data were analyzed for statistical significance using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Means between two groups were analyzed by Student's *t*-test. The differences between values were evaluated through a one-way analysis of variance followed by pair-wise comparison with the Student–Newman–Keuls test. The chi-square test was used to compare categorical variables. Also, $p < 0.05$ was considered statistically significant. All values are means ± SEM.

RESULTS

Study Subjects

The general characteristics of the study subjects are shown in **Table 1**. No difference in general characteristics, including median age, exposure period, and smoking status, were found between three groups. Pulmonary function (%FVC predicted, $\%FEV_1$ predicted, $\%FEV_1$/FVC radio) in SP was significantly decreased compared to SS and HW.

Multiple Cytokines Altered in Serum of SP

To profile the cytokines in the peripheral blood from patients with silicosis, a blinded screening using serum of SP, SS, and HW by protein microarray analysis was performed. As shown in the heat maps, 46 kinds of cytokines were different between SP and SS, and 18 kinds of cytokines were different between SP and HW. Fifty-six kinds of cytokines were different among HW, SS, and SP groups (data shown in Tables S1–S3 in Supplementary Material, respectively). Among those cytokines, there was a marked increase of IFN-γ, IL-7, TNF-α, IL-10, IL-13, IL-6, IL-5, and IL-8 in SP ($n = 5$) compared to SS ($n = 5$) (**Figure 1A**). There was also a marked increase of

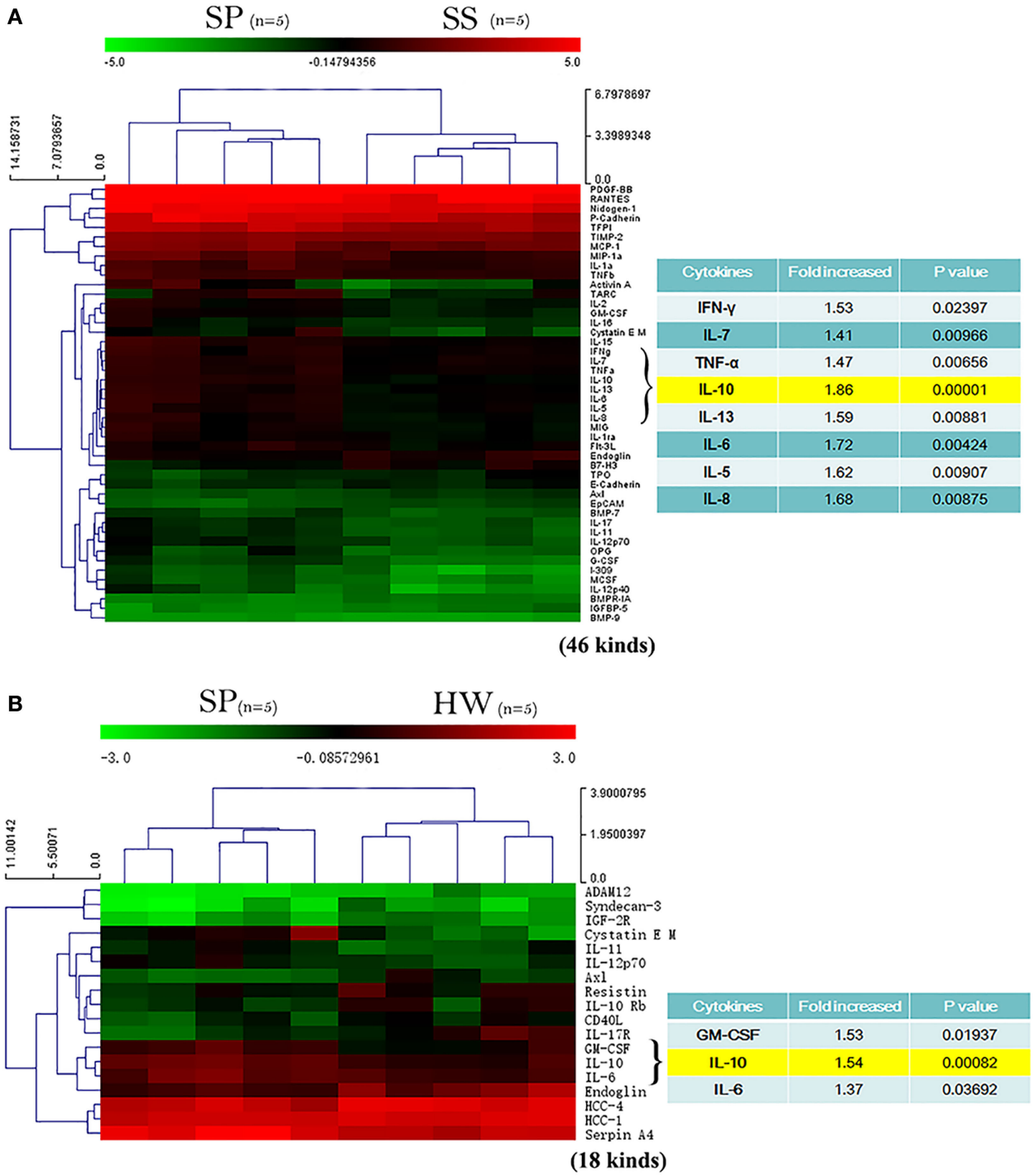
TABLE 1 | Characteristics of study participants.

	HW (male, $n = 19$)	SS (male, $n = 19$)	SP (male, $n = 19$)	*p*-Value
Age (years)	52.26 ± 0.82	52.32 ± 0.69	53.26 ± 1.00	0.65[a]
Exposure period (years)	30.21 ± 0.83	30.00 ± 0.92	28.05 ± 0.93	0.18[a]
%FVC predicted	90.81 ± 2.50	91.28 ± 3.46	67.41 ± 6.82#,*	0.001[a]
$\%FEV_1$ predicted	90.79 ± 3.15	95.45 ± 3.83	72.23 ± 6.20#,*	0.002[a]
$\%FEV_1$/FVC radio	84.90 ± 1.55	88.88 ± 1.49	71.91 ± 3.21#,*	<0.001[a]
Smoking, *N* (%)				0.94[b]
Never	7 (36.8)	8 (42.1)	8 (42.1)	
Past	1 (5.3)	1 (5.3)	2 (10.5)	
Current	11 (57.9)	10 (52.6)	9 (47.4)	

*HW, healthy workers with exposure to silica dust; SS, subjects under surveillance; SP, silicosis patients; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s. Values are expressed as means ± SEM, #p < 0.05 compared with HW, *p < 0.05 compared with SS.*

[a]*Calculated by one-way analysis of variance.*

[b]*Calculated by chi-square test.*



Cytokines	Fold increased	P value
IFN-γ	1.53	0.02397
IL-7	1.41	0.00966
TNF-α	1.47	0.00656
IL-10	1.86	0.00001
IL-13	1.59	0.00881
IL-6	1.72	0.00424
IL-5	1.62	0.00907
IL-8	1.68	0.00875

Cytokines	Fold increased	P value
GM-CSF	1.53	0.01937
IL-10	1.54	0.00082
IL-6	1.37	0.03692

FIGURE 1 | A profile of cytokines in serum of silicosis patients (SP) was detected by protein microarray. **(A)** Heat map analysis between SP and SS. Image showed a marked increase of IFN-γ, IL-7, TNF-α, IL-10, IL-13, IL-6, IL-5, and IL-8 in SP (n = 5) compared to SS (n = 5). Red represents upregulated protein expression, and green represents downregulated protein expression. **(B)** Heat map analysis between SP and workers exposed to silica dust. Image showed a marked increase of GM-CSF, IL-10, and IL-6 in SP (n = 5) compared to healthy workers (HW) (n = 5). Red represents upregulated protein expression, and green represents downregulated protein expression.

GM-CSF, IL-10, and IL-6 in SP (n = 5) compared to HW (n = 5) (**Figure 1B**). The levels of IL-10 showed significance difference between SP and SS (p = 0.00001). The levels of IL-10 were significantly different between SP and HW (p = 0.00082). These results suggested that IL-10 might play a critical role in the pathogenesis of silicosis.

Serum Level of IL-10 and IL-10-Producing CD1d^{high}CD5^{+} Bregs Were Increased in SP

The sample size was enlarged ($n = 19$), and the Bio-Plex array was used to further confirm the variation of serum IL-10 in SP. The Bio-Plex suspension array system permitted the simultaneous measurement of 27 human cytokines, including interleukins, chemokines, colony-stimulating factors, growth factors, interferon, and tumor necrosis factor (data of 27 cytokines shown in Table S4 in Supplementary Material). The results showed that the serum concentrations of IL-10 were significantly increased in SP compared to SS and HW ($p = 0.03$, $p = 0.017$) (**Figure 2A**). To explore the possibility that the increased IL-10 was produced by B cell and/or T cell populations, peripheral B10s and IL-10-producing non-B lymphocytes were investigated from all participants. The percentage of CD19^{+}IL-10^{+} B cells was observed to significantly increase in SP (2.02 ± 0.23%) compared to the SS group (1.46 ± 0.14%) and the HW group (1.21 ± 0.11%) (**Figures 2B,F**). However, there were no obvious differences in the percentage of CD19^{-}IL-10^{+} lymphocytes between the three groups (**Figures 2B,E**). These data suggested that increased serum IL-10 in SP might contribute to B10s, but not IL-10-producing non-B lymphocytes.

Emerging evidence indicates that CD1d^{hi}CD5^{+} B cells are responsible for most of the IL-10 production by B cells (17). We investigated peripheral IL-10-producing CD1d^{hi}CD5^{+} B cells in all participants. Our flow cytometry results showed that a proportion of cells highly expressed CD1d in CD19^{+} B cells (**Figure 2D**). The percentage of IL-10^{+}CD1d^{hi}CD5^{+} B cells significantly increased in SP (0.89 ± 0.18%) compared to the SS (0.39 ± 0.06%) and HW (0.24 ± 0.05%) groups (**Figures 2C,G**). These data suggested that IL-10^{+}CD1d^{hi}CD5^{+} B cells might play a critical role in the pathogenesis of silicosis.

Serum Levels of Chemokines Were Increased in SS

Of the 27 cytokines detected by the Bio-Plex assay, some chemokines tended to increase in the early stages of silicosis.

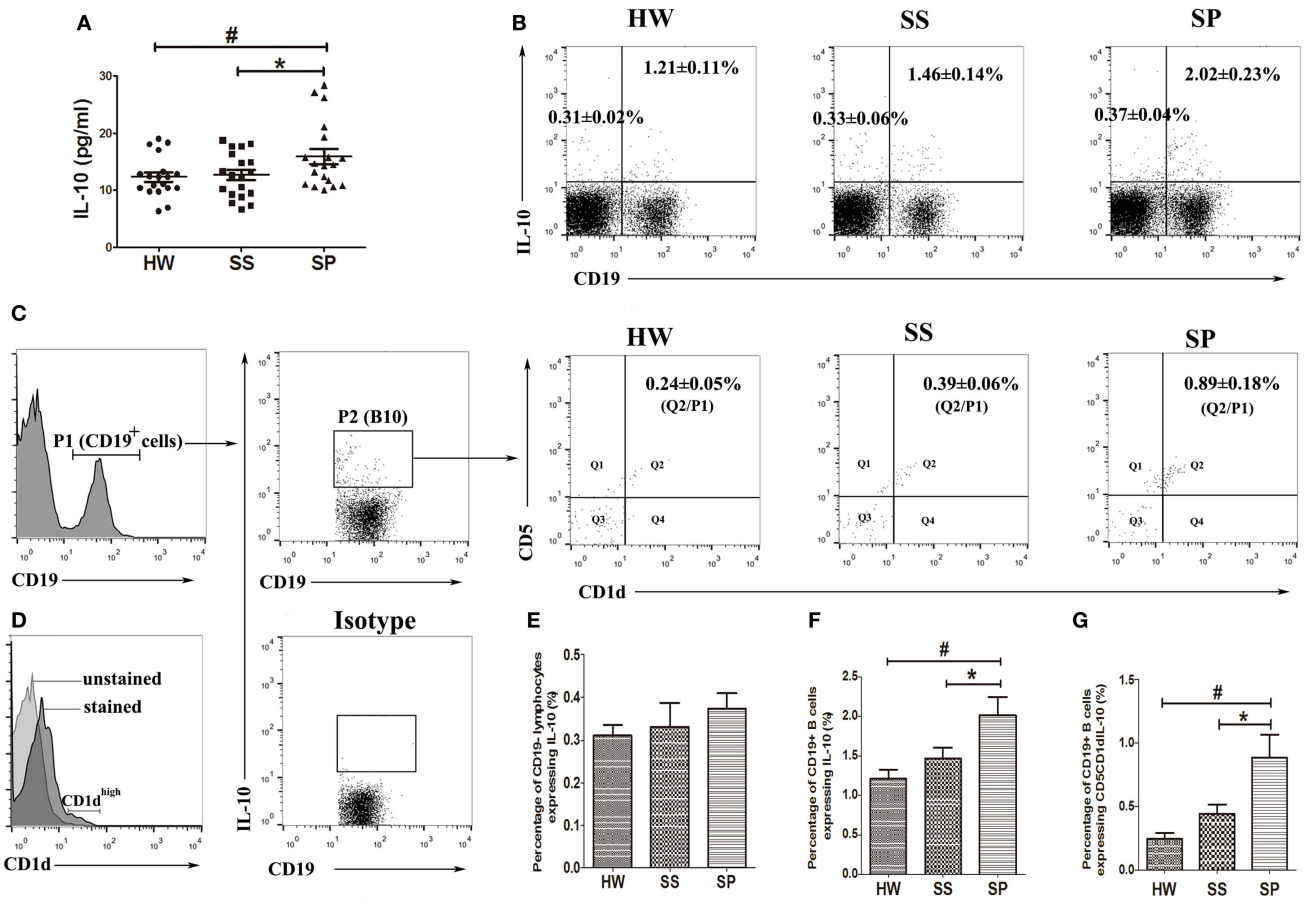


FIGURE 2 | Level of IL-10 in serum, IL-10-producing B cell (B10), and IL-10^{+}CD1d^{high}CD5^{+} B cell subsets in the peripheral blood were assayed in all participants. **(A)** The level of IL-10 in serum was assayed by the Bio-Plex assay. **(B,E,F)** Percentages of CD19^{+}IL-10^{+} B cells and CD19^{-}IL-10^{+} cells in the peripheral blood were assayed by flow cytometry. **(C,G)** Percentage of IL-10^{+}CD1d^{high}CD5^{+} B cells in the peripheral blood was assayed by flow cytometry. **(D)** The overlay graph of CD1d unstained and CD1d stained histograms [$n = 19$ per group, $^{\#}p < 0.05$ compared with healthy workers (HW); $^{*}p < 0.05$ compared with SS]. Error bars indicate mean ± SEM.

Compared with HW, the serum concentration of MCP-1 in SS and SP was significantly increased ($p = 0.008$, $p = 0.002$) (**Figure 3A**). In addition, the serum concentration of interferon gamma-IP-10 was increased in SS and SP compared to HW (**Figure 3B**). The serum concentrations of MIP-1α and MIP-1β were increased in SS compared to HW and SP (**Figures 3C,D**), but these differences were not statistically significant. These results suggested that some chemokines might initially be secreted by activated macrophages.

Tregs, Not Inflammatory Cytokines, Were Increased in SP

To investigate whether Bregs could affect the production of inflammatory cytokines and maintain Tregs in SP, inflammatory cytokines were detected using the Bio-Plex assay, and Tregs in PBMCs were analyzed by flow cytometry. The results showed no significant difference in the serum concentrations of inflammatory cytokines IL-1β, IL-6, and TNF-α between the three groups (**Figures 4A–C**). The percentage of $CD4^+$ T cells expressing Foxp3, which were identified as Tregs, increased significantly in SP (5.92 ± 0.36%) compared with that of SS (3.88 ± 0.38%) and HW (3.91 ± 0.33%) ($p < 0.001$, $p < 0.001$) (**Figures 4D,E**). These results suggested that Bregs might inhibit the production of inflammatory cytokines and maintain Tregs in SP.

Th2 Cytokines, Not Th1 Cytokines, Were Increased in SP

To investigate effects of Bregs on the Th1/Th2 immune response, the levels of Th1/Th2 cytokines were assayed in all participants. There was no significant difference in the levels of Th1 cytokine IFN-γ, IL-2, and IL-12 (p70) in serum between the SP, SS, and HW groups (**Figures 5A–C**). In contrast, the serum concentration of IL-4 was significantly increased in SP compared to SS and HW ($p = 0.015$, $p = 0.039$) (**Figure 5D**). The serum concentration of IL-5 was significantly increased in SP compared to SS ($p = 0.012$) and obviously increased compared to HW ($p = 0.098$) (**Figure 5E**). The serum concentration of IL-13 was significantly increased in SP compared to HW ($p = 0.011$) and obviously increased compared to SS ($p = 0.056$) (**Figure 5F**). These results suggested that Bregs might inhibit the secretion Th1 cytokines and promote the secretion Th2 cytokines to modulate Th1/Th2 immune responses in SP.

DISCUSSION

IL-10 is a pleiotropic cytokine produced by a number of leukocyte populations that impacts immune regulation and tissue homeostasis (27, 28). B10s play an important role in maintaining immune homeostasis (29). In this study, we found that the serum concentration of IL-10 was significantly increased in SP

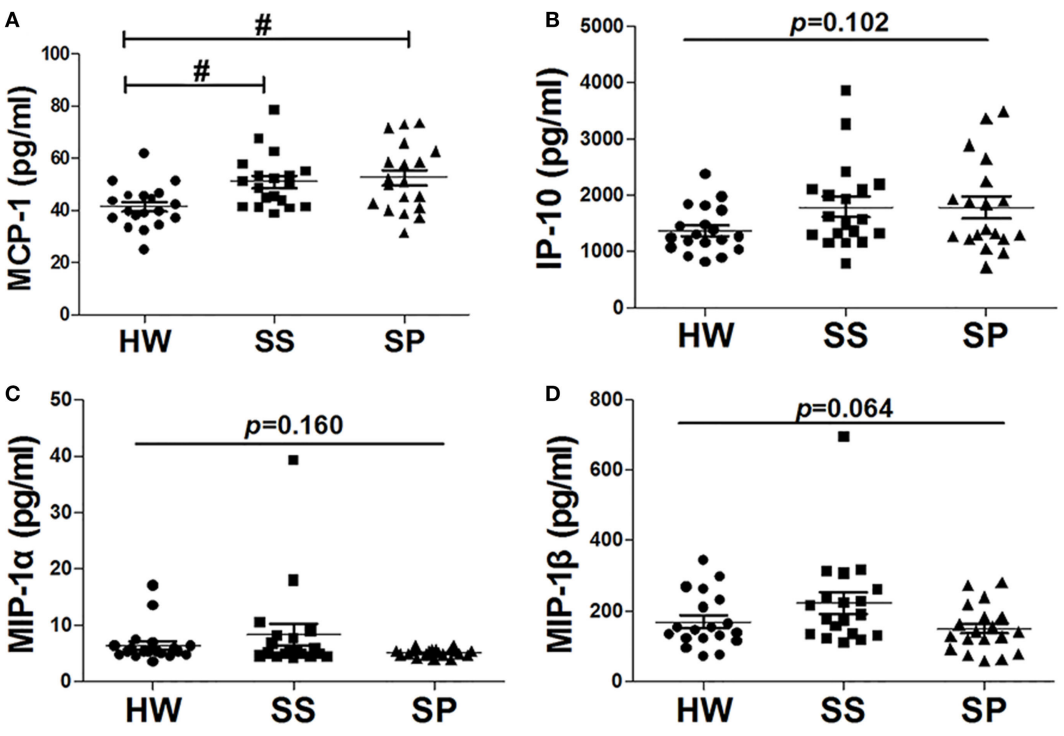


FIGURE 3 | Chemokines in serum were detected in all participants. **(A)** Level of monocyte chemoattractant protein-1 (MCP-1) in serum was assayed by the Bio-Plex assay. **(B)** The level of inducible protein-10 (IP-10) in serum was assayed by the Bio-Plex assay. **(C)** The level of macrophage inflammatory protein-1 (MIP-1)α in serum was assayed by the Bio-Plex assay. **(D)** The level of MIP-1β in serum was assayed by the Bio-Plex assay [$n = 19$ per group, $^{\#}p < 0.05$ compared with healthy workers (HW); $^{*}p < 0.05$ compared with subjects under surveillance (SS)]. Error bars indicate mean ± SEM.

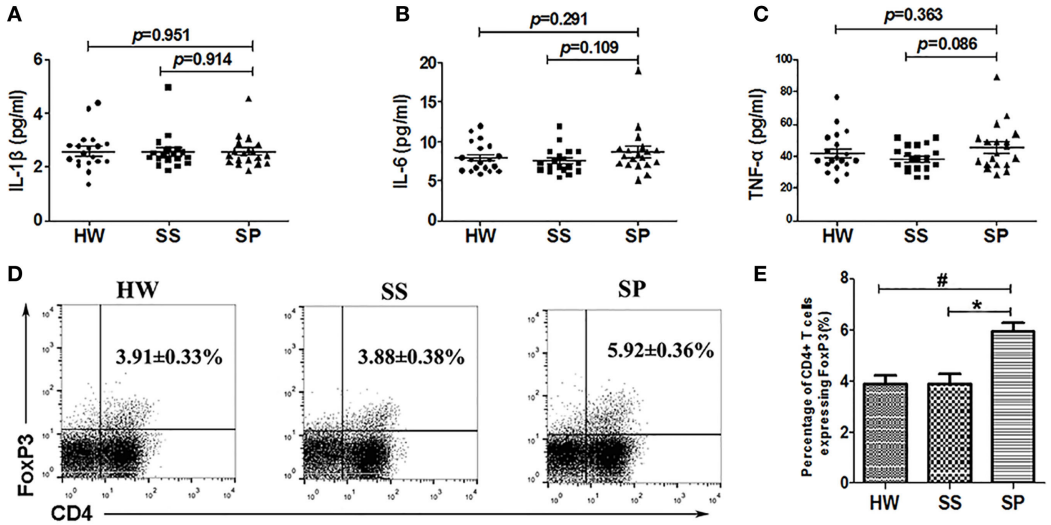


FIGURE 4 | Levels of inflammatory cytokines in serum and regulatory T cells (Tregs) in the peripheral blood were assayed in all participants. **(A)** The level of IL-1β in serum was assayed by the Bio-Plex assay. **(B)** The level of IL-6 in serum was assayed by the Bio-Plex assay. **(C)** The level of TNF-α in serum was assayed by the Bio-Plex assay. **(D,E)** Percentage of $CD4^+Foxp3^+$ Tregs in the peripheral blood was assayed by flow cytometry [$n = 19$ per group, $^{\#}p < 0.05$ compared with healthy workers (HW); $^{*}p < 0.05$ compared with subjects under surveillance (SS)]. Error bars indicate mean ± SEM.

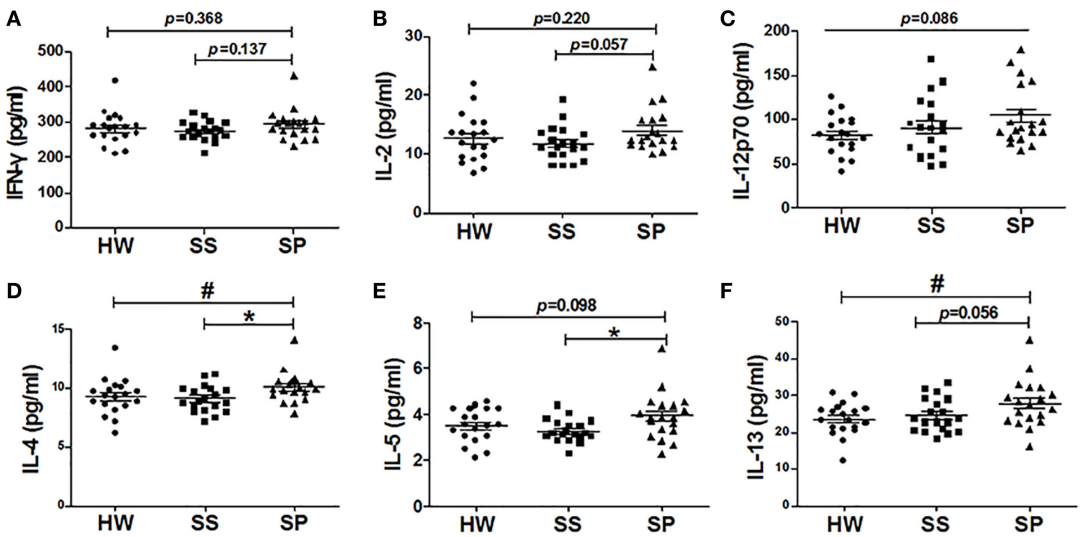


FIGURE 5 | Levels of Th1/Th2 cytokines were assayed in all participants. **(A)** The level of IFN-γ in serum was assayed by the Bio-Plex assay. **(B)** The level of IL-2 in serum was assayed by the Bio-Plex assay. **(C)** The level of IL-12 (p70) in serum was assayed by the Bio-Plex assay. **(D)** The level of IL-4 in serum was assayed by the Bio-Plex assay. **(E)** The level of IL-5 in serum was assayed by the Bio-Plex assay. **(F)** The level of IL-13 in serum was assayed by the Bio-Plex assay [$n = 19$ per group, $^{\#}p < 0.05$ compared with healthy workers (HW); $^{*}p < 0.05$ compared with subjects under surveillance (SS)]. Error bars indicate mean ± SEM.

compared to SS and HW by protein microarray analysis and the Bio-Plex assay. We compared the frequencies of IL-10-secreting B cells and IL-10-secreting non-B lymphocytes in all participants and determined that the frequency of B10s, not IL-10-producing non-B lymphocytes, was higher in SP than in SS and HW groups.

Regulatory B cells are important suppressors of inflammation and other autoimmune responses, producing the negative regulatory cytokines, IL-10 and TGF-β (30). IL-10 and TGF-β-producing B cells mediate tolerance in acute allergic airway inflammation (31). However, IL-10 is considered the hallmark cytokine of Bregs. B10s were the first Bregs to be recognized and were termed "B10" cells (32). Emerging evidence indicates that $CD1d^{hi}CD5^+$ B cells are responsible for most IL-10 production by B cells (17, 33). The function of Bregs was mediated by IL-10 and dependent on the expression of CD1d (20, 34). A Bregs'

deficiency leads to increased allergic airway Inflammation (35). Our results showed that the percentage of IL-10^{+}CD1d^{hi}CD5^{+} B cells significantly increased in SP compared to SS and HW groups. The increase of B10s and IL-10^{+}CD1d^{hi}CD5^{+} B cells in SP might be the results of effective immune response to lung injury. IL-10^{+}CD1d^{hi}CD5^{+} B cells might play a critical role in modulating immune homeostasis of SP (**Figure 6**).

The pathogenesis of silicosis involves alveolar cell injury and activation followed by release of cytokines and recruitment of cells into the areas of silica dust deposition (10, 36, 37). Chemokines such as MCP-1, MIP-1, and IP-10 are chemotactic cytokines that recruit specific groups of monocytes/macrophages and lymphocytes, which are involved in many chronic lung diseases (38–41). MCP-1 is a potent chemotactic factor for blood monocytes and produced by various inflammatory cells, which has been considered a potential biomarker for the progress of coal worker's pneumoconiosis (42). Our results showed that the levels of MCP-1 and IP-10, especially MCP-1, were significantly increased in SS and SP compared to HW. These results suggested that chemokines were involved in the initial stages of silicosis.

It is now widely appreciated that B cells can suppress inflammation and negatively regulate immune responses, in part through the secretion of anti-inflammatory IL-10 (19, 26, 43). The production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, by monocytes and macrophages is suppressed by IL-10 (39). In our previous study, we found that the levels of secreted TNF-α and IL-6 proteins in BALF clearly increased in B10-deficiency mice injected with an anti-CD22 antibody (26). Our data revealed that the serum concentrations of IL-1β, IL-6, and TNF-α were not significantly different between the SP, SS, and HW groups. B10s might partly restrain inflammation of silicosis by downregulating the production of pro-inflammatory cytokines. In addition, it has recently been shown that B10s are crucial for the maintenance of Tregs function (44–47). It was reported in mice that tumor-induced B10s could convert resting CD4^{+} non-Tregs into Tregs (48). Another study showed that B10s promoted Tregs accumulation and function by stimulating Foxp3 expression (49). In our previous study, insufficient B10s reduced the frequency of Tregs, whereas Treg depletion did not influence B10 accumulation in silicosis of mice. B10s might act earlier than Tregs during silica-induced lung inflammation and fibrosis (26). Our present data showed that Tregs significantly increased in SP compared to SS and HW. These results indicated that Bregs might maintain Tregs function, which is involved in suppressing inflammation and regulating Th immune responses in SP (**Figure 6**).

Regulatory B cells have been found to have regulatory properties in inducing primary T cell proliferation and in generating and maintaining CD4^{+} effect T cells (50). It was reported that B cell depletion therapy was associated with changes in T cell repertoire and function in patients with idiopathic thrombocytopenic purpura (51). Our previous studies had demonstrated insufficient B10 could exacerbate Th1 immune response and elevate levels of IFN-γ in silicosis of mice (26). Studies have shown B10s can influence the development of Th2 cells (52, 53). In the current study, the levels of Th1 cytokines IFN-γ, IL-2, and IL-12 (p70) in serum were found to have no significant difference among the three groups. The levels of Th2 cytokines IL-4, IL-5, and IL-13 in serum significantly increased in SP compared to SS and/or HW. These results suggested that increased levels of IL-10/IL-10-producing CD1d^{hi}CD5^{+} Bregs might inhibit the

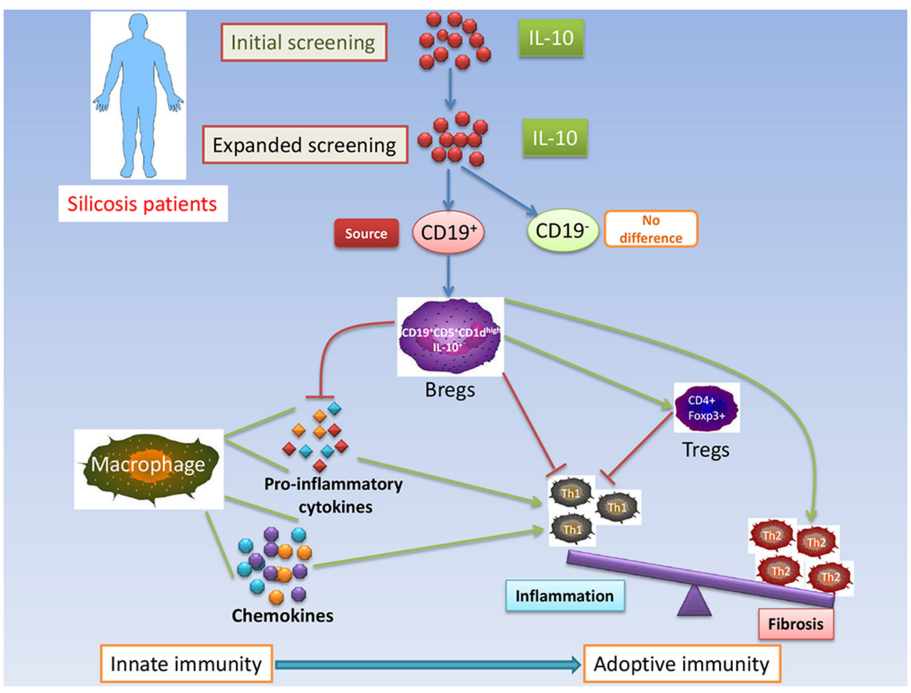


FIGURE 6 | Schematic representation of IL-10-producing CD1d^{high}CD5^{+} regulatory B cells (Bregs) in modulating immune homeostasis in silicosis patients.

production of Th1 cytokines and maintain secretion of Th2 cytokines to regulate the Th1/Th2 balance toward a Th2 phenotype in SP (**Figure 6**).

The results of the present study show that increased level of inhibitory cytokine IL-10 and number of IL-10-producing $CD1d^{hi}CD5^{+}$ Bregs may maintain Tregs and regulate Th1/Th2 polarization, suggesting that IL-10-producing Bregs may play a critical role in modulating immune homeostasis in SP. However, how B cells differentiate into IL-10-producing Bregs and what functions the cells play in silicosis progression remain to be further investigated.

AUTHOR CONTRIBUTIONS

JC and YC conceived and designed the research; YC, CL, YL, HZ, WG, BL, JS, and BY performed experiments; YC, CL, FL, DW, and JC analyzed data and interpreted results; YC prepared figures and drafted the manuscript; YC, CL, FL, DW, and JC edited and revised the manuscript; all the authors approved the final manuscript version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00110/full#supplementary-material.

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Lipopolysaccharide Attenuates Induction of Proallergic Cytokines, Thymic Stromal Lymphopoietin, and Interleukin 33 in Respiratory Epithelial Cells Stimulated with PolyI:C and Human Parechovirus

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Epidemiological studies based on the "hygiene hypothesis" declare that the level of childhood exposure to environmental microbial products is inversely related to the incidence of allergic diseases in later life. Multiple types of immune cell-mediated immune regulation networks support the hygiene hypothesis. Epithelial cells are the first line of response to microbial products in the environment and bridge the innate and adaptive immune systems; however, their role in the hygiene hypothesis is unknown. To demonstrate the hygiene hypothesis in airway epithelial cells, we examined the effect of lipopolysaccharide (LPS; toll-like receptor 4 ligand) on the expression of the proallergic cytokines thymic stromal lymphopoietin (TSLP) and interleukin 33 (IL33) in H292 cells (pulmonary mucoepidermoid carcinoma cells). Stimulation with the TLR ligand polyI:C and human parechovirus type 1 (HPeV1) but not LPS-induced TSLP and IL33 through interferon regulatory factor 3 (IRF3) and NF-κB activity, which was further validated by using inhibitors (dexamethasone and Bay 11-7082) and short hairpin RNA-mediated gene knockdown. Importantly, polyI:C and HPeV1-stimulated TSLP and IL33 induction was reduced by LPS treatment by attenuating TANK-binding kinase 1, IRF3, and NF-κB activation. Interestingly, the basal mRNA levels of TLR signaling proteins were downregulated with long-term LPS treatment of H292 cells, which suggests that such long-term exposure modulates the expression of innate immunity signaling molecules in airway epithelial cells to mitigate the allergic response. In contrast to the effects of LPS treatment, the alarmin high-mobility group protein B1 acts in synergy with polyI:C to promote TSLP and IL33 expression. Our data support part of the hygiene hypothesis in airway epithelia cells *in vitro.* In addition to therapeutic targeting of TSLP and IL33, local application of non-pathogenic LPS may be a rational strategy to prevent allergies.

Keywords: TSLP, IL33, HMGB1, hygiene hypothesis, innate immunity

INTRODUCTION

The hygiene hypothesis declares that a lack of early childhood exposure to environmental microorganisms and pathogens increases susceptibility to allergic diseases by suppressing the establishment of immune tolerance (1). Epidemiological data and experimental evidence showed that exposure to environmental pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), are associated with decreasing the incidence of allergic diseases in later life (2–4). Similarly, muramic acid, a constituent of peptidoglycan of bacteria in the environment was found inversely associated with respiratory wheezing in rural school children (5). Those reports suggest that environmental microbes modulate allergic response.

Innate immunity is a rapid host defense response against invading pathogens, this response is essential to establish antigen-specific adaptive immunity to further eradicate pathogens and instruct the immune memory (6). Human epithelial cells form the largest primary physical barrier against environmental microbes and provide protection to the host via TLR-mediated responses of innate immunity (7–9). TLR signaling in skin and airway epithelial cells promotes the expression of proallergic cytokines, such as thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony-stimulating factor, interleukin-25 (IL25), and IL33, which are crucial for the initiation of the Th2 allergic immune cascade (7, 10–14). Epithelial cells command the innate and adaptive immune responses in atopic diseases (15). The implication of epithelial cells expressing the proallergic cytokines TSLP and IL33 in hygiene hypothesis is unknown.

Childhood exposure to environmental microorganism, such as viral infection can exacerbate asthma severity. Viral infection with rhinovirus, human metapneumovirus and respiratory syncytial virus can induce TSLP expression in airway epithelial cells (9, 16, 17). In particular, rhinovirus infection has been found associated with TSLP levels in the airways of young children (18). Rhinovirus infection can induce IL33 secretion in human bronchial epithelial cells to promote Th2 inflammation and exacerbate asthma severity in patients (19). This finding agreed with the observation of higher IL33 levels in the sera of patients with allergic rhinitis than normal controls (20). In addition, human parechovirus (HPeV), a small, round-structured, non-enveloped virus with a single-stranded and positive-sense RNA genome, belongs to the *Picornaviridae* (21). Nosocomial infection or outbreaks in neonate hospital departments seem to play a large role in HPeV infection (22, 23). Similar to rhinovirus, HPeV also causes respiratory disease in children, with high prevalence (24). It would be interested to understand whether HPeV1 acts like rhinovirus on prompting allergy.

Virus infection also can increase and activate TLR3 signal pathway (25). Among the various TLR ligands, only polyI:C (double-stranded RNA, TLR3 ligand) can stimulate high levels of TSLP expression, which is enhanced by the addition of IL4, IL13, or tumor necrosis factor α (26). Other TLR ligands, such as LPS (TLR4 ligand), CpG (TLR9 ligand), Pam_3CSK_4 (TLR2 ligand), and flagellin (TLR5 ligand), failed to induce TSLP expression in epithelial cells (16, 26). Similarly, IL33 mRNA expression could be induced by IFN-γ, the TLR9 ligand ODN2006, or polyI:C but not LPS in human nasal epithelial cells with allergic rhinitis (20, 27).

The immunoregulatory effect of the LPS/TLR4 axis in immune cells, such as dendritic cells and myeloid-derived suppressor cells was revealed in an animal model of asthma, which suggested that the dose of LPS is critical for the T helper 1 (Th1)/Th2 cell balance. Increased doses of LPS and antigens induce Th1 responses and inhibit allergic inflammation; however, reduced doses of LPS induce Th2 responses and promote airway inflammation (28–31). In addition to LPS, the TLR2 ligand Pam_3CSK_4 blocks the development of asthma (32). Therefore, TLRs in immune cells play roles during allergic airway responses.

The LPS failure to induce expression of TSLP and IL-33 prompted us to explore the mechanism by which LPS downregulates allergic cytokine production in response to polyI:C stimulation in airway epithelial cells. We established an *in vitro* model of the hygiene hypothesis in human airway epithelial mucoepidermoid pulmonary carcinoma cells (H292 cells) and used polyI:C treatment to mimic double-stranded viral RNA during replication to trigger inflammation (15, 26). We used our previously isolated and characterized clinical virus isolate, HPeV1 (33), to address whether LPS regulates virus-mediated allergic inflammation. The effects of LPS on polyI:C- and HPeV1-stimulated TSLP and IL33 mRNA expression were measured. Mechanistically, we also examined how LPS signaling subverts the polyI:C and HPeV1 signal axis in airway epithelial cells.

The non-histone nuclear protein high-mobility group protein B1 (HMGB1) is a damage-associated molecular pattern (DAMP) or called alarmin, which is released outside of the cells while cell activation, injury, or death (34). The HMGB1-mediated airway inflammation disease was characterized in the clinical and experimental asthma (35). In addition, HMGB1 from airway epithelial cells with respiratory syncytial virus infection primes epithelial cells and monocytes to inflammation stimuli in the airway (36). Multiple receptors were identified to be interacted with HMGB1, such as the receptor of advanced glycation end products (RAGE) or integrins, etc. (34). In addition, HMGB1 may act as an endogenous TLR2/4 ligand to trigger inflammatory responses (34, 37, 38). Thus, in this study, we also investigated whether HMGB1 regulates the TSLP and IL33 expression in polyI:C-stimulated airway epithelial cells.

MATERIALS AND METHODS

Cells

The human mucoepidermoid pulmonary carcinoma cell line NCI-H292 (BCRC, 60732) was cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO_2 atmosphere. The human bronchial epithelial cell line NL-20 (ATCC-CRL-2503) was cultured in Ham's F12 medium (Invitrogen, Carlsbad, CA, USA) with 10 ng/ml epidermal growth factor, 0.001 mg/ml transferrin, 500 ng/ml hydrocortisone, and 4% FBS and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO_2 atmosphere. A549 human lung

epithelial carcinoma cells (ATCC: CCL-185), WS1 human fetal skin normal fibroblasts (BCRC: 60300), and HEK-293T cells (ATCC: CRL-3216) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere. The human primary nasal epithelium from three donors was isolated and cultured according to the previous report (39). The study was approved by the Institutional Review Board of Kaohsiung Veterans General Hospital (Protocol number: VGHKS98-VT8-06) and conformed to the current ethical principles of the Declaration of Helsinki. Written informed consents were obtained from all donors.

Reagents

The double-stranded RNA, polyI:C, from Sigma-Aldrich (#P1530) and InvivoGen (#tlrl-picw) were used. Our testing results showed that the two polyI:C products of polyI:C have the similar activity of TSLP induction in H292 cells (Figure S1A in Supplementary Material). So, in this study, the polyI:C from Sigma-Aldrich was used.

Lipopolysaccharide from *Escherichia coli* 0111:B4 (#L2630), *Escherichia coli* 055:B5 (#L2880), *Escherichia coli* 026:B6 (#L-8274), *Escherichia coli* 0127:B8 (#L4516), *Klebsiella pneumoniae* (L4268), *Salmonella enterica serotype enteritidis* (#L7770), *Salmonella enterica serotype minnesota* (#L6261), *Pseudomonas aeruginosa 10* (#L9143), dexamethasone (#D4902), and Bay 11-7082 (#B5556) were all from Sigma-Aldrich (St. Louis, MO, USA) (40). We tested these eight different LPS in the TSLP and IL33 induction. Except the LPS from *Escherichia coli* 0127:B8, other seven types of LPS were not able to promote TSLP and IL33 expression; moreover, the polyI:C-induced TSLP and IL33 expression were attenuated by all of the tested LPS (Figures S1B,C in Supplementary Material). Based on the results of statistical analysis, the LPS from *Escherichia coli 0111:B4* was chosen in this study.

Recombinant human IL4 and HMGB1 were from Peprotech and R&D system, respectively (Rocky Hill, NJ, USA and Minneapolis, MN, USA). The expression vectors of IRF3, IRFs 5D, and IRF3 5A were described in our previous report (41). TurboFect transfection reagent (Thermo Scientific) was used for transient transfection following the manufacturer's protocol.

HPeV1 and Virus Titration

The strains of HPeV1 KVP6 (accession no. KC769584) were isolated by the Virology Group, Department of Microbiology, Kaohsiung Veterans General Hospital and propagated in Vero cells (ATCC: CCL-81) (33). To determine the virus titers, culture medium from HPeV1-infected cells were harvested for plaque-forming assays. Various virus dilutions were added to 6-well plates with 80% confluent Vero cells and incubated at 37°C for 2 h. After adsorption, cells were gently washed and overlaid with 1% agarose containing MEM supplemented with FBS. After 7 days' incubation at 37°C, cells were fixed with 10% formaldehyde, then stained with 1% crystal violet for further plaque counting.

Treatment

The treatment condition of polyI:C and IL4 or HPeV1 infection was evaluated with the TSLP induction in various cell types. H292 cells (1×10^6) in 6-well plates were either transfected by 2.5 μg polyI:C with TurboFect or directly incubated with 30 μg/ml polyI:C-contained medium. The TSLP mRNA level was highly induced at 3 h post-stimulation and then declined at 6 h in both treatments; particularly, polyI:C transfection showed greater induction level of TSLP than just adding polyI:C (Figure S2A in Supplementary Material). Therefore, the method of polyI:C (2.5 μg) transfection was set for the allergic cytokine induction in this study. This treatment condition was confirmed in another cell types in A549 cells, which TSLP mRNA was promoted with polyI:C stimulation at 3 h, 6 h, and 12 h, and then declined at 24 h (Figure S2B in Supplementary Material). Similar results were observed in the NL20 cells (Figure S3A in Supplementary Material). The TSLP protein level was detected by immunoblotting, which showed the increased protein level of TSLP in H292 cells at 3–6 h and in A549 cells at 2–3 h after polyI:C transfection; then, the TSLP protein level was decreased in later time points (Figure S2C in Supplementary Material).

The TSLP induction by different concentration of recombinant IL4 was measured in H292 cells. Only the concentration of 20 ng/ml, but not 1 and 10 ng/ml, of IL4 was able to induce TSLP, the level was peaked at 3 h post-stimulation (Figure S2D in Supplementary Material). The TSLP induction by 20 ng/ml of IL4 had also detected in WS1 human fetal skin normal fibroblasts with a time course-dependent manner (Figure S2E in Supplementary Material).

HPeV1 infection-mediated innate immune activation in A549 cells was revealed in our previous report (33). Here, we also found TSLP induction in A549 cells infected with HPeV1 at multiplicity of infection (MOI) = 5. Two induction peaks were observed at 2 and 36 h post infection (hpi) (Figure S2F in Supplementary Material).

Before LPS treatment, the cell culture medium was replaced with serum-free RPMI medium for 1 h, and then various concentrations of LPS were added to the cells and incubated for 2 h for short-term LPS treatment. LPS-treated cells were stimulated with polyI:C or infected with HPeV1 for the indicated times. In certain case, the cells were stimulated by recombinant IL4 (20 ng/ml) or HMGB1 (1 μg/ml). For long-term LPS treatment, H292 cells were incubated with LPS (30 μg/ml) for 8 or 16 days, or LPS (0.3 μg/ml) for 60 days, and the LPS-containing growth medium was refreshed every 2 days. Before polyI:C stimulation, long-term LPS-treated H292 cells were incubated with serum-free medium for 1 h followed by LPS treatment for 2 h, then stimulated with polyI:C for 3 h. In the inhibitor treatment group, dexamethasone or Bay 11-7082 was added to cells, and cells were incubated for 2 h before stimulation with polyI:C.

Cell Proliferation Assay

WST-1 assay (Roche, Basel, Switzerland) was used to monitor cell proliferation (42, 43); H292 cells were trypsinized and resuspended in culture medium, then plated at 5×10^3 cells per well in 96-well plates and incubated overnight. After LPS treatment followed by polyI:C transfection, cells were incubated with 10 μl WST-1 reagent for 2 h. The cell viability was quantified by multi-well spectrophotometry (Anthos, Biochrom, Cambridge,

UK). The absorbance at 450 nm was monitored, and the reference wavelength was set at 620 nm.

Quantitative Real-time PCR

Total RNA was extracted by using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The cDNA was synthesized from 5 μg total RNA by using a Superscript III reverse transcriptase kit (Thermo Fisher Scientific) with oligo (dT) primers. Real-time PCR involved 10 ng total cDNA and SYBR green master mix (Applied Biosystems, Carlsbad, CA, USA) with the ABI StepOne Plus Real-Time PCR System (Applied Biosystems) (44). The primers for qPCR are in Table S1 in Supplementary Material. The relative mRNA level was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GADPH), as a loading control.

Immunoblotting

Cells were lysed in 2% SDS buffer [2% SDS, 50 mM Tris–HCl (pH 7.5), 20 mM *N*-ethylmaleimide plus complete protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche)]. Whole cell extracts (WCEs) were homogenized by sonication for 10 s with a sonicator (Soniprep 150, MSE, London, UK) (45). Protein concentrations were determined by *DC* Protein Assay (Bio-Rad). In total, 100 μg WCEs were separated by 10 or 12% SDS-PAGE, then transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA), incubated with primary antibody overnight at 4°C, then horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) for 90 min and bands were detected by using the ECL reagent (Advasta, Menlo Park, CA, USA) with the BioSpectrum Image System (UVP, Upland, CA, USA). Protein or phosphor-protein levels were normalized to that of β-actin or corresponding total protein, respectively; and represented as fold changes compared with the control. The primary antibodies against interferon regulatory factor 3 (IRF3; #sc-9082), NF-κB p65 (#sc-372), and TSLP (#sc33791) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against RIG-I (#4520), IκBα (#4814), TBK-1 (#3504), phospho-TBK-1 (Ser172, #5483), and phospho-NF-κB p65 (Ser468, #3039 and Ser536, #3033) were from Cell Signaling (Danvers, MA, USA). Anti-phospho-IRF3 (#ab76493) and anti-β-actin (#MA5-15739) antibodies were from Abcam (Cambridge, UK) and Thermo Fisher Scientific (Waltham, MA, USA), respectively.

NF-κB Luciferase Reporter Assay

Cells cultured in 12-well plates were transfected with NF-κB-Luc reporter plasmids (46) by TurboFect. pRL-TK (Promega), encoding *Renilla* luciferase under a herpes simplex virus thymidine kinase promoter, was an internal control. Twenty-four hours post-transfection, cells were stimulated with polyI:C. Cell lysates were collected for the dual-luciferase assay (Promega). Firefly luciferase activity was normalized relative to that of *Renilla* luciferase.

Immunofluorescence Assay

H292 cells were fixed in 4% paraformadehyde for 20 min and permeabilized with 0.5% Triton X-100 for 15 min, washed three times with PBS, then incubated with 10% skim milk in PBS for 15 min to block non-specific antibody binding. To detect the cellular location of NF-κB p65 or IRF3, cells were incubated with antibodies against NF-κB p65 or IRF3 (1:500 in PBS) at 4°C overnight, then with the secondary antibody biotinylated goat anti-rabbit IgG (1:500 in PBS, Thermo Fisher Scientific) at room temperature for 90 min, then Alexa Fluor 568 streptavidin (1:500 in PBS, Thermo Fisher Scientific) for another 90 min at room temperature. Nuclear counterstaining involved staining with 4′,6-diami-dino-2-phenylindole (DAPI) for 10 min at room temperature. Fluorescence signals were observed by fluorescence microscopy (ZEISS, Observer A1, Oberkochen, Germany). Anti-HPeV VP0 antibody was used to detect HPeV1-infected H292 cells (33).

NF-κB p65 and IRF3 Knockdown

Short hairpin RNA (shRNA) specific to NF-κB p65 and IRF3 was obtained from the National RNAi Core Facility, Institute of Molecular Biology/Genomic Research Center, Academia Sinica. HEK293T cells were transfected with shRNA lentivirus by using pMD.G, pCMV-ΔR8.91, PLKO.1 puro scramble control (shCtrl) or PLKO.1 puro shNF-κB p65 for 24 h, then culture medium was refreshed. Media containing lentivirus was harvested at 72 h post-transfection. H292 cells were infected with shNF-κB p65 or shCtrl lentivirus and infected cells were selected with puromycin (1 μg/ml) for 3 days.

Statistical Analysis

Significant differences between groups were analyzed by two-tailed Student *t*-test with the software GraphPad Prism 5 (La Jolla, CA, USA). Data are presented as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

LPS-Attenuated PolyI:C-Induced TSLP and IL-33 Expression and Cell Damage

To investigate the hygiene hypothesis in airway epithelial cells *in vitro*, we used H292 cells because the gene expression profile of H292 airway epithelial cells is similar to that of primary nasal epithelial cells from healthy human controls on stimulation with house dust mite extracts (47). In studies of cell morphology, polyI:C stimulation induced damage in H292 cells at 12 h post-transfection (**Figure 1A**), which is similar to previous findings (48). We noted that LPS treatment for 2 h dose-dependently protected cells against death induced by polyI:C, with high-dose (30 μg/ml) but not low-dose (3×10^{-8} μg/ml) LPS conferring normal cell morphology (**Figure 1A**). PolyI:C-induced H292 cell death was inhibited by LPS treatment for 2 h or 8 days (**Figure 1B**). The data suggest a cross-regulation between TLR4 and TLR3 signaling pathway.

Gene expression of the allergic inflammation cytokines TSLP, IL33, and IL25 was measured in H292 cells. The TSLP and IL33 expression were significantly increased with polyI:C and IL4 treatment, by approximately three- to fivefold and

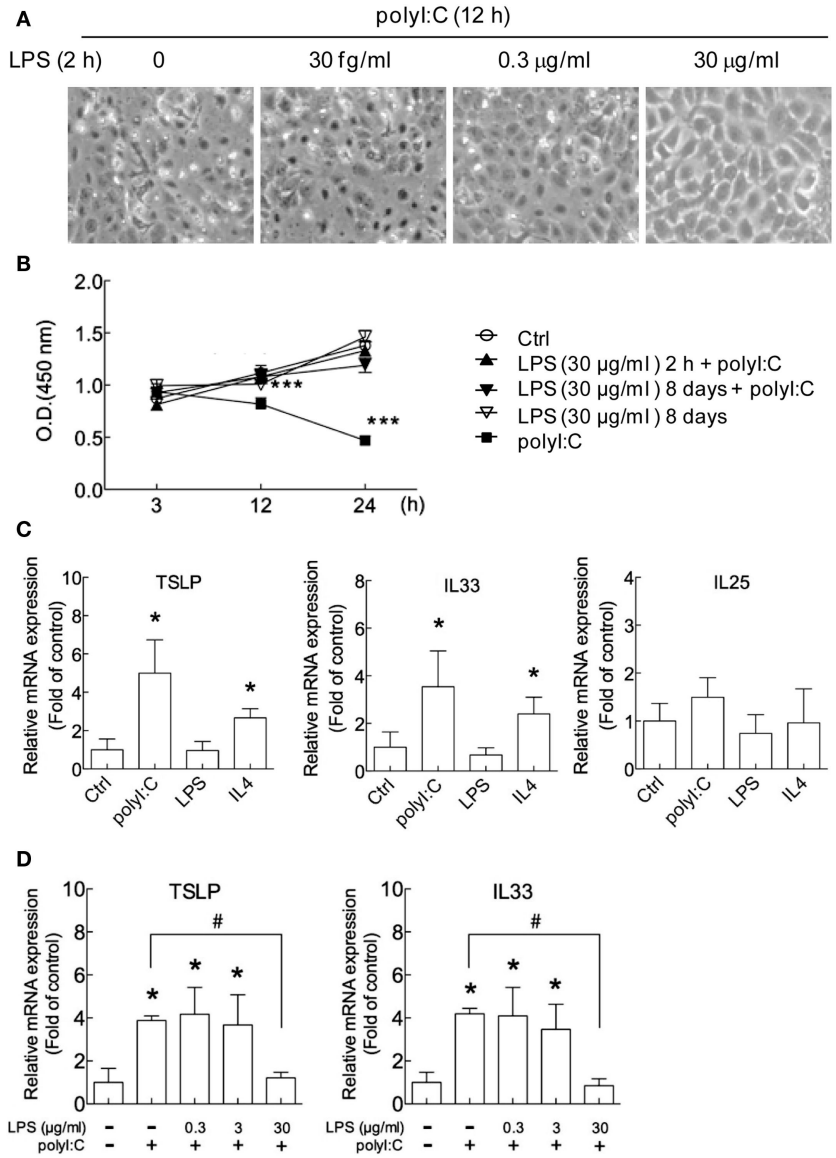

FIGURE 1 | PolyI:C-induced cell damage and allergic cytokine expression are attenuated by lipopolysaccharide (LPS) in airway epithelial cells. **(A)** H292 cells were treated with LPS (3×10^{-8}, 0.3 and 30 μg/ml) for 2 h followed by polyI:C (2.5 μg) transfection for 12 h. Representative images from three independent experiments show that cell morphology under an inverted microscope. **(B)** H292 cells were treated with control medium (Ctrl) or 30 μg/ml LPS for 2 h or 8 days then stimulated with polyI:C (100 ng) for 3, 12, or 24 h. Cell viability was analyzed by WST-1 assay. Results are representative of three independent experiments. **(C)** H292 cells were treated with polyI:C (2.5 μg), LPS (30 μg/ml), or IL4 (20 ng/ml) for 3 h, and thymic stromal lymphopoietin (TSLP), interleukin 33 (IL33) and IL25 mRNA expression was analyzed by RT-qPCR. **(D)** H292 cells were pretreated with LPS (0, 0.3, or 30 μg/ml) for 2 h, then stimulated with polyI:C for 3 h. The expression of TSLP and IL33 were monitored by RT-qPCR and normalized to the internal control GAPDH; fold induction over controls is presented. Data of RT-qPCR values are mean ± SD from three independent experiments. Two- tailed student *t*-test, $^{*}P < 0.05$, $^{***}P < 0.001$ compared to controls; $^{\#}P < 0.05$.

twofold, respectively (**Figure 1C**) but not LPS. PolyI:C modestly increased IL25 expression, and IL4 has no effect (**Figure 1C**). PolyI:C and IL4-induced TSLP expression was also determined in A549 and WS-1 cells, respectively (Figures S2A–E in Supplementary Material). The polyI:C and IL4-induced TSLP and IL33 expression were also shown in NL20 cells, an immortalized, non-tumorigenic human bronchial epithelial cell line (Figures S3A–D in Supplementary Material). Low-dose LPS (0.3 and 3 μg/ml) had no significant effect on the polyI:C-stimulated mRNA level of TSLP and IL33, but high-dose LPS (30 μg/ml) suppressed the increased TSLP and IL33 level (**Figure 1D**). The reduction of polyI:C-induced TSLP expression by LPS with a dose-dependent manner was also showed in the human primary nasal epithelial cells (Figures S4A,B in Supplementary Material). These data suggest that LPS concentration is critical for its inhibitory effect.

LPS Treatment Inhibits PolyI:C-Triggered IRF3 Activation

TLR-mediated innate immune responses are elegantly regulated by several adaptive signaling proteins and transcription factors. In the TLR3 signaling axis, activation of the transcription factors IRF3 and NF-κB p65/50 by I kappa B kinase (IKK) and IKK-related kinases, such as TBK-1, IKKϵ, IKKα, and IKKβ, is required for transcription of downstream cytokines (6, 49). In H292 cells, this pathway could be activated by polyI:C, the IRF3 phosphorylation was detected at 3 h after stimulation (**Figure 2A**). And the conspicuous increases of phospho-TBK1, -IRF3, and -NF-κB p65 (Ser456 and Ser536) were shown at 12 h (**Figure 2A**). The protein level of NF-κB p65 increasing and IκBα degradation was also noted (**Figure 2A**). The NF-κB activation by polyI:C was further validated by luciferase reporter assay (**Figure 2B**). Those data indicate the competent innate immunity in H292 cells.

To determine the mechanism why LPS-attenuated polyI:C-induced TSLP and IL-33 expression, IRF3, and NF-κB were investigated, PolyI:C-induced IRF3 phosphorylation was inhibited by high-dose (30 μg/ml) but not low-dose LPS treatment in H292 cells (**Figure 2C**). By contrast, the lowest dose of LPS (0.3 μg/ml) enhanced polyI:C-mediated IRF3 phosphorylation approximately 2.4-fold as compared with polyI:C stimulation alone. PolyI:C stimulation induced an 11-fold decrease in IRF3 phosphorylation with 30 μg/ml LPS treatment as compared with 0.3 μg/ml LPS treatment (**Figure 2C**). Although the total NF-κB p65 level was not significantly changed, as compared with that observed with 0.3 μg/ml LPS treatment, 3 and 30 μg/ml LPS treatment blocked the polyI:C-induced IκBα degradation by approximately twofold, which suggests that the polyI:C-mediated activation of the NF-κB pathway was downregulated with high-dose LPS (**Figure 2C**). The nuclear translocation of IRF3 and NF-κB confirmed that LPS significantly interfered with polyI:C-induced IRF3 and

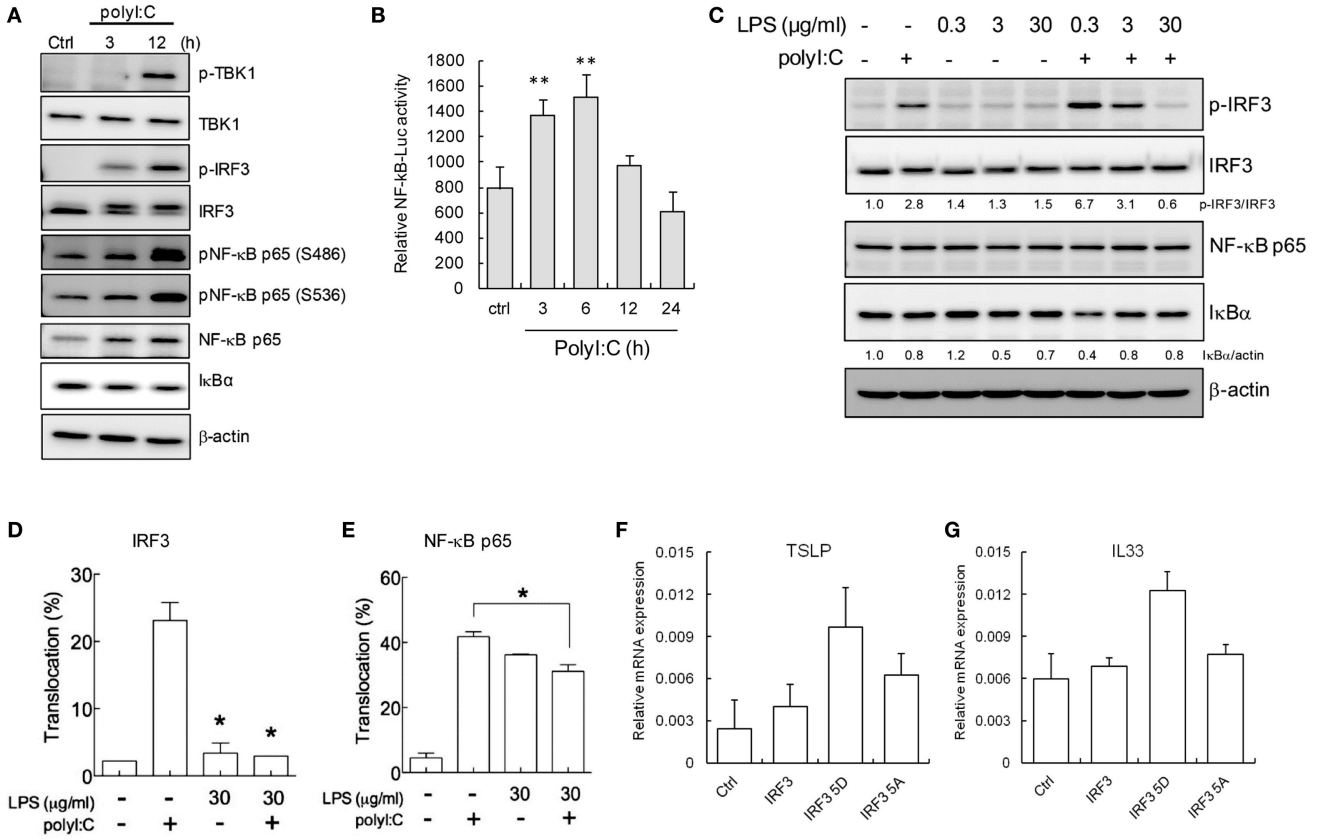

FIGURE 2 | LPS downregulates polyI:C-triggered interferon regulatory factor 3 (IRF3) and NF-κB activation. **(A)** H292 cells were stimulated with polyI:C (2.5 μg) for 3 and 12 h. Total cell extracts underwent SDS-PAGE and immunoblotting with specific antibodies. Data shown are representative of three different experiments. **(B)** Dual-luciferase assay of NF-κB luciferase reporters. H292 cells transfected with Luc reporter (0.6 μg) and pRL-TK (0.06 μg) were treated with polyI:C (2.5 μg) for 3–24 h. Data are mean ± SD from three independent tests. $^{**}P \leq 0.01$ vs. untreated group. **(C)** H292 cells were pretreated with doses of LPS as indicated for 2 h and then stimulated with polyI:C (2.5 μg) for 3 h. Cell extracts underwent SDS-PAGE and immunoblotting with specific antibodies. The signaling proteins expression level was analyzed by density meter and normalized to untreated control. Data shown are representative of three different experiments. **(D,E)** IRF3 and NF-κB p65 nuclear translocation was measured by immunofluorescence assay in H292 cells treated with LPS (30 μg/ml) for 2 h, then polyI:C stimulation for 3 h. Nuclear translocation rate is shown as mean ± SD from three observed fields. $^{*}P < 0.05$ compared to controls. **(F,G)** H292 cells were transfected with control vector, wild-type IRF3, IRF3 5D (constitutive active IRF3), and IRF3 5A (dominate negative IRF3) for 48 h. The expression of TSLP and IL33 mRNA were measured by RT-qPCR and normalized to the internal control GAPDH. Values represent the average of three independent experiments ± SD.

NF-κB activation (**Figures 2D,E**). The representative immunofluorescence images are shown in Figure S5 in Supplementary Material. IRF3 activity-associated TSLP and IL33 expression was further validated by ectopic expression of constitutive active IRF3 (IRF3 5D) but not dominant negative IRF3 mutant (IRF3 5A) (**Figures 2F,G**).

Blocking IRF3 or NF-κB Activation Inhibits TSLP and IL-33 Induction

Based on the findings in **Figures 1** and **2**, blocking IRF3 and NF-κB activity by LPS treatment might be the key mechanism underlying the hygiene hypothesis. We further validated the role of NF-κB and IRF3 in TSLP and IL33 induction by a pharmatheutical approach. The NF-κB inhibitor dexamethasone (50) significantly downregulated TSLP and IL33 mRNA expression in H292 cells stimulated with polyI:C (**Figure 3A**), and immunoblotting data confirmed the inhibitory effect of dexamethasone in interfering with NF-κB activity by inhibiting IκBα degradation (**Figure 3B**). Although phospho-IRF3 level was enhanced in polyI:C-stimulated cells with dexamethasone treatment (**Figure 3B**), this phenomenon did not alter the inhibitory activity of dexamethasone (**Figure 3A**). Bay 11-7082 is an IKK inhibitor that targets IKKα/β, TBK1, IRAK1/4, and TAK1 to modulate NF-κB and IRF3 activity (51). PolyI:C-stimulated TSLP and IL33 mRNA expression was inhibited in H292 cells treated with Bay 11-7082 (**Figure 3C**). Bay 11-7082 effectively inhibited polyI:C-induced IRF3 phosphorylation and IκBα degradation (**Figure 3D**).

The indispensable activity of IRF3 and NF-κB in TSLP and IL33 induction was further demonstrated by shRNA knockdown. IRF3 knockdown efficiency was confirmed by RT-qPCR, and

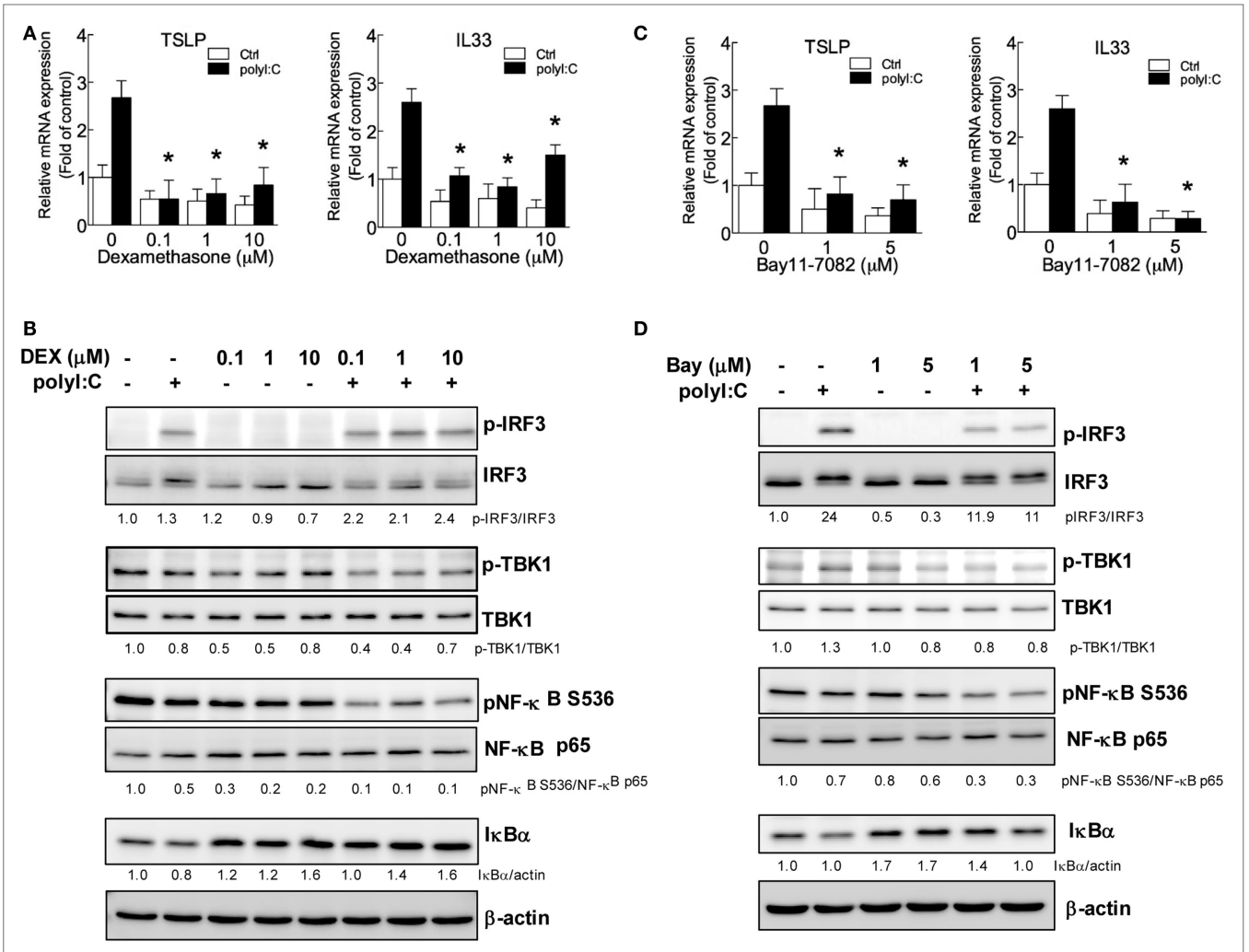

FIGURE 3 | **IRF3 and NF-κB inhibitors suppress polyI:C-stimulated TSLP and IL33 expression**. H292 cells were treated with NF-κB or IRF3 inhibitors dexamethasone (0.1, 1, and 10 μM) or Bay 11-7082 (1 and 5 μM) for 2 h, then challenged with polyI:C (2.5 μg) for 3 h. **(A,C)**. The mRNA expression of TSLP and IL33 was measured by RT-qPCR. Data are mean ± SD from three independent experiments. *$P < 0.05$ compared to untreated controls with polyI:C stimulation. **(B,D)** Immunoblotting analysis with the indicated antibodies. Data shown are representative of three different experiments. The proteins expression level was measured by density meter; the value is normalized to untreated control.

immunoblotting showed that polyI:C failed to induce IRF3 protein expression and phosphorylation in H292 cells with IRF3 knockdown (**Figure 4A**). Importantly, polyI:C-induced TSLP and IL33 expression was inhibited with IRF3 knockdown (**Figure 4B**). The mRNA and protein levels of NF-κB p65 were not increased in knockdown cells with polyI:C stimulation (**Figure 4C**). In addition, polyI:C-induced TSLP and IL33 expression was inhibited with NF-κB p65 knockdown, by ~40 and 50%, respectively (**Figure 4D**). These data validated that the activity of IRF3 and NF-κB is associated with TSLP and IL33 induction by polyI:C.

LPS-Attenuated HPeV1-Mediated TSLP and IL33 Expression and Signaling

To determine the TSLP and IL33 induction in H292 cells with viral infection, the cell was infected by HPeV1 for various times. The TSLP and IL33 levels were significantly induced at 48 hpi (**Figure 5A**). The expression of HPeV VP1 positive strain (+) and negative strain (−) genes showed HPeV1 replication in H292 cells (**Figure 5B**). HpeV1-induced TSLP expression was also shown in A549 cells (Figure S2F in Supplementary Material). To evaluate whether LPS regulates HPeV1 infection-mediated allergic inflammation, H292 cells were treated with LPS before HPeV1 infection (**Figure 5C**). Immunofluorescence assay showed no significant change of the HPeV1 infectivity in H292 cells with or without LPS treatment (**Figure 5D**); whereas, compare to untreated control cells, LPS significantly reduced TSLP and IL33 level in H292 cells infected with HPeV1 (**Figure 5E**). Again, the data of HPeV1 VP1 gene expression indicated no effect of LPS in HPeV1 replication (**Figure 5F**). Immunoblotting analysis showed that LPS downregulated the phosphorylation of IRF3, TBK1, and NF-κB in HPeV1-infected H292 cells (**Figure 5G**). Taken together, these data suggest that LPS modulates HPeV1 infection-triggered allergic cytokines expression.

Long-term LPS Treatment Inhibits TSLP and IL33 Production Pathways

The short-term treatment with high level LPS showed inhibitory effect of allergic cytokines response in epithelial cells (**Figures 1–5**), whereas the effect of long-term treatment of LPS remained to be explored. Thus, to establish long-term LPS-treated H292 cells, cells were incubated with LPS (30 μg/ml) and subcultured every 2 days with LPS-containing medium. On day 8 of LPS treatment, cells were stimulated with polyI:C (**Figure 6A**). Similar to short-term LPS treatment, with long-term LPS treatment, with polyI:C, the increased mRNA expression of TSLP and IL33 was inhibited (**Figure 6B**). The polyI:C-induced TSLP protein level was inhibited by LPS treatment (Figure S6A in Supplementary Material). Furthermore, the protein levels of TLR3 downstream adaptor signaling proteins, phosphorylated TBK1, IRF3 were inhibited with high-dose (30 μg/ml), long-term LPS treatment and polyI:C stimulation for 3 and 12 h. The IκBα degradation

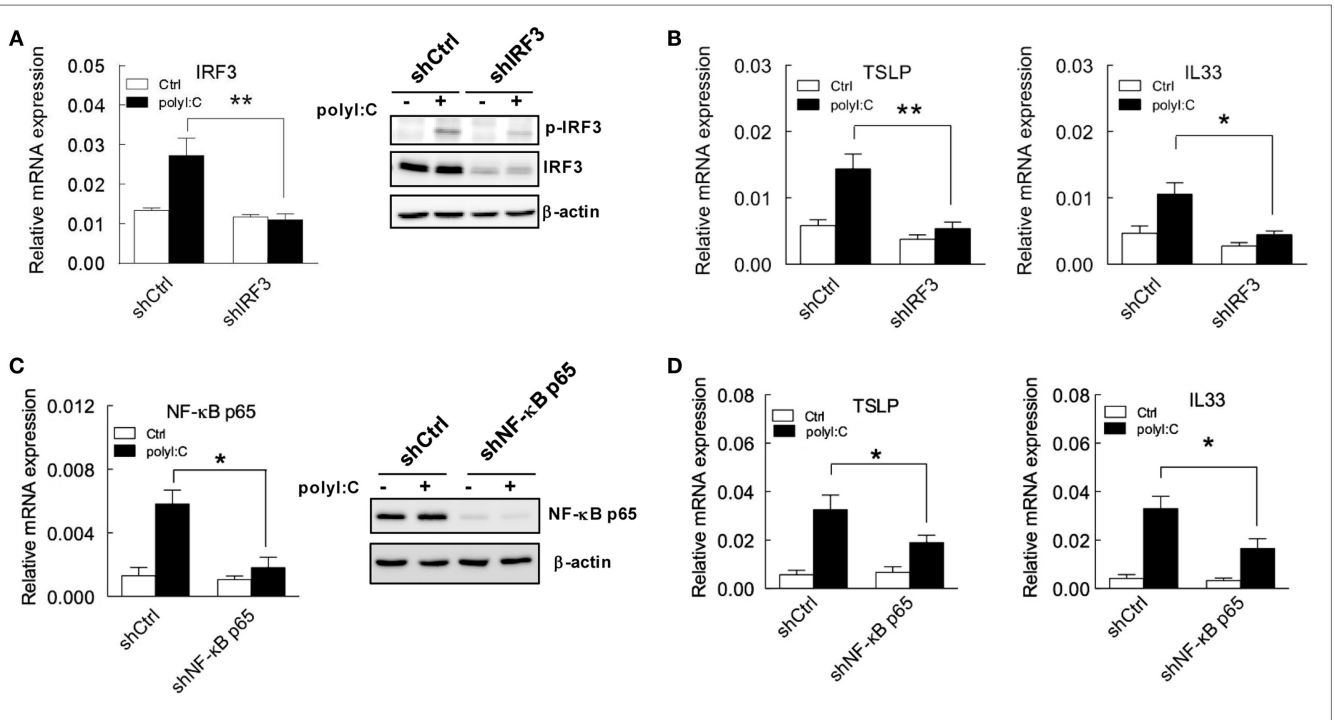

FIGURE 4 | TSLP and IL33 induction is inhibited in H292 cells with IRF3 and NF-κB p65 knockdown with polyI:C stimulation. **(A,C)** H292 cells were infected with lentivirus carrying IRF3 and NFκB p65 shRNA. After puromycin selection, the mRNA and protein expression of IRF3 and NF-κB p65 was analyzed by RT-qPCR (left panel) and immunoblotting (right panel), respectively. Data shown are representative of three different experiments. **(B,D)** TSLP and IL33 mRNA expression was measured by RT-qPCR in H292 cells with IRF3 and NF-κB knockdown that were treated with polyI:C. Data are mean ± SD from three independent experiments. *$P < 0.05$, **$P < 0.01$.

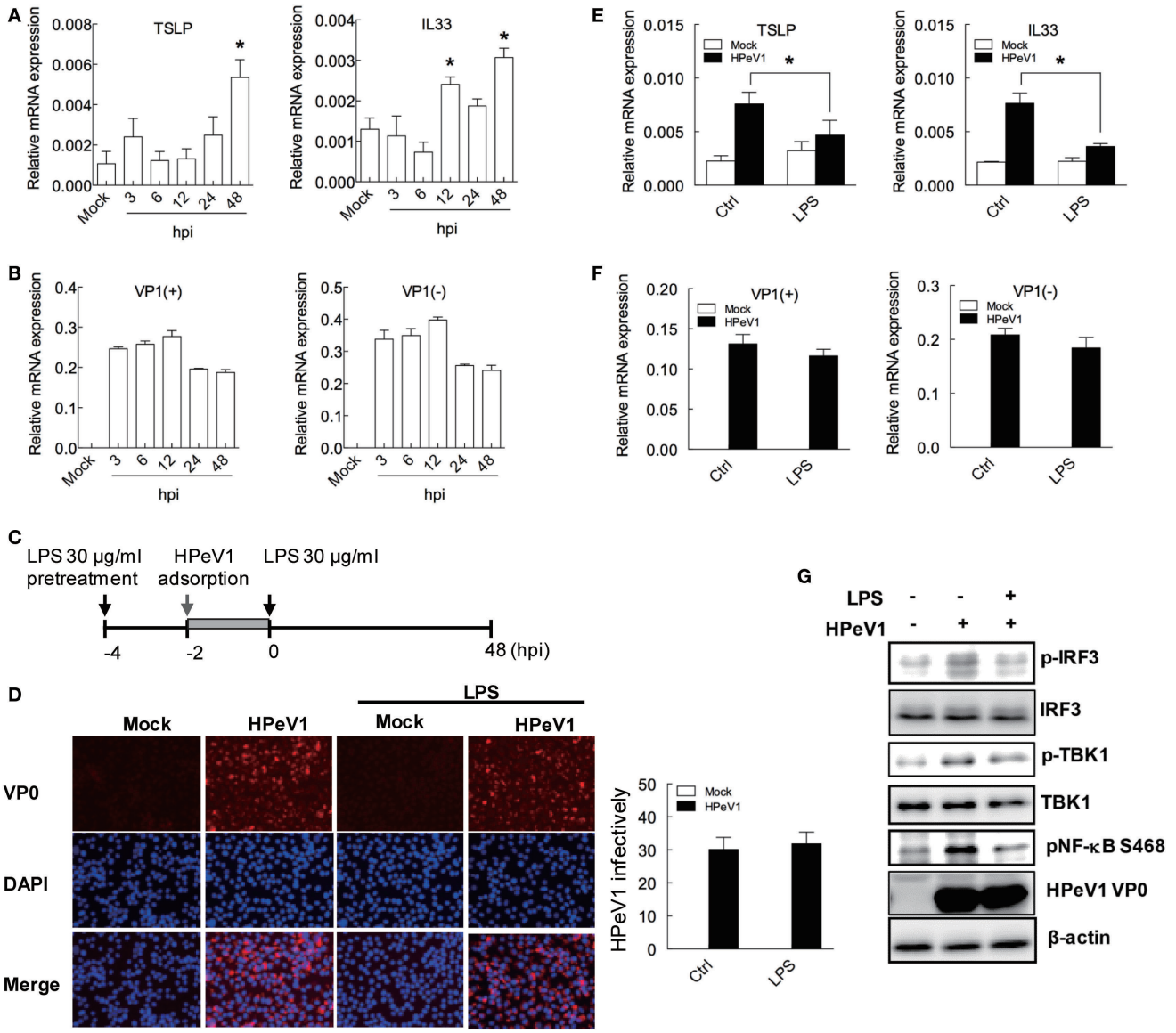

FIGURE 5 | LPS-attenuated HPeV1-mediated TSLP and IL33 expression and signaling. **(A,B)** TSLP and IL33, HPeV1 VP1 (+), and HPeV1 VP1 (–) expression were measured in H292 cells with mock or HPeV1 infection at various times. Data are mean ± SD from three independent experiments. **(C)** The schematic shows LPS treatment and HPeV1 infection in H292 cells. **(D)** Left panel: H292 cells, pretreated or untreated with LPS, were subjected to HPeV1 infection at multiplicity of infection (MOI) = 2.5. HPeV1 infectivity was analyzed by immunofluorescence assay with anti-HPeV0 antibody. HPeV1-infected cells show red fluorescence, and DAPI staining (blue color) show cell nuclei. The merged images show overlapping anti-HPeV VP0 and DAPI staining. Results are representative of three independent experiments. Right panel: HPeV1 infectivity was calculated from three observation fields. **(E,G)** TSLP and IL33, HPeV1 VP1 (+) and HPeV1 VP1 (–) expression was measured in H292 cells with or without HPeV1 infection (Mock) and with (control medium) or without LPS treatment. **(F)** H292 cells were pretreated with or without LPS (30 μg/ml) then infected with or without HPeV1 (MOI = 2.5), then underwent immunoblotting with specific antibodies. Data are mean ±SD from three independent experiments. *$P < 0.05$ compared to controls.

was also downregulated by high-dose LPS treatment in H292 cells stimulated with polyI:C for 12 h (**Figure 6C**).

We also determined whether the basal mRNA level of signaling proteins was changed with long-term LPS stimulation. Compared with untreated control cells, cells with long-term LPS treatment showed significantly downregulated basal mRNA level of NF-κB p65, MyD88, TRAF6, TLR3, NOD1, NEMO, and RIP1 but not NOD2, IRAK1, or MAP3K7 (**Figure 6D**). Similar results were displayed in H292 cells with long-term LPS (30 μg/ml) treatment for 16 days that LPS decreased polyI:C-mediated TSLP and IL33 expression (**Figure 7A**). The polyI:C mediated-protein level of retinoic acid-inducible gene I (RIG-I, a dsRNA receptor), phosphorylated TBK1 and IRF3 were inhibited in H292 cells with LPS (30 μg/ml) treatment for 16 days (**Figure 7B**). Again, the basal mRNA level of singling protein genes were downregulated in those cells (Figure S6B in Supplementary Material). These data

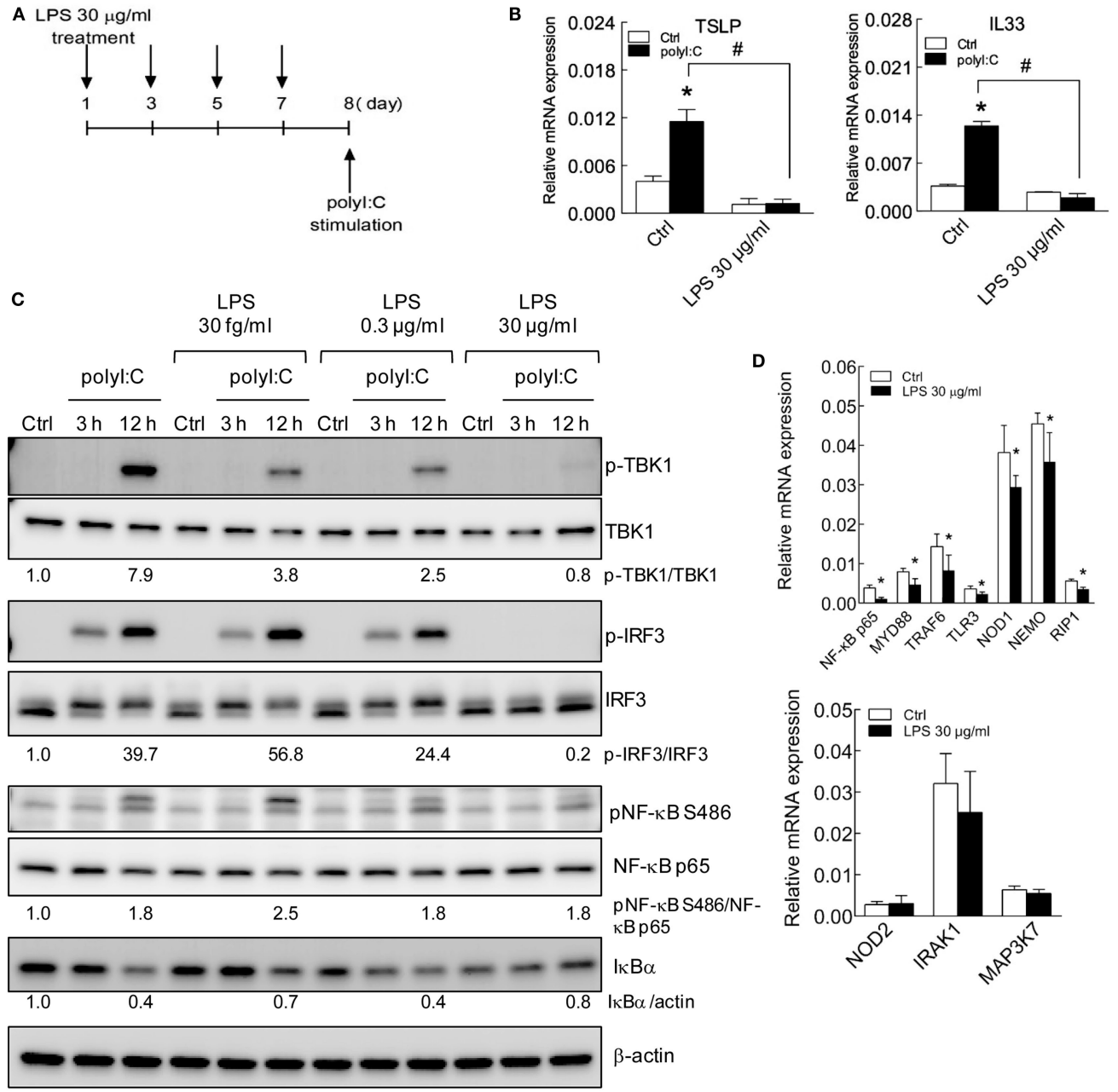

FIGURE 6 | **TSLP and IL33 induction is impaired in H292 cells with long-term LPS exposure**. **(A)** The schematic shows long-term LPS treatment of H292 cells. H292 cells were stimulated with LPS 30 μg/ml for 8 days, then with polyI:C (2.5 μg) for 3 h. **(B)** RT-qPCR analysis of mRNA levels of polyI:C-induced TSLP and IL-33 in H292 cells with long-term LPS (30 μg/ml) treatment. **(C)** H292 cells were treated with various doses of LPS for 8 days, then stimulated with polyI:C (2.5 μg) for 3 h. Immunoblotting analysis with the indicated antibodies. The ratio of signaling protein expression was normalized to medium control. Results are representative of three independent experiments. **(D)** RT-qPCR analysis of TLR signaling gene expression in H292 cells with long-term LPS treatment. Data are mean ± SD from three independent experiments. $^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$.

suggest a fundamental transcription modulation in LPS-treated cells. Consequently, in these cells, allergic cytokine expression was decreased after polyI:C stimulation.

Our data indicated H292 cells with high level (30 μg/ml) LPS treatment for short term (2 h) and long term (8 and 16 days) were able to modulate polyI:C-induced TSLP and IL33 expression; but low-level (0.3 μg/ml) LPS treatment for 2 h showed no effect against allergic inflammation.

To evaluate the effect of low-level LPS and long-term treated H292 cells on TSLP and IL33 expression, LPS-trained H292 cells were established via maintaining in low-level (0.3 μg/ml) LPS-containing growth medium for 60 days. The mRNA analysis

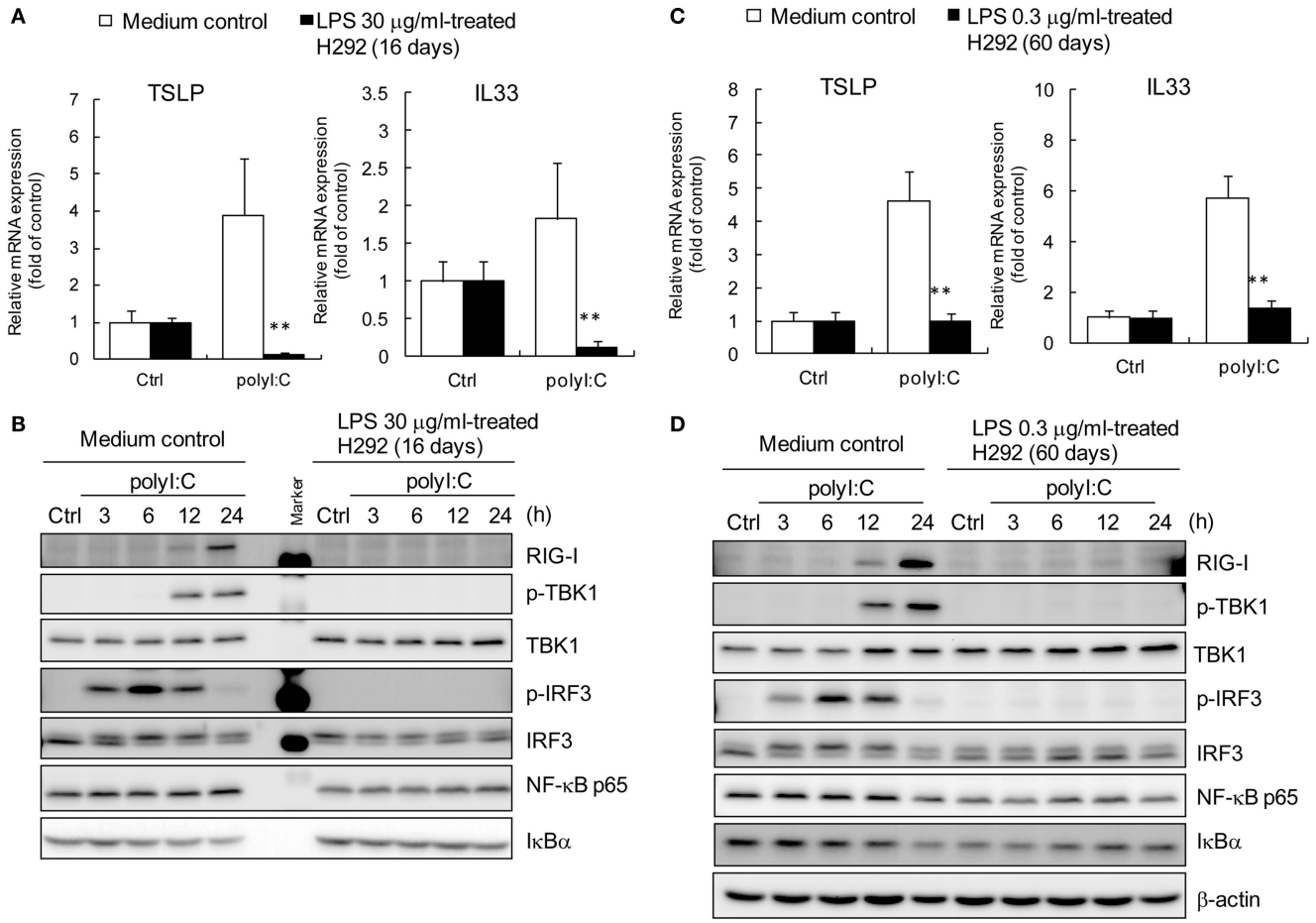

FIGURE 7 | Defective activation of RIG-I-IRF3 signaling axis in H292 cells with long-term LPS treatment. LPS-trained H292 cells were established by long-term treatment of high level LPS (30 μg/ml) and low-level LPS (0.3 μg/ml) for 16 days **(A)** and 60 days **(C)**, respectively. RT-qPCR analysis of mRNA levels of polyI:C-induced TSLP and IL-33 are shown. The growth medium cultured cells were as the untreated control group. Data are mean ± SD from three independent experiments. $^{**}P < 0.01$ compared to control. **(B,D)** The whole cell extracts from high level and low-level LPS-trained or untrained H292 cells were harvested at 3, 6, 12, and 24 h after polyI:C stimulation. Immunoblotting analysis was performed with the indicated antibodies. Results are representative of three independent experiments.

showed that polyI:C failed to induced TSLP and IL33 in the LPS-trained H292 cells (**Figure 7C**). In addition, similar to the H292 cells with long-term high-level LPS treatment, the polyI:C-associated RIG-I, phosphorylated TBK1 and IRF3 expression was suppressed (**Figure 7D**).

HMBG1 Acts in Synergy with PolyI:C to Induce TSLP and IL33 Expression

To evaluate the role of HMGB1 in proallergic cytokines expression, the TSLP and IL33 RNA were measured in the H292 cell with HMGB1 stimulation for 3, 24, and 48 h. The HMGB1 alone was not able to induced TSLP and IL33, while polyI:C showed positive induction (**Figures 8A,B**). Although the IRF3 and NF-κB were activated at 24 and 48 h after HMGB1 stimulation (**Figure 8C**); however, those signals might not be enough for downstream TSLP and IL33 expression. We further evaluated that whether HMGB1 plays a similar role to LPS to attenuate the activity of polyI:C-mediated proallergic cytokine expression. Surprisingly, in the polyI:C-stimulated cells, the RNA level of TSLP and IL-33 was increased by HMGB1 pretreatment for 3 h (**Figures 8D,E**). The immunoblotting also showed that HMGB1 enhanced the level of polyI:C-mediated IRF3 phosphorylation (**Figure 8F**). These results suggest that HMGB1 acts in synergy with polyI:C to promote TSLP and IL33 expression, which may be involved in allergic inflammation.

DISCUSSION

In this study, we delineated the cellular and molecular roles of airway epithelial cells in hygiene hypothesis *in vitro*. Treatment of LPS could suppress the levels by subverting the polyI:C- and viral

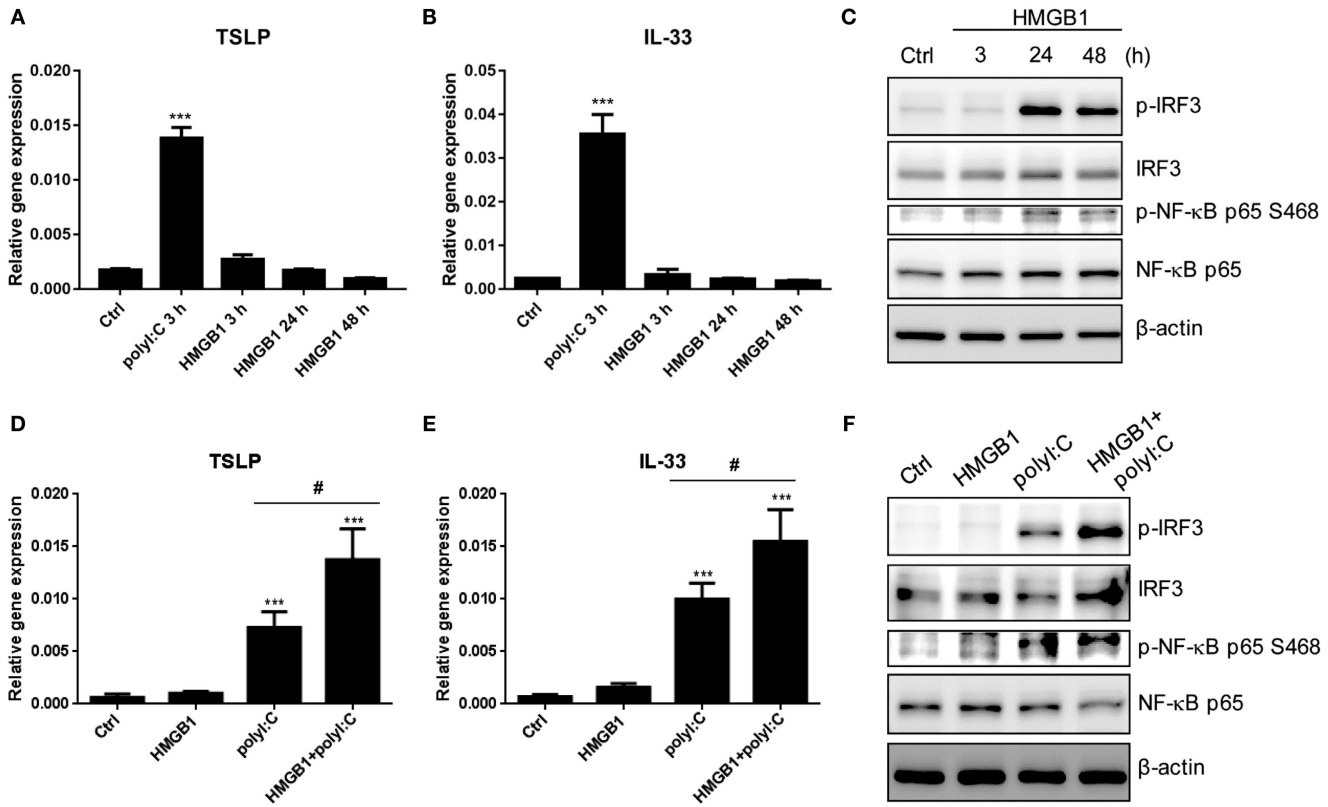

FIGURE 8 | HMGB1 enhances polyI:C-stimulated TSLP and IL33 expression. **(A,B)** RT-qPCR analysis of TSLP and IL33 expression in H292 cells stimulated with polyI:C (2 μg) for 3 h or HMGB1 (1 μg/ml) for 3, 24, and 48 h. Data are mean ± SD from three independent experiments. ***$P < 0.001$ compared to control. **(C)** The whole cell extracts from H292 cells were harvested at 3, 24, and 48 h after HMGB1 (1 μg/ml) stimulation. Immunoblotting analysis was performed with the indicated antibodies. Results are representative of three independent experiments. **(D,E)** H292 cells were pretreated with HMGB1 (1 μg/ml) for 3 h, then stimulated with polyI:C for 3 h. The expression of TSLP and IL33 were monitored by RT-qPCR. Data are mean ± SD from three independent experiments. ***$P < 0.001$ compared to controls; #$P < 0.05$. **(F)** H292 cells were pretreated with HMGB1 for 3 h and then stimulated with or without polyI:C (1 μg) for 3 h. Cell extracts underwent SDS-PAGE and immunoblotting with specific antibodies. Data shown are representative of three different experiments.

infection-mediated TBK1, IRF3, and NF-κB response. Therefore, the level of LPS exposure played a key role in inhibiting TSLP and IL33 expression in epithelial cells. Moreover, the DAMP inflammation factor HMGB1 increased the dsRNA-mediated proallergic cytokines expression. Blocking IRF3 and NF-κB by shRNA gene knockdown or inhibitors treatment suppressed TSLP and IL33 induction, so, in addition to TSLP and IL33, epithelium IRF3 and NF-κB activity could be targets for allergic inflammation therapy (52) (**Figure 9**).

Epithelial cells play key roles in bridging the innate and adaptive immune systems (15). The TLR signaling plays the crucial role in this system; however, TLR3 not only induce host inflammation response but also promote cell apoptosis (48). Physiologically, cell death is a host defense mechanism to restrict virus expanding; the exposed-viral antigen from death cells can trigger a series of immune response activation with cytokines releasing, which is usually associated with the viral pathogenic effects (53, 54). In our study, polyI:C-induced H292 cell death was inhibited by LPS treatment, which might refer that LPS-TLR4 interaction derives a negative regulation or a desensitization mechanism to provide protection against pathogen-mediated cell death (55).

Thymic stromal lymphopoietin is expressed mainly by epithelial cells and epidermal keratinocytes; other types of cells, such as mast cells, smooth muscle cells, fibroblasts, dendritic cells, trophoblasts, and cancer or cancer-associated cells also express TSLP. TSLP expression in the epidermis, epithelium, and submucosa in skin, airway and ocular tissues plays critical role in the pathogenesis of allergic disease (56, 57). IL33 is abundantly expressed in epithelial cells from tissue exposed to the environment, and in fibroblastic reticular cells of lymphoid organ. IL33 expression has been also observed in endothelial cells from blood vessels (58). The roles of TSLP and IL33 in allergy were evaluated in TSLP receptor (TSLPR)- and IL33 receptor-deficient (T1/ST2) mice, respectively; TSLPR- and T1/ST2-knockout mice showed strong Th1 responses with high levels of IL2 and IFN-γ and impaired the Th2 response (59–61). The TSLP- and IL33-mediated Th2 response was demonstrated in TSLP- and IL33 knockout mice, respectively (60, 62). By contrast, lung-specific expression of a *Tslp* transgene induced Th2-mediated airway inflammation and hyperactivity (60). Epithelial cell-derived TSLP mediates chemotactic activity in dendritic cells and airway smooth muscle cells (63, 64), which may be associated with the

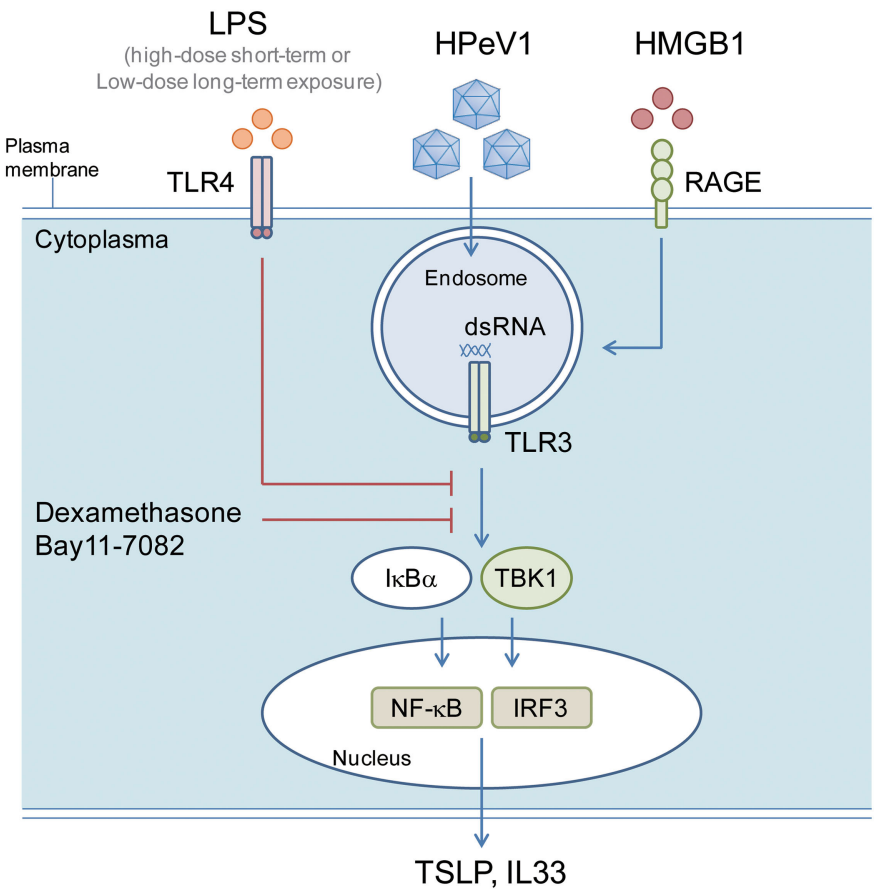

FIGURE 9 | LPS inhibits polyI:C- and virus-induced proallergic cytokines expression via targeting TBK1, IRF3, and NF-κB response. A schematic model shows the possible mechanism of the LPS modulating TSLP and IL33 in airway epithelial cells. The polyI:C or HPeV1 activated TLR signaling is inhibited in the cells with short- and long-term LPS exposure, or inhibitors (Dexamethasone and Bay11-7082) treatment, which lead to a defective expression of TSLP and IL33.

development of allergy. During allergic inflammation initiation, polyI:C- or dsRNA-induced TSLP and IL33 derived from epithelium are critical to activate dendritic cells to produce IL4, IL5, and IL13 and to promote Th2 responses. In turn, the positive feedback provided by IL4, IL5, and IL13 upregulates TSLP and IL-33 production in various cell types, which may aggravate allergic inflammation (Figure S7 in Supplementary Material) (10, 16, 56, 65–67). Thus, blocking TSLP and IL33 activity by antibody or signaling inhibitors may be considered for atopic disease therapy (68, 69). Alternatively, inhibition of TSLP and IL33 levels in epithelial cells may effectively attenuate allergic responses.

In this study, we demonstrated that TSLP and IL33 were induced by polyI:C and HPeV1 stimulation in H292 cells, which supported result from the previous study of primary bronchial epithelial cells (70). Nevertheless, our model also serves as a cell line-based platform for anti-allergy drug screening. We found that dexamethasone and Bay 11-7082 inhibited TSLP and IL33 expression by downregulating IRF3 and NF-κB activities. Coincidentally, the essential roles of the IRF3 and NF-κB pathways in TSLP and IL-33 induction were demonstrated in other research models [(16, 71–75), Schuijs et al. (76) #5788]. Moreover, we found that LPS-modulated IRF3 and NF-κB activation in the polyI:C or HPeV1/TLR3 axis could be the causal mechanism of hygiene hypothesis. Our findings are supported by a current study that farm dust and LPS attenuated house dust mite-mediated allergic response in respiratory epithelial cells (76).

The dose of LPS is one of the critical factors in the establishment of airway allergy. However, the used- dose of LPS *in vitro* experiments was varied. In primary cells, such as macrophages, dendrite cells, or embryonic fibroblast, 100 ng/ml–1 μg/ml of LPS is sufficient to activate innate immunity (77–80). However, in the macrophage cell line (JA774A.1) or lung carcinoma epithelial cells (A549 and BEAS-2B cells), 10–100 μg/ml of LPS was used to change cellular behaviors (40, 55). In our model, TSLP and IL33 production were downregulated in H292 cells with 30 μg/ml of LPS, short-term treatment or 0.3 μg/ml of LPS, long-term treatment. Moreover, 4–12 μg/ml of LPS also attenuate TSLP expression in human primary nasal epithelial cells. We thought that our data are not conflicting in certain with the previous reports; moreover, it also suggests that the stimulation dose of LPS and time course were critical factors in the inhibition of allergic cytokine induction.

We had tried to test our hypothesis in an experimental mouse model of allergic rhinitis; however, the animal model was extremely hard to conduct and there were several points, which are difficult to overcome in our system. First, mice always sneezed and rubbed their noses until the fluid flowed out after reagent solutions, such as LPS were dropped into their nasal cavity, so the scheduled incubation period of 1–3 h became unreliable and the stimulating dosage unreliable too. Second, when the reagent solutions stimulated the nasal cavity, we cannot differentiate whether the effects came from stimulation of the dendritic cells or epithelial cells. We thought that establishment of a proper animal model would be required for further translation approach.

Even though, the effect of LPS dose was investigated in mice reported by others, that low-dose LPS induced Th2 responses, but high-dose LPS with antigen treatment resulted in Th1 responses (29). Therefore, the level of LPS exposure can determine the skew of Th1/Th2 responses and provide a potential mechanistic explanation for epidemiological findings on LPS exposure and asthma prevalence involving the activation of antigen-containing dendritic cells (29). However, in this mouse model, only endpoint analysis was accessed; it would be of interest to investigate the immunoregulatory effect in mice receiving low-level LPS for an extended/long-term period. In addition, the physical effective dose of LPS may be varied among LPS from various bacteria (40); so, the different allergic regulation activity may be observed in mice challenged with various types and dose of bacterial LPS.

We show that short- and long-term LPS exposure had similar inhibitory effects in polyI:C/TLR3-axis-mediated TSLP and IL33 expression, with certain signaling-protein gene transcripts downregulated in H292 cells after treatment with high-dose LPS for 8 and 16 days. We also found that low-dose LPS-suppressed expression of TLR signaling genes was substantial in H292 cells treated with LPS for 2 months. Therefore, LPS silenced the TLR-mediated innate immunity, which may occur at the gene transcription level and be controlled by chromatin modification activity (81, 82). Thus, determining whether the epigenetic regulation of the host response to LPS is involved in the hygiene hypothesis would be of interest.

A recent study proposed a hypothesis about early life airway exposure to microbial pathogens and the development of asthma, which suggests that specific microbial colonization and co-infection of bacteria and viruses such as *Streptococcus* and *Corynebacterium*, respiratory syncytial virus and human rhinoviruses in infants was a strong predictor of persistent asthma developing by 5 years of age. Additionally, antibiotic treatment reduced this risk by disrupting microbial colonization (83, 84). The hypothesis that infections trigger asthma suggests an additional opinion on the hygiene hypothesis and refers to the sophisticated regulation of specific microbes and a host defense system that affect the occurrence of atopy. Therefore, our data raise important issues as to whether different LPS resources from various microbes have different inhibitory outcomes.

The LPS from *Escherichia coli* 0111:B4 was mainly used in this study, but LPS derived from other bacteria, such as *Klebsiella pneumoniae* or *Salmonella enteric*, were also tested. Although, the inhibitory effect of all these LPS on polyI:C were observed, but the implication of the pathogenic bacteria infection in allergy remains to be explored. Moreover, determining whether the inhibitory effect of LPS could be demonstrated with other bacterial components, such as peptidoglycan, teichoic acid, or phosphorylcholine in airway epithelial cells would be of interest (85).

High-mobility group protein B1 act as endogenous danger signals to promote and exacerbate the inflammatory response (37, 38, 86, 87). Given the relevance of HMGB1 as a ligand for TLRs (88, 89), we tested the activity of HMGB1 in our *in vitro* model. We found that HMGB1 itself was not able to induce TSLP and IL33 expression. However, in the polyI:C-stimulated cells, the level of TSLP and IL33 expression and IRF3 phosphorylation was enhanced by HMGB1; which is contrary to LPS treatment on the regulation of allergic inflammation. We thought that certain additional activation signaling derived from HMGB1 receptor other than TLR4, such as RAGE, might contribute to dsRNA-mediated allergic inflammation (90). Thus, it would be important to future evaluate whether HMGB1 would be a therapeutic target for allergy (91).

To the best of our knowledge, our study is the first to delineate a cellular and molecular mechanism of the hygiene hypothesis via TLR signaling in epithelial cells. In addition, we also revealed HPeV1 elicits allergic inflammation. Varying the concentrations or treatment time course of TLR4 agonist could regulate TLR3-associated allergic inflammation for a new strategy to combat allergic diseases.

AUTHOR CONTRIBUTIONS

T-HL, H-YK, and T-HC conceived and designed the experiments. T-HL and C-CC performed the experiments. T-HL, C-CC, H-YK, and T-HC analyzed the data. H-HS, N-CH, J-JC, and H-YK contributed reagents, materials, and analysis tools. T-HL and T-HC wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00440

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REVIEW
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The ST2/IL-33 Axis in Immune Cells during Inflammatory Diseases

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Il1rl1 (also known as ST2) is a member of the IL-1 superfamily, and its only known ligand is IL-33. ST2 exists in two forms as splice variants: a soluble form (sST2), which acts as a decoy receptor, sequesters free IL-33, and does not signal, and a membrane-bound form (ST2), which activates the MyD88/NF-κB signaling pathway to enhance mast cell, Th2, regulatory T cell (Treg), and innate lymphoid cell type 2 functions. sST2 levels are increased in patients with active inflammatory bowel disease, acute cardiac and small bowel transplant allograft rejection, colon and gastric cancers, gut mucosal damage during viral infection, pulmonary disease, heart disease, and graft-versus-host disease. Recently, sST2 has been shown to be secreted by intestinal pro-inflammatory T cells during gut inflammation; on the contrary, protective ST2-expressing Tregs are decreased, implicating that ST2/IL-33 signaling may play an important role in intestinal disease. This review will focus on what is known on its signaling during various inflammatory disease states and highlight potential avenues to intervene in ST2/IL-33 signaling as treatment options.

Keywords: IL-33, ST2, IL1RL1, graft-versus-host disease, cardiac diseases, lung diseases

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INTRODUCTION

In 1989, the Il1rl1 gene product, which has also been given the alias ST2 and defined as the IL-33 receptor, as it binds to IL-33, was discovered (1, 2). It belongs to the IL-1-receptor superfamily. The literature has been misnaming ST2 as "suppressor of tumorigenicity 2," when in fact the original name was "growth stimulation expressed gene 2" (2) and has recently been renamed by the original discoverer, Shin-ichi Tominaga, as "serum stimulation-2" (3), as it was first discovered to function as a mediator of type 2 inflammatory responses (4). IL1RL1 is located on chromosome 2q12.1 in humans, while the gene "suppressor of tumorigenicity 2," also called ST2, is located on chromosome 11p14.3-p12 in humans. In this review, we will call ST2 as the IL-33 receptor or Ilr1rl gene product.

ST2 has two main splice variants due to differential promoter binding: a membrane-bound form (ST2), which promotes NF-κB signaling, and a soluble form (sST2), which prevents its signaling. It was not until 2005 that the ligand for ST2, the cytokine IL-33, was identified through database searching for genes homologous to other IL-1 superfamily members (5, 6). IL-33 has been identified as a mediator of various inflammatory diseases such as asthma, cardiovascular diseases, and allergic diseases (6). Besides being secreted, IL-33 can be found in the nucleus of human high endothelial venules (7), lung airway epithelium, keratinocytes, fibroblastic reticular cells, and some epithelial cells of the stomach and salivary glands (8). Due to the presence of a *N*-terminal domain nuclear localization sequence and a homeodomain-like helix-turn-helix motif, IL-33 is able to bind heterochromatin, potentially giving IL-33 transcriptional regulatory capacity (7).

Dysregulation of ST2/IL-33 signaling and sST2 production have been implicated in a variety of inflammatory diseases such as cardiac disease (9–12), inflammatory bowel disease (IBD) (13–16), graft-versus-host disease (GVHD) (17–24), small bowel transplant rejection (25), and type 2 diabetes (26–29). The purpose of this review is to highlight the function of both ST2 and sST2, ST2/IL-33 in regard to different immune cells, and sST2 production and ST2 signaling in inflammatory diseases.

TWO MAIN ISOFORMS OF ST2: SECRETED AND MEMBRANE BOUND ON IMMUNE CELLS WITH OPPOSITE ROLES

The ST2 gene is located on human chromosome 2q12.1 and is approximately 40 kb long. Homologs of ST2 are found in the genomes of mouse, rat, and fruit fly. ST2 has four splice isoforms from a single transcript dependent on the promoter being used: ST2, a membrane receptor; sST2, a soluble factor; ST2V, a variant form of ST2; and ST2LV, another variant form of ST2, which are differentially regulated through alternative promoter binding (30–32). Little is known about ST2V other than it is expressed highly in gastrointestinal organs (33). ST2LV lacks the transmembrane domain found in ST2; is secreted by eye, heart, lung, and liver tissues; and is found during later stages of embryogenesis (34). Other information on ST2LV is currently lacking.

By cloning the *Il1rl1* gene in rat and sequencing sST2 and ST2 cDNAs, it was found that sST2 and ST2 have different exon 1 sequences (30). Mapping the promoter regions for *Il1rl1* showed that the transcription start site for sST2 is in a proximal promoter region while the transcription start site for ST2 is in a distal promoter region, 15 kb upstream from the sST2 proximal promoter (30) (**Figure 1**). Three to four GATA transcription factors have been identified at the distal promoter region within 1,001 bp, two of which were conserved between human and mouse *Il1rl1* genes (32, 35). These GATA elements binding to the distal promoter lead to ST2 expression. The transcription factor PU.1 also binds to the distal promoter near the GATA elements in both human mast cells and basophils (36). PU.1 and GATA2 cooperatively transactivate the distal ST2 promoter inducing expression of ST2, but not sST2 (36). Loss of PU.1 significantly decreased ST2 expression (36). Conversely, a PMA-responsive element has been found near the proximal promoter region of ST2 in the mouse fibroblast line NIH 3T3 (37). Similarly, activated human fibroblast line TM12, which only uses the proximal promoter for *Il1rl1* transcription, led to sST2 expression (32). These data further suggest that the distal promoter is used to transcribe ST2 and the proximal promoter is used to transcribe sST2. To verify these results and find other transcription factors important in ST2 and sST2 expressions, ChIP-seq experiments should be performed.

ST2

ST2 was first found in serum-stimulated BALB/c-3T3 cells in the presence of cycloheximide (38). It contains an extracellular domain, which binds IL-33 with the help of IL-1 receptor accessory protein (IL-1RAP), a transmembrane domain, and an intercellular domain called a Toll/interleukin-1 receptor (TIR) domain. Due to the presence of the TIR domain, ST2 has been classified as a member of the IL-1 receptor superfamily. ST2 is expressed on cardiomyocytes (39) and a large variety of immune cells, including T conventional cells, particularly type 2 (40), regulatory T cells (Tregs) (41), innate helper 2 cells [innate lymphoid cell type 2 (ILC2)] (42), M2 polarized macrophages (43), mast cells (44), eosinophils (45), basophils (46), neutrophils (46), NK (47), and iNKT cells (47). Signaling through ST2 in immune cells induces type 2 and Treg immune responses, IgE production, and eosinophilia (5, 40–42, 48).

sST2

sST2 protein lacks the transmembrane and cytoplasmic domains contained on ST2 and contains a unique nine amino acid *C*-terminal sequence (35). *In vitro*, sST2 production has been shown to be enhanced by pro-inflammatory cytokines (IL-1β and TNF-α) in human lung epithelial cells and cardiac myocytes. In humans, sST2 can be not only produced spontaneously by cells in the lung, kidney, heart, and small intestine (49) but also produced after activation with IL-33 in mast cells (50) or anti-CD3/anti-CD28 in both CD4 and CD8 conventional T cells (51). In a murine GVHD model, it has recently been shown that intestinal Th17 and Tc17 cells produced large amounts of sST2 following alloreactivity (51). This enhanced sST2 presence has been shown

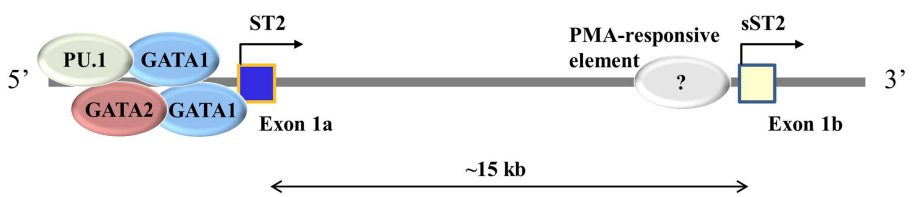

FIGURE 1 | Different promoter usage dictates ST2 and sST2 expressions. ST2 consists of two main splice isoforms: ST2 and sST2. These isoforms are splice variants of each other regulated by alternative promoter bindings, the distal promoter for ST2, and the proximal promoter for sST2. Exon 1 varies between ST2 and sST2 depending on the promoter being bound. In immune cells, GATA1, GATA2, and PU.1 have been shown to bind to the distal promoter. The proximal promoter has not been well studied; it is thought that a PMA-responsive element induced sST2 transcription (37).

to inhibit the production of the type 2 cytokines IL-4 and IL-5 but not the type 1 cytokine IFN-γ (52).

ST2/IL-33 SIGNALING

The Membrane-Bound Form of ST2 Signals through MyD88/NF-κB

Upon IL-33 binding, the membrane-anchored ST2 forms a heterodimer along with IL-1RAP (53, 54) leading to the dimerization of the TIR domain. This leads to the recruitment of the TIR domain binding protein MyD88 and subsequent IL-1R-associated kinase activation, which can activate MAP kinases and NF-κB pathways (**Figure 2**) (5, 6). In regards to ST2/IL-33 signaling, how ST2/IL-33 signals specifically to either the MAPK or NF-κB is currently unclear. However, downstream events of ST2 do seem to occur differentially, as TRAF6 is required for NF-κB activation and induction of type 2 cytokines but TRAF6 is not needed for IL-33-induced ERK (a MAPK protein) activation (55). How TRAF6-independent activation of ERK occurs after IL-33 binding ST2 is currently unknown.

A recent report has shown that signaling through ST2/IL-33 in colonic Tregs helps to promote Foxp3 and GATA3 expressions while also promoting Treg function through enhancing TGF-β1-mediated differentiation (41). This enhancement is caused by phosphorylation of GATA3, which recruits more GATA3 and RNA polymerase II to the *Foxp3* promoter (41). GATA3 binds to the ST2 promoter, enhancing ST2 on the surface of both Th2 cells (56, 57) and Tregs (41, 57). IL-33 has been shown to drive NF-κB and p38 signaling in Tregs, leading to the selective expansion of ST2$^+$ Tregs (58). As this effect is observed in Tregs in a non-diseased setting, independent of outside inflammatory responses, we believe that the ST2/IL-33-GATA3-Foxp3 pathway to be canonical. Conversely, in a non-canonical MyD88-dependent pathway (59), IFN regulatory factor (IRF) 1 signaling can inhibit Tregs by binding to the *Foxp3* promoter and preventing *Foxp3* transcription in murine T cells (60); however, this signaling leading to IRF1 activation through MyD88 has only been shown to be induced using CpG-B, a TLR9 agonist and a pathway independent from ST2/IL-33 (59). Whether ST2/IL-33 can activate IRF1 in a MyD88-dependent pathway and whether this ST2/IL-33-IRF1 activation can affect Treg function are currently unknown. We have highlighted the different ST2 signaling pathways in **Figure 2**.

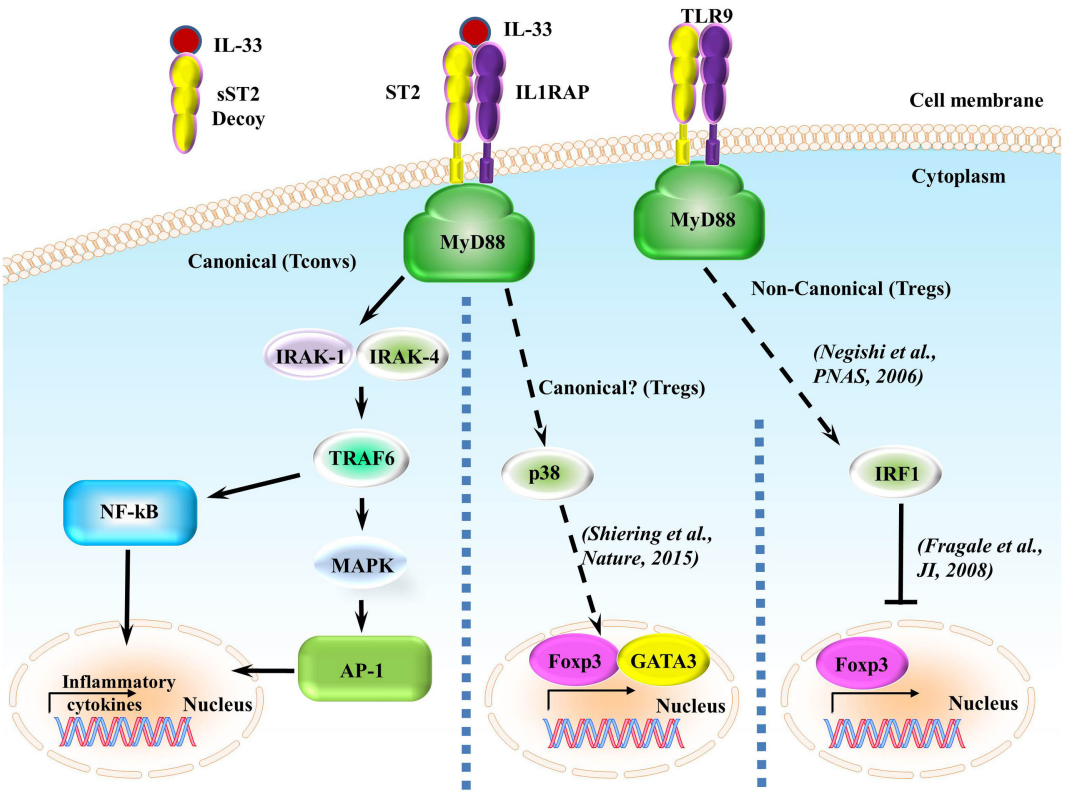

FIGURE 2 | ST2/IL-33 signaling pathway. IL-33 either binds to the ST2/IL-1 receptor accessory protein (IL-1RAP) heterodimer, recruiting MyD88 to its intracellular domain, or the sST2 decoy receptor, which does not signal. MyD88 binding recruits IL-1R-associated kinase (IRAK) and TRAF6, leading to either the NF-κB or AP-1 pathway being activated. NF-κB and AP-1 activations promote inflammatory cytokine expressions. On regulatory T cells (Tregs), ST2/IL-33 signaling has been shown to promote Foxp3 and GATA3 expressions, while also promoting Treg function and expansion through enhancing TGF-β1-mediated differentiation though a p38-dependent mechanism. It has recently been shown that IFN regulatory factor (IRF) 1, which can be activated through MyD88 signaling, can inhibit Tregs by binding to the Foxp3 promoter and preventing Foxp3 transcription.

Unlike IL-1RAP, the single immunoglobulin domain IL-1R-related molecule (SIGIRR or TIR8) SIGIRR can form a complex with ST2 upon IL-33 stimulation and can inhibit ST2/IL-33-mediated signaling both *in vitro* and *in vivo* (6, 61). IL-33 binding to ST2 has also been shown to negatively regulate ST2 through ST2 polyubiquitination, internalization, and degradation (62).

The Soluble Form, (s)ST2, Is a Decoy Receptor and Does Not Signal

sST2 acts as a decoy receptor to sequester free IL-33, preventing ST2/IL-33 signaling. This was shown using a thymoma cell line transfected to express ST2, but not sST2, in the presence of added IL-33. When these thymoma cells were pretreated with sST2, they showed suppressed NF-κB activity (63). Another group used IL-33-treated cardiomyocytes and observed blocked prohypertrophic effects of angiotensin II or phenylephrines in the presence of sST2 (64). Blocking NF-κB signaling in lung alveolar epithelial cells and cardiac myocytes with the specific NF-κB inhibitor CAPE prevented sST2 production by these cells (49). In a human endotoxin model, healthy donors injected with LPS (2 ng/kg) had increased sST2 in their plasma within 24 h of injection (49). Fibroblast growth factor 2 enhanced sST2 production in the human breast adenocarcinoma cell line MCF-7 through MEK/ERK signaling (65). Lysophosphatidic acid has also been shown to increase sST2 production by human bronchial epithelial cells in an NF-κB- or JNK-dependent manner (66). Enhanced sST2 plasma circulation has been correlated with pulmonary fibrosis (67), acute myocardial infarction (39), subclinical brain injury and stroke (68), celiac disease (69), gastric cancer (70), HBV-related acute-on-chronic liver failure (71), HIV progression (72), and GVHD (17–24).

IL-33 Regulation and Release

During cell stress or damage, IL-33 is released in either a full length or a cleaved form. Unlike IL-1β, however, IL-33 is not cleaved *via* caspase-1, and cleavage is not necessary for secretion nor biological activity of released IL-33, further suggesting its role as an alarmin (73, 74). Surprisingly, caspase-1, caspase-3, or caspase-7 processing actually leads to IL-33 inactivation (75, 76). Inactivation of IL-33 *via* caspases is therefore thought to alleviate the immune response, rather than enhance it. Other proteins are able to cleave IL-33, such as the neutrophil serine proteases cathepsin G and elastase, mast cell-derived serine proteases, tryptase, and chymase, and these proteins, unlike caspases, increase the biological activity of cleaved IL-33 10–30 times compared to that of full-length IL-33 (74, 77, 78).

IL-33 is expressed mainly by non-hematopoietic cells, including endothelial cells, adipocytes, fibroblasts, and intestinal and bronchial epithelial cells (8, 79, 80); however, some hematopoietic cells like dendritic cells (DCs) have also been show to express IL-33 when activated (5). In many non-hematopoietic tissues, IL-33 is constitutively expressed. Constitutive expression of IL-33 in epithelial cells suggests that IL-33 is used as an alarmin in response to infection or injury (8). Further suggesting IL-33 as an alarmin, IL-33 is released by damaged or necrotic cells (8), leading to activation of the immune system through ST2/IL-33 signaling (8, 81).

IL-33 can be found in the nucleus due to a nuclear localization sequence in the *N*-terminus, leading to binding of heterochromatin in the nucleus (7). Nuclear IL-33 can bind directly to NF-κB, sequestering it and preventing NF-κB signaling in HEK293RI cells, causing a downregulation of pro-inflammatory signaling (82). Further evidence of IL-33 having the ability to repress gene transcription is described because there is a structural similarity between a part of the IL-33 protein and the Kaposi sarcoma herpes virus motif latency-associated nuclear antigen (82). This mimicry allows IL-33 to bind to the H2A–H2B chromatin dimer and regulate the compaction of chromatin through nucleosome–nucleosome interactions. Recent discoveries have shown that nuclear IL-33 can bind to multiple sites in the promoter regions of ST2 in human endothelial cells and that knockdown of IL-33 increased sST2 levels (83). Loss of the nuclear localization domain of IL-33 led to non-resolving lethal inflammation (84). However, IL-33$^{-/-}$ mice fail to develop autoimmune disease, and no one has shown whether nuclear IL-33 has been found in immune cells. These results indicate that nuclear IL-33 could act as a moderator of inflammation, but more evidence is needed to confirm the extent of the ability of nuclear IL-33 to moderate inflammation.

ST2/IL-33 AND IMMUNE CELLS

ST2 Signaling on Lymphoid Cells

Th2 Cells

ST2 was first shown both *in vitro* and *ex vivo* to be preferentially expressed on murine Th2 cells (**Figure 3**; **Table 1**) expressing predominantly IL-4, IL-5, or IL-10, but not IFN-γ or IL-2 (40, 85). Its expression is independent of IL-4, IL-5, and IL-10, as loss of any of these cytokines does not affect ST2 expression on Th2 cells (40). ST2 expression on Th2 cells is dependent on GATA3 signaling (86) and is enhanced by IL-6, IL-1, TNF-α, and IL-5 (4). Given that ST2 expression in Th2 cells is independent of IL-4 and dependent on GATA3 signaling, it makes sense that ST2 expression occurs late during Th2 differentiation (4). IL-33 stimulation of Th2 cells *in vitro* increased the amount of IL-5 and IL-13 produced (5). Antigen-specific ST2^{+} Th2 cells were shown to produce more IL-5 and IL-13 compared to non-antigen-specific Th cells and ST2$^{-/-}$ Th2 cells (87). Interestingly, IL-33 polarization of antigen-stimulated murine and human naïve CD4^{+} T cells leads to high IL-5 production but no IL-4 production, independent of GATA3 and STAT6 induction but dependent on MAPK and NF-κB signaling (88, 89). Adoptive transfer of these cells into naïve IL-4$^{-/-}$ mice still triggered airway inflammation (88). *In vivo* administration of IL-33 led to an increase in the number of lymphocytes circulating in the blood and increased type 2 cytokine secretions in the thymus, spleen, liver, and lung (5). IL-33 has also been shown to be a chemoattractant for Th2 cells, as adoptive transfer of Th2 cells into ST2 knockout (KO) followed by IL-33 administration into the footpad of these mice led to the accumulation of the transferred Th2 cells (90). Loss of ST2 on Th2 during infection with the helminthic parasite *Nippostrongylus brasiliensis* did not affect Th2-mediated clearance of the infection

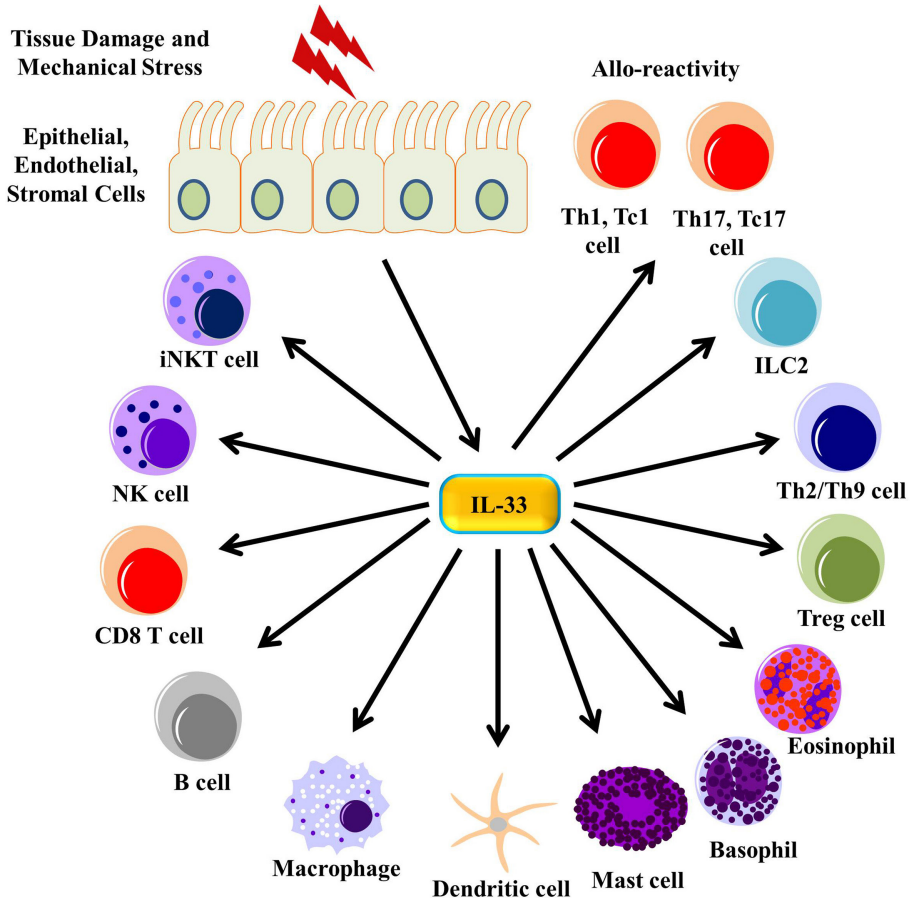

FIGURE 3 | IL-33 signaling on immune cells. Tissue damage and mechanical stress to epithelial, endothelial, and stromal cells lead to the release of IL-33 from these cells. IL-33 then signals through many different immune cells, enhancing their function.

nor was recruitment of Th2 cells in a murine model of asthma dependent on ST2, indicating that ST2 is not necessary for Th2 function (91). Recently, it was shown that human and murine Th2 cells do not produce sST2 *in vitro* (51).

Th9 Cells

IL-9-producing Th9 cells are the newest T cell subset to be described, polarized through TGF-β and IL-4 signaling (92, 93). When used separately on naïve T cells, TGF-β alone would cause Treg development, while IL-4 would induce Th2 cell differentiation. It has been found that the PU.1 gene is a Th9-specific transcription factor, which could induce IL-9 production in cells under Th2- or Th9-stimulating condition *in vitro* (94). Human or mouse PU.1-deficient T cells have diminished IL-9 production. Furthermore, IRF4 binds directly to the IL-9 promoter and is required for the development of Th9 cells, just like PU.1 (95). However, unlike PU.1, IRF4 is also required for the development of other Th cell subsets, including Th2 and Th17 cells (96, 97). Studies have shown that Th9 cells primarily secrete IL-9 to mediate the immune response in several diseases, such as asthma, autoimmune diseases, and parasitic infections (98), and IL-9 is associated with impaired Th1 immune response in patients with tuberculosis (99). Treatment of *in vitro* polarized human Th2 cells with TGF-β and IL-33 increases their IL-9 and ST2 expressions (100, 101).

Regulatory T Cells

ST2/IL-33 signaling in Tregs was first suggested to enhance their protective ability in an experimental colitis model in which IL-33 treatment ameliorated colonic tissue injury and colitis symptoms (41). IL-33 was shown to increase both ST2 and Foxp3 levels and expand Tregs in mice with colitis. ST2/IL-33 signaling in Tregs has also been shown to increase Treg frequency and decrease IL-17 and IFN-γ productions in an experimental autoimmune encephalomyelitis (EAE) model (102, 103). $ST2^+$ Treg expansion is helped by IL-33 signaling in DCs, as IL-33 has been shown to stimulate DC production of IL-2, which selectively expands $ST2^+$ Tregs (104). In a model of GVHD, treatment of mice daily with IL-33 from 10 days pretransplantation to day 4 posttransplantation enhanced the frequency of $ST2^+$ Tregs, which persisted after irradiation, leading to disease amelioration through prevention of T conventional cell accumulation in target GVHD

TABLE 1 | ST2 and sST2 expressions and their regulation in immune cells.

Cell type	ST2	ST2 regulation	sST2	sST2 regulation	Reference
Mast cell	+	Constitutively expressed	+	Strongly induced by IL-33 and weakly by Ag or SCF	(44, 50, 137–139)
Basophil	+	Induced by IL-3 stimulation	+	Released after IL-3 stimulation	(46, 145)
Eosinophil	+	Weakly constitutively expressed but strongly induced after IL-33 stimulation	?		(45)
Th2	+	Constitutively expressed and enhanced by IL-6, IL-1, TNF-α, and IL-5	–		(40, 51, 85)
Th1	–		+	Released after CD3 stimulation or alloactivation	(51)
Th17	–		+	Released after CD3 stimulation or alloactivation	(51)
Regulatory T cell (Treg)	+	Constitutively expressed only on Tregs expressing GATA3; enhanced by IL-33	–		(41, 58)
Th9	+	Constitutively expressed and enhanced by IL-33	?		(100, 101)
Innate lymphoid cell type 2	+	Constitutively expressed and enhanced by IL-33	–		(42, 109)
Dendritic cell	+	Weakly constitutively expressed but strong induction after rapamycin treatment	?		(134, 149)
Neutrophil	+	Weakly constitutively expressed	?		(46, 153)
Macrophage	+	Weakly constitutively expressed but enhanced by IL-4 and IL-13	+	Constitutively expressed	(43, 134)
B1 B cell	+	Constitutively expressed	?		(128)
iNKT cell	+	Constitutively expressed	?		(47, 130)
NK cell	+	Constitutively expressed	?		(47, 130)
Tc1 T cell	+	Weakly constitutively expressed	+	Released after CD3 stimulation or alloactivation	(51, 123, 124)
Tc17 T cell	–		+++	Released after CD3 stimulation or alloactivation	(51)

+, expressed; –, not expressed; ?, unknown; Ag, antigen; SCF, stem cell factor; ST2, serum stimulation-2; sST2, soluble serum stimulation-2.

organs (58). Treatment of mice receiving a heart transplant with IL-33 has been shown to prolong graft survival through increase of Treg and myeloid suppressor-derived cell numbers (105, 106). Similarly, mice treated with IL-33 after skin transplantation had increased Treg numbers in the graft, decreased IFN-γ and IL-17 production, increased IL-10 production, and increased skin graft survival (107). This group also showed that ST2/IL-33 signaling can convert Foxp3$^-$ CD4 cells into Foxp3$^+$ CD4 Tregs in the periphery. We have shown that in a murine model of allogeneic hematopoietic stem cell transplantation (allo-HCT), transplanting ST2 KO Tregs with wild-type (WT) T conventional cells worsens GVHD compared to mice receiving WT T conventional cells and Tregs (51), further indicating the enhanced suppressive effect of ST2$^+$ Tregs. Conversely to the enhanced protective effect of Tregs through ST2/IL-33, it has been reported that IRF1 is downstream of MyD88 (108) and negatively regulates *Foxp3* transcription (60, 108), although whether ST2/IL-33 signaling increases IRF1 expression, leading to decreased Treg function, has yet to be studied. Given that ST2/IL-33 has been shown to enhance, rather than impair Treg function, upregulation of IRF1 through MyD88 signaling is probably independent of ST2/IL-33. These data show that IL-33 signaling on Tregs increases their immunomodulatory function and could be further studied for their potential clinical benefits in a variety of diseases.

Innate Lymphoid Cells Type 2

Innate lymphoid cells type 2 were first discovered in the mouse and human mesenteries and found to be lineage marker negative, c-Kit positive, Sca-1 positive, IL-7Rα positive, and ST2 positive (42, 109). These cells have been shown to play a protective role against helminth infection and regulate metabolic homeostasis (110). In humans ST2$^+$, ILC2s were later found in the lung and gut (111), and these ILC2s produced IL-5 and IL-13. During ILC2 activation, ST2 is upregulated in a GATA3- and Gfi1-dependent manner (112, 113). Treatment of Rag2 KO mice with IL-33 induced IL-5 and IL-13 production, whereas Rag2 and common gamma chain double KOs, which still have mast cells and basophils (both of which express ST2 and secrete type 2 cytokines), did not increase IL-5 or IL-13 production, indicating that this increase is due to ILC2 stimulation with IL-33 (42). ST2/IL-33 signaling enhancement was shown to expand ILC2s *in vivo* (42, 114). This group also found that ILC2s are major producers of type 2 cytokines after *Nippostrongylus brasiliensis* infection. It was also shown using the *N. brasiliensis* infection model that loss of both IL-33 and IL-25 signaling on ILC2s completely abrogated the early response against this infection due to impaired expansion of ILC2s and lack of IL-13 production, and adoptive transfer of WT ILC2s rescued this phenotype (42). During lung inflammation, ILC2s produce IL-9 (115), and IL-33 can promote cytokine production by ILC2s (116). Recently, it was shown that in a murine eosinophilic airway inflammation model that T-bet regulates IL-9 production by IL-33-stimulated ILC2s (117). ST2/IL-33 signaling in ILC2s is also important for protection against lung infection, as blocking ST2 signaling during influenza infection in mice lowered ILC2 frequency and number in the lung and resulted in diminished lung function, loss of airway epithelial integrity, and impaired respiratory tissue remodeling (118). Histological examination of influenza-infected lungs from anti-ST2-treated

mice showed severe damage similar to that seen in a similar experiment where ILC2s were depleted (118). ILC2s have been recently reported to home to the skin in humans, where activation induces upregulation of ST2 (116). ST2/IL-33 signaling of ILC2s in the murine skin has been shown to not only promote atopic dermatitis (AD)-like inflammation (116, 119) but also promote skin wound repair (120). However, overstimulation of ILC2s with IL-33 during tissue remodeling of the liver after chemical injury promoted liver fibrosis (121). Also, signaling through ST2/IL-33 on ILC2s during breast cancer has been shown to promote breast cancer growth and metastasis (122). These data indicate that beneficial or harmful ST2/IL-33 stimulation in ILC2s is dependent on certain disease states.

CD8 T Cells

CD8 T cells have been shown to either express ST2 or produce sST2 (51, 123, 124). Although CD8 T cells express low levels of ST2, loss of either IL-33 or ST2 impaired the CD8 T cell response to LCMV infection (124). ST2/IL-33 signaling has also been shown to enhance CD8 T cell antitumor activity (125). During GVHD, however, IL-33 treatment during peak inflammation significantly increased GVHD severity and mortality in part through increased expansion of Tc1 cells (126). Given that IL-33 can increase type 1 responses when IL-12 levels are high (127), IL-33 treatment during peak inflammation was deleterious in this case.

B Cells

ST2 has been shown to be expressed on B1 B cells but not B2 B cells, leading to enhanced proliferation capacity and IgM, IL-5, and IL-13 productions both *in vitro* and *in vivo*; neutralizing IL-5 almost completely abolished this effect (128). Recent studies have also shown that IL-33 treatment in mice increases circulating IL-10-producing B cells that are neither conventional B1 nor B2 B cells (129). Adoptive transfer of these IL-33-treated, IL-10-producing B cells prevented spontaneous colitis in IL-$10^{-/-}$ mice without affecting Treg frequency (129).

iNKT Cells and NK Cells

ST2/IL-33 signaling in murine iNKT cells causes their expansion and activation (130). Mice treated with IL-33 had twice as many iNKT cells in the spleen and liver compared to untreated mice (130). Unexpectedly, ST2 signaling in iNKT cells induced IFN-γ instead of IL-4 upon TCR engagement, which synergized in the presence of IL-12 (47, 130). This effect was also seen in Vα24^{+} human iNKT cells (47). NK cells constitutively express ST2, and ST2/IL-33 signaling increases IFN-γ levels synergistically with IL-12 (47, 130). Loss of ST2 in Ly49H^{+} NK cells did not affect their development but did impair their ability to expand and protect against MCMV (131). These data have yet to translate to human disease.

sST2 Expression in Lymphoid Cells

Th1 and Th17 Cells

Although much of the research on ST2/IL-33 signaling in T conventional cells has been devoted to type 2 signaling, recent studies have come out looking at ST2/IL-33 signaling in type 1- and type 17-mediated diseases. Blockade of IL-33 with 200 μg anti-IL-33 every other day from day 0 until day 18 post-$MOG_{35–55}$ injection during MOG-induced EAE ameliorated the disease in part through decreased IL-17 and IFN-γ productions, and treatment of 50 μg/kg IL-33 during this same time course enhanced IL-17 and IFN-γ productions (102). However, the amount of IL-33 given here is not physiological, so caution must be advised when interpreting these data. Conversely, another group using the same EAE model found that treatment with 1 μg IL-33 daily from day 12 to day 20 after immunization reduced IL-17 and IFN-γ productions and alleviated the disease (103). Seemingly, timing of ST2/IL-33 signaling affects response, perhaps through differing environments. In a murine model of collagen-induced arthritis, treatment with anti-ST2 antibody reduced both IFN-γ and IL-17 productions (132). In a murine model of rheumatoid arthritis, treatment with an sST2-Fc fusion protein attenuated disease and decreased production of IFN-γ, TNF-α, and IL-6 (133). Recently, we were the first to show that both murine and human Th1 and Th17 cells produce sST2 *in vitro* and *in vivo* after allo-HCT (51). Blocking ST2 with a blocking antibody *in vivo* decreased sST2 production in intestinal T cells 10 days after allo-HCT while maintaining ST2. Recipients of ST2$^{-/-}$ T cells, compared to WT T cells, showed lower frequencies of T cells expressing the Th1 transcription factor T-bet and the Th17 transcription factor RORγt and their associated cytokines IFN-γ and IL-17, respectively, while increasing the expressions of the Th2 transcription factor GATA3 and the Treg transcription factor Foxp3 and their associated cytokines IL-4 and IL-10, respectively (51). Importantly, anti-ST2 treatment did not lead to loss of immunomodulatory ST2^{+} Tregs but rather maintained them in the intestine. On the basis of our findings, we have suggested that increased sST2 production affects the normal balance of pathogenic Th1/Th17 cells and immunomodulatory Th2/Treg cells by promoting the Th1/Th17 response and dampening the ST2-mediated Th2/Treg response through sequestering IL-33 (51).

Tc1 and Tc17 Cells

We were also the first to demonstrate that CD8 T cells, particularly Tc1 and Tc17 cells but not Tc2 cells, produce significant amounts of sST2 *in vitro* and after allo-HCT due to alloreactivity (51). sST2 secretion by donor T cells significantly increased as GVHD progressed. Similar to CD4 T cells, blocking ST2 with a blocking antibody decreased sST2 production by Tc1 and Tc17 cells *in vivo* after allo-HCT (51). Our data indicate that sST2 secretion by Tc1 and Tc17 cells sequester free IL-33, preventing ST2/IL-33-mediated Th2/Treg responses. In patients with early HIV infection, sST2 levels were strongly correlated with CD8 T cell count and their expressions of the activation markers HLA-DR and CD38 (72). However, it is not known if sST2 was produced from the CD8 T cells themselves or if sST2 is only a marker of gut damage and disease progression. While our study was the first to show that preventing sST2 secretion from CD8 T cells prevented disease pathogenesis, further studies are warranted to determine their role in other disease pathogeneses.

Myeloid Cells

Macrophages

Macrophages, mast cells, basophils, eosinophils, and DCs all have been shown to express ST2 (43–46, 134). IL-33 amplifies the expression of M2 markers on murine macrophages (43, 135). Bone-derived human macrophages have been shown to constitutively express both ST2 and sST2; however, skewing these macrophages toward an M2 phenotype using IL-4 and IL-13 increased the expression of ST2 while not affecting sST2 expression (136). ST2/IL-33 signaling has been shown to enhance the activation of macrophages by upregulating the LPS receptor components TLR4 and MD2, soluble CD14, and MyD88 (135).

Mast Cells

ST2/IL-33 signaling on both murine and human mast cells has been shown to promote their survival through upregulation of B-cell lymphoma-X large in the peritoneum (137). ST2/IL-33 signaling also promotes mast cell activation and maturation, as IL-33 treatment of $CD34^+$ mast cell precursors accelerated their maturation *in vitro* and induced GM-CSF, IL-5, IL-13, CXCL8, CCL17, CCL22, and CCL2 secretions (138, 139). These cytokine and chemokine secretions may be NFAT and AP-1 signaling dependent (140). It is well documented that mast cells can produce a variety of type 2 cytokines after ST2 signaling (141–143); however, ST2/IL-33 signaling on mast cells during airway inflammation has also been shown to promote a Th17 response (144).

Basophils and Eosinophils

ST2/IL-33 signaling promotes not only type 2 cytokine secretions such as IL-4 and IL-13 but also IL-8 in synergy with IL-3 or Fcε receptor activation on basophils (145). Basophils can also release sST2 after activation *via* IL-3 and C5a or anti-FcεRIα antibody, while IL-33 prevents sST2 release (145). IL-33 induces the degranulation of eosinophils and production of superoxide (45); controls their responsiveness to Siglec 8 (146); and increases IL-13, TGF-β, CCL3, CCL17, and CCL24 in the lungs during airway inflammation (147). Treatment with anti-ST2 antibodies prevented the upregulation of CD11b and decreased survival of eosinophils (148).

Dendritic Cells

Dendritic cells express low basal levels of ST2 on their cell surfaces (134); however, activation of DCs with rapamycin strongly upregulates ST2 through autocrine IL-1β signaling (149). Treatment of DCs with IL-33 has been shown to increase surface levels of MHC-II, CD40, CD80, CD86, OX40L, and CCR7 (134, 150, 151). ST2/IL-33 signaling in DCs also increases their productions of IL-4, IL-5, IL-13, CCL17, TNF-α, and IL-1β (150). In the presence of naïve $CD4^+$ T cells, IL-33-activated DCs induce IL-5 and IL-13 but not IL-4 and IFN-γ from the T cells (134, 151). Interestingly, sST2 has also been shown to be internalized by DCs, suggesting a non-canonical method of action for sST2 (152). It is currently unknown whether sST2 can be internalized by other immune cells. IL-33-activated murine DCs have recently been shown to be required for *in vitro* and *in vivo* expansion of $ST2^+$ Tregs through DC IL-2 production (104), which could be used for therapeutic benefit against inflammatory diseases through expansion of Tregs both *in vitro* and *in vivo*. ST2 expression on host hematopoietic cells, including DCs, and non-hematopoietic cells was not implicated in the severity of GVHD as recipient ST2 KO bone marrow chimeras did not modify GVHD severity (51).

Neutrophils

While ST2 has been shown to be present on neutrophils (46, 153), not much is known about the role of ST2 on neutrophils. It has been shown that IL-33-treated murine and human neutrophils do not downregulate CXCR2 induced by the activation of TLR4 through the inhibition of GRK2 (153). IL-33 injected into the ears of mice induced neutrophil recruitment to the skin (154); however, it is not clear if ST2/IL-33 signaling on the neutrophils directly led to their migration.

ST2/IL-33 IN INFLAMMATORY DISEASES

Gastrointestinal Diseases

Inflammatory Bowel Disease

It is believed that IBD starts with a dysregulated immune response to either food or commensal gut bacteria, leading to the production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1, and IL-8. Expression of these cytokines along with chemokine release leads to attraction of T cells, specifically type 1 T cells, to the intestines. Continual damage of the gut mucosa by these type 1 cells and other immune cells such as macrophages, neutrophils, and DCs leads to the release of various alarmins and other proteins. sST2 was found to be significantly increased in both the gut mucosa and the serum in both patients and experimental models of IBD (13–16). However, in IBD patients, ST2 expression remained similar to that of healthy patients (13). In the lamina propria of active ulcerative colitis (UC) patients, ST2 predominately came from $CD11b^+$ and $CD4^+$ cells (14). These findings suggest that increased sST2 production by lymphocytes or the gut mucosa could either lead to development of IBD, particularly UC, or that these proteins are markers for disease severity.

ST2/IL-33 signaling has been shown to enhance epithelial proliferation and mucus production in the gut (5), suggesting that the increase in IL-33 in the colonic mucosa in active UC could be beneficial. However, in multiple mouse models of IBD, use of ST2 KO mice led to amelioration of IBD compared to WT controls. These results were verified using an IL-33 KO. Using bone marrow chimeras, it was shown that ST2 signaling in non-hematopoietic cells was responsible for IBD. This was due to ST2/IL-33 signaling impairing epithelial barrier function and delayed wound healing. Lack of ST2 signaling in hematopoietic cells did not prevent UC development. A ST2 blocking antibody confirmed the findings from the KO experiments (155). Crohn's disease (CD), however, shows opposite results from UC. In a trinitrobenzene sulfonic acid-induced IBD model, which mimics the pathology of human CD, administration of recombinant IL-33 (rIL-33) into mice ameliorated colonic tissue injury and clinical symptoms (156). Protection was shown to be through upregulation of type 2 cytokines, $Foxp3^+$ Tregs, and CD103 DCs, which promote Treg development. In patient colons with active IBD, Treg levels in the lamina propria are increased compared to healthy controls

and function normally (157, 158). It has recently been shown that colonic Tregs preferentially express ST2 and that signaling through ST2/IL-33 promotes both Treg accumulation and maintenance in the intestine and enhances their protective function (41). However, treatment with rIL-33 to promote Treg-mediated protection may be time dependent, as rIL-33 treatment at onset of a DSS-induced colitis model exacerbated disease severity. rIL-33 treatment during recovery or chronic phases ameliorated DSS-induced colitis (159). Given these data, selective treatment of ST2$^+$ Tregs with IL-33 could provide therapeutic benefits.

Graft-versus-Host Disease

Graft-versus-host disease is a common occurrence in patients who undergo allo-HCT as treatment for both malignant and non-malignant diseases of the blood and bone marrow. The pathogenesis of GVHD has been well documented and is now thought to occur in three steps: (1) activation of antigen-presenting cells caused by tissue damage from the conditioning regimen leading to the release of pro-inflammatory cytokines and danger signals, (2) alloactivation of donor T cells leading to their proliferation and differentiation into type 1 and type 17 T cells, and (3) tissue destruction by alloreactive T cells through release of cytolytic molecules leading to donor cell apoptosis, mainly in the mucosal tissues (160). Discovering prognostic and diagnostic biomarkers for GVHD has been successful with sST2 being one of the most validated to date (17–24). Blocking sST2 with a blocking antibody during the peritransplant period decreased GVHD morbidity and mortality in both minor histocompatibility and humanized murine models (**Figure 4**). Importantly, the ST2-blocking antibody, which inhibits the full length of ST2 and not specifically sST2, maintained protective ST2-expressing T cells while also not impairing the graft-versus-leukemia activity (51), suggesting that addition of anti-ST2 or a ST2 small molecule inhibitor could show efficacy in reducing GVHD-related morbidity and mortality in patients. Using IL-33 as a treatment seems to be time dependent, as injections with IL-33 during the peak inflammatory response in a murine model led to increased morbidity and mortality in mice due to increased migration and increased pro-inflammatory cytokine production (126). IL-33 treatment preconditioning, however, increased the number of ST2$^+$ Tregs, which persisted after irradiation in a murine model. This led to decreased GVHD severity and mortality. Adoptive transfer of ST2$^+$ versus ST2$^-$ Tregs showed that GVHD protection is increased by ST2$^+$ and not ST2$^-$ Tregs (58). Given that IL-33 is pleiotropic, IL-33 treatment for GVHD seems to be dependent on both timing and the state of inflammation present.

Other Gut Diseases

ST2/IL-33 signaling has been implicated in protection from various infections, which could impact the gut. Studies have shown that treatment of mice with rIL-33 led to epithelial cell hyperplasia in the gut along with infiltration of eosinophils and mononuclear cells in the lamina propria (42, 161). These effects are thought to be mediated by IL-13, which becomes overexpressed after IL-33 treatment (5). Treatment of mice with IL-33 after *Trichuris muris* infection increased parasite clearance through increased Th2 cytokine response (161). Other infections that can impact the gut, including *Toxoplasma gondii* (162), *Leptospira* (163),

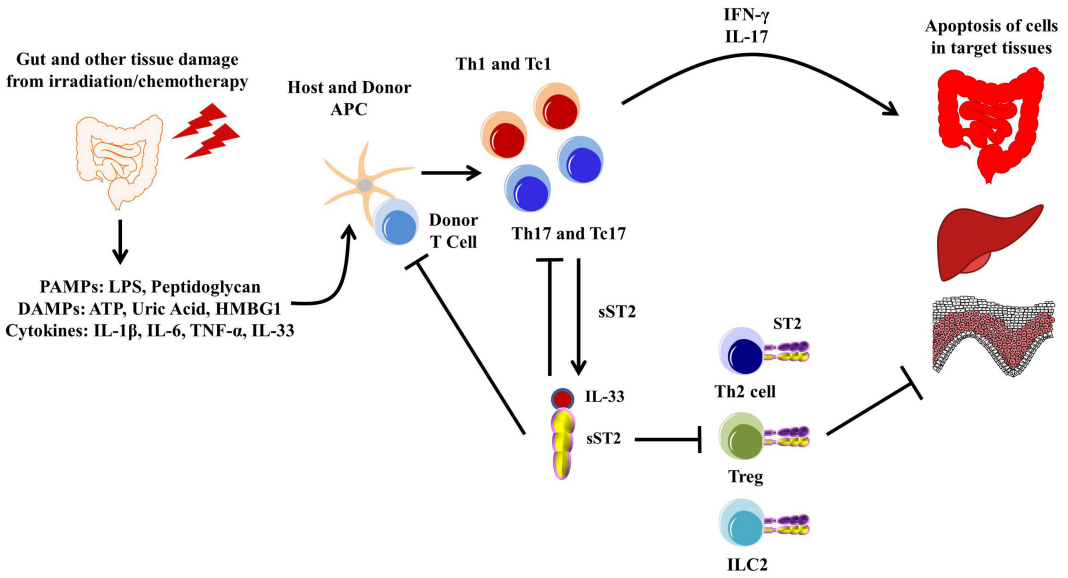

FIGURE 4 | Pathogenesis of graft-versus-host disease (GVHD). The gut and other issues are damaged during irradiation or chemotherapy, leading to the release of various DAMPs, PAMPs, and cytokines, including IL-33. These DAMPs, PAMPs, and cytokines activate both host and donor antigen-presenting cells (APCs), which then activate the donor T cells. The APCs are also secreting various cytokines that promotes T cell differentiation toward type 1 and type 17 responses. These activated type 1 and type 17 T cells are able to secrete various pro-inflammatory cytokines, leading to apoptosis of healthy tissue, mainly in the gut, liver, and skin, which can be exacerbated by free IL-33. Furthermore, sST2 is produced by both type 1 and type 17 T cells, and while this may sequester free IL-33 from the type 1 and type 17 T cells, sST2 can also prevent the potential beneficial effects from ST2/IL-33 signaling in Th2 cells, Tregs, and lymphoid cell type 2 (ILC2s).

and *Pseudomonas aeruginosa* (164), have shown that the loss of ST2 or high sST2 levels led to higher morbidity and mortality with increased Th1 cytokine profiles. Recent studies have shown that gut epithelial barrier dysfunction and immune activation independently predict mortality during treated HIV infection (165). A later study showed that patients during the early stage of HIV infection, defined as being within 180 days of the date of infection, had higher levels of sST2 in their plasma and was highly correlated with CD8 T cell count and levels of gut mucosal damage, but not with viral load or CD4 T cell count (72).

sST2 increase has also been implicated during small bowel transplant rejection (25). Patients who had rejection of small bowel transplants had higher serum levels of sST2 during rejection compared to that during rejection-free time points and that rejection increased allograft ST2 expression. An increase in sST2 in the allograft was predicted by Pathway and Network Analysis caused by TNF-α and IL-1β signaling (25). However, these data do not implicate sST2 as a mediator of disease but rather a biomarker of occurring transplant rejection.

Lung Diseases

Asthma and Allergy

Asthma is characterized by varying levels of airway hyperresponsiveness, mucus secretion, bronchoconstriction, and chronic inflammation, affecting 300 million people worldwide (166). ST2/IL-33 signaling in mast cells, ILC2s, eosinophils, basophils, Th2, and Th9 cells drive allergic asthma (141–143, 147, 167–170). Activating ST2 signaling in bone marrow-derived mast cells and basophils *in vitro* shows strong type 2 cytokine production, including IL-4 and IL-13 (171). Treatment of mice with IL-33 alone induced airway hyperresponsiveness and goblet cell hyperplasia through IL-4, IL-5, and IL-13 induction in the lungs (172). RAG2$^{-/-}$ mice treated with IL-33 also induce this phenotype, implicating the importance of the innate immune system in generating airway inflammation (171). IL-33 has also been shown to be a chemoattractant for Th2 cells, as injection of IL-33 into the footpad of ST2$^{-/-}$ mice, which were adoptively transferred with WT polarized Th2 cells led to local accumulation of the transferred Th2 cells (90). After ovalbumin (OVA)-induced acute allergic lung inflammation, levels of IL-33, ST2, and sST2 are significantly increased in the lung (172). While activation of the innate immune system in part through ST2/IL-33 signaling establishes airway inflammation, ST2^{+} T cells maintain this inflammation. Indeed, injecting mice with a ST2 blocking antibody during the resolution phase after OVA-induced allergic inflammation, when Th2 cells but not eosinophils are present in the lung, reduced airway hyperresponsiveness and mucus production (173). Loss of both IL-4 and IL-5 during OVA-induced airway inflammation did not abolish airway hyperreactivity, which was abolished only after anti-CD4 treatment (174), suggesting that ST2/IL-33 signaling in CD4 T cells may be critical for efficient antigen-induced airway inflammation (175). Blockade of either IL-33 or addition of sST2 before OVA-induced allergic airway inflammation showed reduced total cell counts and eosinophil counts in the bronchoalveolar lavage fluid and decreased IL-4, IL-5, and IL-13 (40, 52, 176).

Non-Allergic Lung Disease

During idiopathic pulmonary fibrosis, patients with stable disease versus healthy controls had similar levels of serum sST2; however, during exacerbations of fibrosis, there was over a sixfold increase in serum sST2 in the exacerbation group compared to both healthy controls and stable disease groups, which correlated with measurements of inflammation (67). Patients with acute respiratory distress syndrome had significantly higher sST2 levels at day 0, which were associated with worse prognosis and mortality (177). Serum levels of sST2 were also increased in patients with chronic obstructive pulmonary disease compared to control patients (178). Patients with moderate or severe H1N1 influenza infection also had significantly increased serum sST2 compared to patients with mild H1N1 infection or rhinovirus infection (179). Idiopathic pneumonia syndrome (IPS) occurs in 5–10% of patients who undergo allo-HCT. In a cohort of 42 patients with IPS without infection, sST2 was significantly higher in the serum of patients with IPS compared to healthy controls and patients with human rhinovirus and parainfluenza occurring at approximately the same time after transplantation (180).

Skin Diseases

Atopic Dermatitis

Atopic dermatitis is a Th2-driven disease and patients with AD show higher IgE levels and eosinophilia in the skin and blood (181). ST2/IL-33 signaling has been recently implicated in AD, as transgenic mice expressing IL-33 under the keratin 14 promoter had spontaneous AD-like inflammation (119). This led to increased IL-5 producing ILC2s. Mice receiving topically applied OVA, house dust mites, or staphylococcal enterotoxin B led to upregulation of both sST2 and IL-33 expressions (182). Patients with AD have higher IL-33 expression levels in their skin lesions compared to healthy controls (182). However, in a small cohort of 71 adults and 61 children with AD, serum sST2 levels were not significantly elevated compared to adult controls, in contrast to sST2 expression in patient skin lesions (182, 183). Currently, phase I–II clinical trials are being conducted using novel anti-IL-33 antibodies.

Psoriasis and Vitiligo

Unlike AD, psoriasis is driven by Th1 and Th17 cytokines (184). However, psoriatic skin still shows increased IL-33 expression compared to healthy skin in patients (154). ST2$^{-/-}$ mice had reduced cutaneous inflammatory responses compared to WT mice in a phorbol ester-induced murine model of psoriasis (154).

Patients with vitiligo are characterized by the disappearance of their melanocytes. In lesions of patients with vitiligo, both ST2 and IL-33 levels were increased, and serum levels of IL-33 were increased (185). As ST2 signaling in psoriasis and vitiligo is relatively new, not much else has been published as of yet.

Scleroderma and Chronic GVHD

Scleroderma is characterized by the fibrosis and hardening of the skin and connective tissues, measured by the modified Rodnan skin score (MRSS) test (186). In a two cohort study, serum sST2 levels were increased in patients with scleroderma compared to healthy controls, which, when combined with Spondin-1, best

described longitudinal change in MRSS, using mixed linear models (187). This was validated using three other independent cohorts (187).

Chronic GVHD can affect multiple organs, with skin involvement being one of the most common. In a large study of chronic GVHD, plasma samples were collected from patients at day +100 post-allo-HCT. A four-biomarker panel, which included sST2, correlated strongly with chronic GVHD diagnosis, severity, and non-relapse mortality (21).

Cardiac Diseases

sST2 was found to be upregulated after mechanically stimulating cardiomyocytes and stimulating with IL-1β (39). Inducing myocardial infarction *via* coronary artery ligation increased sST2 in the serum of mice compared to unoperated controls (39). This observation was also seen in patients, as those who suffered myocardial infarction had elevated serum sST2 levels 1 day postevent (39). In a cohort of over 800 patients with acute ST-elevation myocardial infarction, sST2 levels 1 day postevent correlated with 30-day mortality independent of age, blood pressure, heart rate, infarct territory, and time from symptom onset to treatment (11). sST2 levels of patients with non-ischemic congestive heart failure at time of entry to the study also correlated with both brain natriuretic peptide levels (BNP), which is routinely used in the clinic and serum noradrenaline levels (12). This study also found that changes in serum sST2 were an independent predictor of mortality. sST2 levels are correlated with impaired epicardial coronary flow and risk of death or congestive heart failure within 30 days of presentation, independent of BNP (188). These data show the value of sST2 as a biomarker in cardiac diseases.

Functional analysis of ST2/IL-33 signaling and sST2 production has shown that treatment of cultured rat neonatal cardiomyocytes with rIL-33 blocked angiotensin II or phenylephrine induced hypertrophy, while addition of sST2 or blocking of ST2 with an antibody reversed this effect (64). When using $ST2^{-/-}$ or WT mice to look at *in vivo* response to pressure overload by transverse aortic constriction, $ST2^{-/-}$ mice had more left ventricular (LV) hypertrophy, more chamber dilation, reduced fractional shortening, more fibrosis, and impaired survival compared with WT mice (64). Treatment of WT mice with rIL-33 reduced fibrosis and hypertrophy and increased survival in WT mice (64). This reduction in damage when treating with IL-33 may be due to inhibited apoptosis in cardiomyocytes (189).

In an atherosclerosis model in which mice deficient for the ApoE protein fed a high-fat diet, treatment with rIL-33 reduced aortic atherosclerotic plaque development and increased levels of type 2 cytokines in the serum (190), which have an atheroprotective effect. Mice treated with sST2 developed significantly larger atherosclerotic plaques (190). These data indicate that ST2/IL-33 signaling may have a protective effect, while sST2 plays a deleterious role in cardiac diseases.

Obesity and Metabolic Complications

Accumulation of visceral adipose tissue (VAT) due to obesity leads to inflammation, insulin resistance, and development of type 2 diabetes (191), leading to the reduction and function of Tregs in the VAT (192), which have been shown to be enriched for ST2 expression (193, 194). IL-33 is critical for the development and maintenance of these VAT Tregs (194). *In vitro* culturing of murine adipocytes with IL-33 induced IL-5 and IL-13 production, decreased expression of genes associated with adipogenesis and lipid metabolism, and reduced lipid storage (195). $ST2^{-/-}$ mice fed a high-fat diet had increased body weight and fat mass and impaired glucose regulation and insulin secretion compared to high-fat diet WT controls (195). IL-33 treatment to genetically obese diabetic mice led to reduced adiposity, lower fasting glucose levels, improved glucose and insulin tolerance, accumulation of Th2 cells and M2 macrophages in their adipose tissue, and increased the proportion of $ST2^{+}$ Tregs in the VAT (193, 195). However, ST2/IL-33 signaling may only help obesity-related insulin resistance. An age-associated insulin resistance model showed that fat-resident Treg depletion protected against insulin resistance, and these findings were confirmed using an anti-ST2 antibody (196).

ST2 and IL-33 are produced by white adipose tissue and in preadipocyte and adipocyte cell cultures in humans (79), while sST2 expression has been shown to be increased in omental and subcutaneous human adipose tissues (197). In a large, population-based study, sST2 levels in the plasma of patients strongly correlated with markers of diabetes, after adjusting for age and gender (28). In another study separating 525 patients into normal, prediabetic, and diabetic groups, plasma sST2 levels were only significantly increased in the diabetic group compared to prediabetic and normal groups (29). In a multicenter, cross-sectional study of 180 patients with metabolic syndrome with normal LV ejection fraction, LV mass index was independently associated with serum sST2 concentrations. Increased sST2 associated with increased likelihood of LV hypertrophy and increased systolic blood pressure (198). New-onset posttransplantation diabetes mellitus (PTDM) is a common occurrence after allo-HCT. Serum sST2 levels from three cohorts collected at engraftment and day 30 showed elevated sST2 levels at both time points and that high sST2 levels predicted PTDM and non-relapse mortality, independent of conditioning and high-grade GVHD (27). These data suggest that high sST2 levels correlate with obesity and type 2 diabetes and metabolic complications even when sST2 is already elevated by alloreactivity.

POTENTIAL THERAPEUTIC BENEFIT OF TARGETING ST2/IL-33 SIGNALING

The clinical usefulness of targeting either sST2 excess of secretion or ST2/IL-33 excess signaling or use of sST2 as a biomarker for diseases has been a hot topic in the last few years, as shown by the increase in translational studies devoted to ST2/IL-33 and sST2. Manipulation of ST2/IL-33 signaling or blocking sST2 secretion or sequestration of IL-33 is highly disease dependent. Several new antibodies that inhibit IL-33 binding to ST2 are currently being tested in phase I–II clinical trials for patients with asthma and chronic obstructive pulmonary disease. Using either an antibody or small molecule inhibitor is an attractive option for therapeutics targeting sST2 in CD, GVHD, or heart disease, while ideally maintaining ST2. However, given the involvement of ST2/IL-33 in a multitude of processes, caution must be afforded.

sST2 usefulness as a clinical biomarker has been studied extensively in both cardiac and allo-HCT patients, showing both prognostic and diagnostic value (11, 12, 17–24, 27, 39, 187, 188). sST2 levels are also increased in patients suffering from intestinal (13–16) and metabolic diseases (27, 28, 197, 198); however, the data from these studies so far are correlative and have not passed the qualification for biomarkers that can be used in clinic (199).

CONCLUSION

ST2/IL-33 signaling in immune cells has recently become a hot target of study. This signaling helps to activate T cells, ILC2s, DCs, B cells, mast cells, basophils, eosinophils, and other immune cells. Most of the work has shown that ST2/IL-33 signaling enhances the type 2 response, although recent studies have shown how ST2/IL-33 signaling enhances the immunomodulatory effects of Tregs. T cells have also been recently shown to produce sST2, which was once thought to be produced only by non-hematopoietic cells. ST2/IL-33 signaling in Tregs, ILC2s, and IL-10-producing B cells protects against inflammation, while sST2 can act either as a biomarker or can play a role in a variety of diseases by sequestering IL-33 and preventing ST2/IL-33 signaling. However, ST2/IL-33 signaling can also lead to progression of various lung and skin diseases such as asthma and AD. Given the complexity between ST2/IL-33 signaling and timing during the immune response and the importance of ST2/IL-33 in various organ systems, several questions and challenges remain. When does ST2/IL-33 signaling affect Treg response more so than the inflammatory response in various diseases? Which mediators can enhance ST2 expression on immunomodulatory cells? Which mediators can reduce or promote sST2 production during disease? A better understanding of the impact of ST2/IL-33 and sST2 during disease and how ST2 and sST2 targeting could affect different organ systems will be critical for the development of therapeutics.

AUTHOR CONTRIBUTIONS

Both BG and SP devised, wrote, and revised the manuscript.

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REVIEW
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The Role of IL-33-Dependent Inflammation in the Tumor Microenvironment

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There is compelling evidence that inflammation contributes to tumorigenesis. Inflammatory mediators within the tumor microenvironment can either promote an antitumor immune response or support tumor pathogenesis. Therefore, it is critical to determine the relative contribution of tumor-associated inflammatory pathways to cancer development. Interleukin-33 (IL-33) is a member of the IL-1 family of cytokines that is released upon tissue stress or damage to operate as an alarmin. IL-33 has been primarily implicated in the induction of type-2 immune responses. However, recent findings have shown a role of IL-33 in several cancers where it may exert multiple functions. In this review, we will present the current knowledge on the role of IL-33 in the microenvironment of different tumors. We will highlight which cells produce and which cells are activated by IL-33 in cancer. Furthermore, we will explain how IL-33 modulates the tumor-associated inflammatory microenvironment to restrain or promote tumorigenesis. Finally, we will discuss the issues to be addressed first before potentially targeting the IL-33 pathway for cancer therapy.

Keywords: cancer, inflammation, tumor microenvironment, interleukin-33, therapy

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INTRODUCTION

Cancer is a heterogeneous disease and represents one of the leading causes of death worldwide. The process generally underlying cancer development was described as the "hallmarks" of cancer (1, 2). These hallmarks include self-sufficient proliferation, insensitivity to antiapoptotic signals, evasion of apoptosis, unlimited replication, maintenance of vascularization, and ability for invasion and metastasis. The so-called tumor microenvironment is also important for tumorigenesis, which supports the hallmarks of cancer. The concept of the tumor microenvironment implies that cancer cells alone are not able to manifest the disease but rather involve resident non-malignant cells or recruit them to participate to tumor development. The interactions between the cancer cells and

Abbreviations: AOM, azoxymethane; BM, bone marrow; DC(s), dendritic cell(s); CAC, colitis-associated (colorectal) cancer; CAF(s), cancer-associated fibroblast(s); CCA, cholangiocarcinoma; cDC(s), conventional dendritic cell(s); CRC, colorectal cancer; CTL(s), cytotoxic T lymphocytes; DSS, dextran sodium sulfate; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cancer; HSPC(s), hematopoietic stem/progenitor cell(s); IBD, inflammatory bowel disease; IL, interleukin; ILC(s), innate lymphoid cell(s); MDSC(s), myeloid-derived suppressor cell(s); MMP, metalloprotease; NK, natural killer cells; NSCLC, non-small-cell lung cancer; pDC(s), plasmacytoid dendritic cell(s); PDGF, platelet-derived growth factor; sST2, soluble form of ST2; TNF, tumor necrosis factor; $T_{reg(s)}$, regulatory T cell(s); VEGF, vascular endothelial growth factor; WT, wild type.

their supporting stroma result in the formation of tumors and local invasions, metastasis, or vascular niches promoting malignancies (3). The tumor microenvironment consists of immune cells, endothelial cells, pericytes, fibroblasts, and smooth muscle cells (4, 5).

In addition to the occurrence of somatic "driver" mutations in transformed cells, long-term exposure to various environmental stresses, and certain diets may contribute to tumor development (6). Meanwhile, it is also widely accepted that inflammation has an important impact on tumorigenesis (4). The developing tumor itself can promote antitumor immune responses, which is mainly executed by cytotoxic T lymphocytes (CTLs), natural killer cells (NK), but also T helper 1 cells. However, local mechanisms of immune suppression specific to the tumor may blunt these tumor-infiltrating immune effectors (7–9). In addition, inflammatory stimuli may also exert a pro-tumorigenic action. Indeed, about 15% of malignancies are associated with microbes and a subsequent state of chronic inflammation (10, 11). *Helicobacter pylori*, human papillomavirus, hepatitis virus B and C, as well as cytomegalovirus and Epstein–Barr virus have been related to gastric cancer, cervical cancer, hepatocellular carcinoma (HCC), and hematologic malignancies, respectively. Patients suffering from inflammatory bowel diseases (IBDs) have an increased risk of developing colorectal carcinomas (12, 13). Therefore, immune cells and inflammatory mediators are important components of the local environment of tumors (14).

Cytokines are central mediators of the interaction between cells in the inflammatory tumor microenvironment (15). Interleukin-33 (IL-33) is a member of the IL-1 family of cytokines. In contrast to other IL-1 family members like IL-1β and IL-18, IL-33 is inactivated upon caspase cleavage (16, 17). While full-length IL-33 is already biologically active, its activity is enhanced ~10-fold upon cleavage by neutrophil serine proteases cathepsin G and elastase (18). Full-length IL-33 is mainly expressed by epithelial and endothelial cells, where it is stored and bound to the chromatin in the nucleus (19, 20). IL-33 may exert a dual function, as damage-associated molecular pattern (DAMP) and cytokine (21), or as nuclear factor modulating gene expression (22). Full-length IL-33 acts as "alarmin" if released extracellularly, subsequent to cell stress or damage. Soluble IL-33 binds to its receptor, a heterodimeric complex comprising IL-1RL1/ST2 (which is encoded by *IL1RL1*) and IL-1 receptor accessory protein (which is encoded by *IL1RAP*). IL-33/ST2 signaling is transduced *via* recruitment of MyD88 and IL-1 receptor-associated kinase-4 (IRAK-4), downstream adaptor proteins shared with other IL-1 family members and with most Toll-like receptors (TLRs) (**Figure 1**). In addition, a soluble form of ST2 (sST2) exists, which is produced by alternative promoter usage, 3′ processing or differential splicing and that may function as a decoy receptor for IL-33 (19, 23–25). IL-33 is primarily known as a driver of type-2 immune responses, triggering the release of Th2 cytokines and thereby promoting allergic reactions (26). Moreover, IL-33 supports tissue repair by coordinating the action of innate lymphoid cells (ILCs) and regulatory T cells (T_{regs}) (27–30). Yet, IL-33 may also be involved in pathologic wound repair and fibrosis (31–34). Finally, recent findings have revealed an important contribution

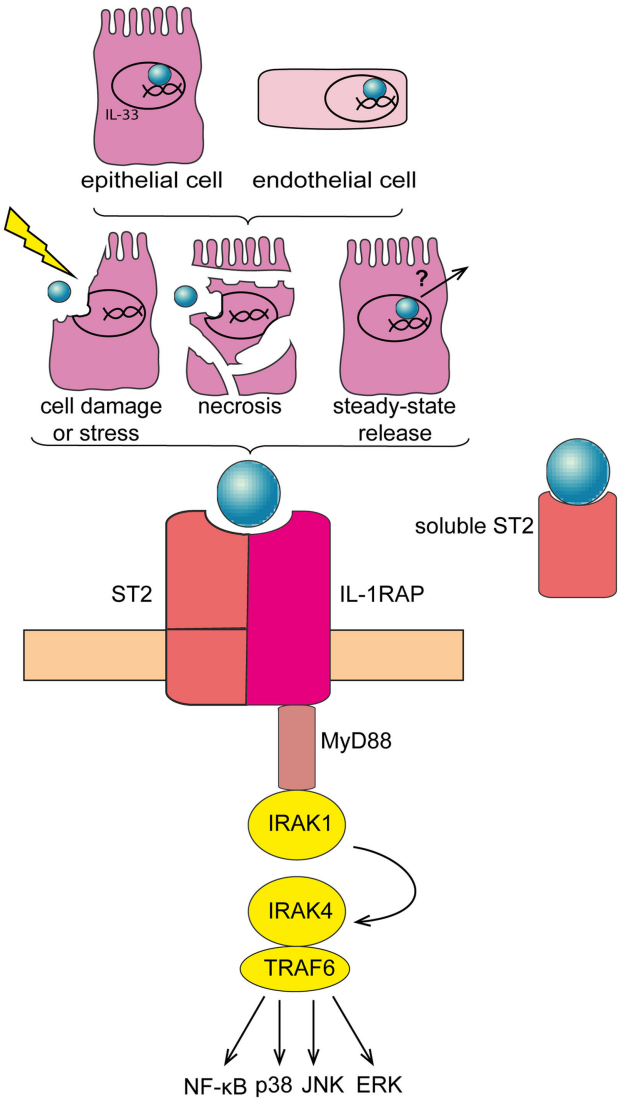

FIGURE 1 | Interleukin-33 (IL-33)/ST2 signaling pathway. IL-33 is mainly expressed in the nucleus of epithelial and endothelial cells. IL-33 is released subsequent to cell damage, stress, or necrosis. Alternatively, IL-33 may be released under steady-state conditions—at low levels—*via* a so far unknown mechanism. Soluble IL-33 binds to its receptor, a heterodimeric complex comprising IL-1RL1/ST2 (ST2) and IL-1 receptor accessory protein (IL1RAP), thereby mobilizing downstream signaling molecules including MyD88 and TRAF-6, and eventually activating NF-κB, p38, JNK, and ERK pathways. Soluble ST2 acts as a decoy receptor for IL-33 to negatively regulate IL-33/ST2 signaling.

of IL-33 to several cancers, where it may exert pro and—less frequently—anti-tumorigenic functions (35).

Here, we review the current knowledge about the role of IL-33 in the microenvironment of different types of tumors. Each cancer type is presented according to the following scheme: (1) short introduction to the specific cancer type; (2) net effect of IL-33/ST2 signaling for tumorigenesis; (3) origin of IL-33- and ST2-expressing cells in the tumor stroma of a particular cancer type; (4) mechanisms of action of IL-33; and (5)

therapeutic opportunities. Furthermore, we discuss the major issues which we believe should be investigated in the future to improve our understanding of the role of IL-33 for tumor initiation/progression, toward the development of therapeutic strategies.

MAIN REVIEW TEXT

IL-33 in Head and Neck Squamous Cancer (HNSCC)

Head and neck squamous cancer represents one of the six leading cancer types worldwide and it arises in the oropharynx, larynx, oral cavity, and hypopharynx. Major risk factors for the development of HNSCC are alcohol consumption and tobacco smoke. However, infection with human papillomavirus (HPV) can also promote tumor development (36).

The tumor microenvironment appears to strongly contribute to HNSCC development. Cancer-associated fibroblasts (CAFs) are the most critical cells in the HNSCC microenvironment, which promote tumor cell proliferation, invasion, and metastatic potential. Therefore, CAFs determine HNSCC tumor aggressiveness. These CAFs release IL-33, which induces epithelial-to-mesenchymal transition of cancer cells, thereby supporting their ability for migration and invasion. Furthermore, IL-33 induces *IL33* gene expression in HNSCC cells *via* a positive feedback mechanism. Increased *IL33* expression in CAFs and in tumor cells is associated with low patient survival (37). In patients with squamous cell carcinoma of the tongue, high expression of IL-33 or ST2 is indicative of worse prognosis. Increased IL-33 levels in these tumors also correlate with microvessels in the stroma (38).

While these data suggest that IL-33 blockade may be beneficial for HNSCC patients, further investigations are required to define downstream key factors dependent on IL-33 for the development of HNSCC. In particular, *in vivo* experiments should be performed to validate these initial data.

IL-33 in Non-Small-Cell Lung Cancer (NSCLC)

Non-small-cell lung cancer is the most common type of lung cancers, representing more than 85% of all lung cancers. NSCLC has a predicted 5-year survival rate of 15.9%. Although the prognosis for NSCLC has only marginally changed the past years, understanding of the cellular and molecular mechanisms underlying the disease has greatly improved (39).

While many studies have shown a critical role of the IL-33/ST2 signaling in inflammatory lung disease, including respiratory allergy, asthma, and chronic obstructive lung disease (19, 40–42), few have investigated the contribution of this pathway to lung cancer. In NSCLC patients, expression of *IL33* and *IL1RL1* was found to be increased in tumor tissue compared to adjacent non-tumor tissue, and this expression was associated with disease clinical stage (43). However, another study reports downregulation of *IL33* and *IL1RL1* in human lung cancer tissue and cells, compared to normal tissue and cells. Furthermore, the authors found that *IL33* and *IL1RL1* transcript levels were inversely correlated with stages of human lung cancers and overall survival. Accordingly, ST2 protein was expressed in low-metastatic, but not in high-metastatic Lewis lung carcinoma (3LL) cells (44).

Mechanistically, *in vitro* stimulation with IL-33 or overexpression of *IL33* in primary NSCLC cells enhanced their growth and metastasis after transfer into immunodeficient mice. Inversely, shRNA-mediated knockdown of *IL33* in primary NSCLC cells limited their proliferation and invasion capacity *in vivo*. Corresponding results were obtained by overexpressing or downregulating *IL1RL1* in NSCLC cells, thereby showing that the IL-33-driven tumor progression in this model relies on ST2. In addition, engagement of IL-33/ST2 signaling in NSCLC cells was found to increase the membrane expression of glucose transporter 1 (GLUT1) and thereby enhance their glucose uptake and lactate production. Finally, knockdown of *SLC2A1/GLUT1* in NSCLC cells diminished their IL-33-mediated proliferation as well as their metastatic potential, *in vitro* (43).

Yet, IL-33 may also trigger death of ST2-expressing lung tumor cells under conditions mimicking the tumor environment *in vitro*, including upon nutrient depletion or under hypoxic/anoxic conditions. This mechanism may possibly select for more malignant, ST2-negative cancer cells (44).

One can only speculate on the reason for the controversial findings in these two studies. Patient cohorts and cohort size were different in the two investigations. Moreover, no information was provided on the therapeutic status of the analyzed patient samples. Indeed, radiotherapy, alone or in combination with chemotherapy, belongs to the standard treatment of NSCLC (45), which can lead to a rapid increase in the expression of IL-33 protein in tissues (46). Thus, careful evaluation of patient therapeutic history is key for the study of IL-33 signaling in human samples. Finally, while one study describes the use of lung cancer cell lines, primary NSCLC cells were used in the other report, which makes a direct comparison difficult.

Therefore, additional studies are necessary to clarify the contribution of IL-33/ST2 signaling to lung cancer, in particular to NSCLC.

IL-33 in Breast Cancer

Breast cancer is one of the major causes of cancer-related death among women worldwide (47). Distant metastasis to the lung, bones, liver, and brain are causal for the death of breast cancer patients (48). The immune system has an ambivalent role in breast cancer as it may either promote or prevent tumorigenesis. Tumor-infiltrating leukocytes in particular have a strong effect on breast cancer development. While NK cells and CTLs mediate an anti-tumorigenic response in the microenvironment of breast tumors, T_{regs} and myeloid-derived suppressor cells (MDSCs) act in an immunosuppressive fashion [reviewed in Ref. (49)].

Several studies point toward a pro-tumorigenic role of IL-33/ST2 signaling in breast cancer. IL-33 and ST2 expression are elevated in human breast cancer tissue compared to normal breast tissue (50, 51). In breast cancer patients, serum levels of IL-33 and of its decoy receptor sST2 were enhanced compared to healthy controls. This positively correlated with the expression of

vascular endothelial growth factor (VEGF), metalloprotease-11 (MMP-11), or platelet-derived growth factor-C, which are markers of poor prognosis in breast cancer (52).

Compared to wild-type (WT) controls, *Il1rl1*-deficiency led to delayed tumorigenesis in mice tested in the syngeneic 4T1 breast cancer model. Moreover, after orthotopic injection of 4T1 cells, *Il1rl1*-deficient mice showed reduced metastatic potential to the lung and liver. This was caused by decreased tumor cell proliferation in *Il1rl1*-deficient mice. Accordingly, administration of IL-33 in this model enhanced tumor cell proliferation in WT mice, through an indirect effect of IL-33 on tumor cells (53, 54).

In the 4T1 breast cancer model, IL-33 was expressed by CD45-positive leukocytes and tumor cells, while ST2 was mainly expressed on T_{regs} and type-2 ILCs (54). Furthermore, there were fewer MDSCs in tumor lesions of *Il1rl1*-deficient mice, while treatment with exogenous IL-33 promoted the accumulation of these suppressor cells in mammary tumors. MDSCs responded to IL-33 by increasing their production of immunosuppressive TGF-β (54). These MDSCs may be recruited from peripheral lymphoid organs to the tumor lesions, where they trigger the generation of $CD4^+$ $FoxP3^+$ T_{regs} (55). Indeed, the percentage of $CD4^+$ $FoxP3^+$ T_{regs}, especially the cells expressing ST2 and IL-10, was found to be enhanced within 4T1 mammary tumors.

In addition to these indirect pro-immunosuppressive mechanisms, IL-33 signaling appears to also blunt tumor surveillance in the murine 4T1 breast cancer model. Indeed, NK cells showed increased IFN-γ production in *Il1rl1*$^{-/-}$ mice injected with 4T1 breast cancer cells, which translated into improved antitumor immunity. Along the same line, administration of IL-33 reduced NK cell function *via* decreased expression of FasL or NKG2D and enhanced programed cell death protein 1 (PD-1) levels. IL-33 also altered the ratio of dendritic cell subsets in 4T1 breast tumor lesions (54).

In other models, IL-33 also showed a direct action on malignant breast cells. Stimulation with IL-33 enhanced proliferation as well as colony formation and size of a ST2-expressing breast cancer cell line. This occurred *via* a mechanism involving MAP3K8 phosphorylation upon engagement of IL-33/ST2 signaling (51).

Together, these studies indicate that the IL-33/ST2 pathway promotes breast tumorigenesis both directly, *via* activation of cancer cells, and indirectly, *via* modulation of antitumor immunity. Thus, the IL-33/ST2 pathway may represent an interesting target for breast cancer therapy.

IL-33 in Hepatocellular Carcinoma (HCC)

Hepatocellular carcinoma is one of the most prevalent cancers and it represents a prominent cause of mortality. Individuals with advanced fibrosis, cirrhosis, and hepatitis B are predisposed to HCC development. Patients with chronic hepatitis B and C infections are most commonly affected. The pathogenesis of HCC is a multistep and complex process, wherein angiogenesis plays an important role (56, 57).

Immuno-histochemical analysis revealed an increase in IL-33-positive tissue in HCC compared to normal liver tissue. In addition, IL-33 serum levels were higher in pre- and post-operative serum samples from HCC patients compared with normal healthy controls (58). However, a different research group found no difference in IL-33 serum levels in HCC compared to liver cirrhosis patients and healthy controls, although levels of sST2 were significantly elevated in their liver cirrhosis and HCC patient groups compared to healthy controls. In HCC patients, serum sST2 levels correlated with overall survival (59).

Yet another study reports reduced levels of IL-33 protein in HCC compared to normal liver, hepatitis, and cirrhosis tissues. Moreover, while IL-33 was observed both in the nucleus and, to a lesser extent, in the cytoplasm of hepatocytes in normal liver tissue, HCC showed only cytoplasmic IL-33 staining. IL-33 expression was negatively associated with HCC histological grade, but not with other parameters including lymph node metastasis. Together, this suggests that cytoplasmic/non-nuclear IL-33 might play a role in HCC development (60).

Although the mechanisms by which IL-33/ST2 signaling may contribute to HCC are still elusive, tumor-infiltrating, IL-33-producing effector-memory $CD8^+$ T cells have been observed in resected HCC tissue, which are associated with prolonged patient survival (61).

IL-33 in Cholangiocarcinoma (CCA)

Cholangiocarcinoma is a malignant neoplasm of the biliary-duct system that represents the second most common primary hepatic malignancy (62). Parasite infections, primary sclerosing cholangitis, biliary-duct cysts, hepatolithiasis, and toxins are known risk factors of this type of cancer (62). Moreover, CCA is also associated with chronic inflammation.

Interleukin-33 has a promoting role for CCA. In a murine model of CCA based on biliary epithelium transduction of constitutively active AKT and Yes-associated protein (YAP) together with bile duct ligation, administration of IL-33 increased biliary tumorigenesis (63). Mechanistically, ST2 protein was expressed in cancer cells and in CAFs, and IL-33 enhanced tumorigenesis through an increase of IL-6 expression in tumor tissue. Moreover, IL-6 was able to substitute for IL-33 in this model (63). IL-6 triggers survival and mitogenic signals in CCA cells, in an autocrine or paracrine fashion (64, 65). Alternatively, administration of IL-33 to mice was found to enhance cholangiocyte proliferation, *via* a mechanism involving an increase in the number of IL-13-producing type-2 ILCs. Activation of this IL-33/ILC2/IL-13 axis in animals with constitutive activation of AKT and YAP in bile ducts induced CCA with liver metastases (66).

Additional *in vivo* experiments for instance using blocking antibodies are necessary to evaluate the relevance of the IL-33/IL-6 or IL-33/IL-13 axis as therapeutic targets in CCA.

IL-33 in Gastric Cancer

Gastric cancer represents the fourth most common type of cancer and is the second leading cause of cancer-mediated death in the world (67). The 5-year overall survival of gastric cancer patients is only around 20% (68). Major risks for the development of gastric cancer are dietary factors, *H. pylori* infections, gastro-esophageal reflux disease, and obesity [reviewed in Ref. (68)]. Inflammation is an important contributor to gastric tumorigenesis, which is exemplified by the well-studied pro-tumorigenic role of *H. pylori* for this cancer. Indeed, bacterial virulence factors of *H. pylori* and

pathogen interaction with the host immune system result in a chronic inflammation that promotes tumorigenesis (69, 70).

Interleukin-33 appears to exert a pro-tumorigenic function in gastric cancer. Human gastric cancer cell lines stimulated with IL-33 showed a dose-dependent increase in cancer cell invasion and migration. These effects were abrogated by knocking-down *IL1RL1*. Engagement of IL-33/ST2 signaling in these gastric cancer cell lines activated ERK1/2 (71), a pathway known to be important for tumor invasion and metastasis (72). Moreover, IL-33 also triggered gastric cancer cells to secrete IL-6 and MMP-3 (71), factors with established pro-tumorigenic properties (73, 74).

Still, additional studies on human gastric cancer tissue and *in vivo* experiments in mice are required to further assess the function of IL-33 for gastric cancer development and progression.

IL-33 in Colorectal Cancer (CRC)

Colorectal cancer represents one of the most common human cancer types, that is fatal in a considerable number of cases (75). CRC development is a multistep process that follows the adenoma-carcinoma sequence as a consequence of the accumulation of alterations in key oncogenes and tumor suppressor genes involved in the Wnt/β-catenin-, KRAS-, MYC-, MAPK-, TGF-β/bone morphogenetic protein-signaling pathways—among others—and TP53 (76–78). Chronic inflammation strongly promotes intestinal tumorigenesis (4), which is also illustrated by the fact that patients suffering from IBD have a higher risk to develop CRC (79, 80). In addition, several cytokines and inflammatory mediators have been shown to be associated with or to promote intestinal tumorigenesis (81).

While most studies have shown a pro-tumorigenic role of IL-33/ST2 signaling in CRC, recent data suggesting a protective role have also been published. We and others found increased levels of *IL33* and *IL1RL1* expression, both on the transcript and protein level, in adenoma and low grade adenocarcinoma of CRC patients, compared to adjacent normal tissue and high grade tumors (82, 83). Similarly, *IL33* transcript levels were found to be higher in stage I–III CRC compared to adjacent normal colonic tissue. However, *IL33* expression was lower in stage IV CRC than in stage I–III intestinal tumors (84). In contrast, another study reports progressively increasing levels of *IL33* and *IL1RL1* transcript from low to high grade and stage of human CRC (85).

In animal models, *Il33* and *Il1rl1* levels are elevated in adenomatous polyps of $Apc^{Min/+}$ mice. In this model of CRC, *Il33* deficiency is associated with decreased growth of intestinal polyps (86). Similarly, in the azoxymethane (AOM)/dextran sodium sulfate (DSS) model of colitis-associated CRC (CAC), we found that IL-33/ST2 signaling promotes tumorigenesis. Indeed, $Il1rl1^{-/-}$ mice treated with AOM/DSS showed delayed CAC development, fewer/smaller tumors and lesions of lower grade, compared to WT controls. Experiments using bone marrow (BM) chimeric mice indicated that IL-33/ST2 signaling was engaged on both the radio-resistant and the radio-sensitive compartment (83). In a different model of CRC, syngeneic MC-38 colon carcinoma cells injected into the cecum of recipient mice showed enhanced growth and liver metastasis when overexpressing IL-33 (84).

Stimulation of human primary CRC cells with exogenous IL-33 resulted in a dose-dependent increase in their invasive potential. In addition, IL-33 overexpression in these CRC cells resulted in an enhanced invasion, a mechanism dependent on signaling through ST2. These *in vitro* findings were confirmed by challenging nude mice with metastatic SW620 cells. Overexpressing *IL33* in SW620 cells injected subcutaneously enhanced their growth, metastasis, and reduced the survival of recipient nude mice, while downregulating *IL33* had an opposite effect (85).

In human and murine CRC tissue, IL-33 is predominantly expressed by epithelial cells but also in endothelial cells and myofibroblasts. ST2 is mainly expressed on transformed epithelial cells, endothelial cells, myofibroblasts, and infiltrating immune cells (82, 83, 86, 87).

Mechanistically, IL-33 enhances the recruitment of $CD11b^{+}F4/80^{+}$ macrophages and $CD11b^{+}Gr\text{-}1^{+}$ MDSCs to CRC tumors. IL-33 also stimulates the secretion of S100A8/9 (a DAMP) and VEGF from these cell populations to support tumor angiogenesis and metastasis (84).

Several studies have reported an accumulation of mast cells in human and mouse intestinal adenomas, which promote polyposis in *Apc* mutant mouse models *via* stimulation of angiogenesis, modulation of T_{reg} function, and mobilization of MDSCs within the tumor microenvironment (88–90). Along these lines, *Il33*-deficient $Apc^{Min/+}$ mice showed reduced accumulation of ST2-expressing mast cells in their polyps, compared to $Apc^{Min/+}$ mice expressing *Il33*. In the same model, abrogation of IL-33/ST2 signaling was associated with reduced expression of proteases and cytokines known to promote polyposis (86).

Stimulation of primary human CRC cells with IL-33 resulted in an increased secretion of IL-6, CXCR4, MMP-2, and MMP-9 (85), which have all been involved in CRC metastasis (91–95). $Apc^{Min/+}$ mice deficient in *Il33* show reduced transcript levels of *Il4* and *Il6*, which drive CRC development (81, 86). In addition, engagement of IL-33/ST2 signaling on radio-resistant cells decreases the integrity of the intestinal barrier and enables translocation of microbial products to the circulation. This was associated with enhanced systemic levels of pro-tumorigenic IL-6. Furthermore, hematopoietic cells isolated from the vicinity of CAC lesions and stimulated with exogenous IL-33 showed upregulated *Il6* transcript levels. Therefore, the IL-33/ST2 axis contributes to colorectal tumorigenesis partly *via* increased production of IL-6 (83).

Epidermal growth factor (EGF) appears to regulate *Il33* expression in intestinal epithelial cells. In tumors of AOM/DSS-challenged mice treated with gefitinib, an inhibitor of epidermal growth factor receptor, *Il33* transcript levels were decreased, compared to untreated controls. This correlated with fewer and smaller tumors in this model. EGF stimulation of an intestinal epithelial cell line increased its expression of ST2 and intracellular IL-33 (87). This suggests a link between EGF and the IL-33/ST2 signaling axis and may provide a target for the treatment of CRC, although this requires further investigation.

In contrast to the above-presented findings, two recent studies reported an anti-tumorigenic role of IL-33/ST2 signaling for intestinal tumorigenesis. In a first report, expression of transmembrane ST2 was found to be lower in human CRC

compared to adjacent, non-tumor tissue, and it progressively decreased during tumor progression from Stage I to Stage IV CRC. While shRNA-mediated knockdown of *Il1rl1* did not alter the proliferation or migration of CT26 CRC cells *in vitro*, these cells developed larger tumors when injected into the flanks of immunocompetent recipient mice. This correlated with a reduction of macrophage recruitment to the tumor tissue, likely due to diminished CCL2 production by tumor cells with abrogated IL-33/ST2 signaling (96). In a second report, IL-33 was shown to promote IgA production, thereby preventing microbial dysbiosis and IL-1α-dependent inflammation in the intestine. Upon AOM/DSS treatment of *Il33*-deficient mice, this translated in augmented secretion of inflammatory cytokines as well as tumors of increased number, size, and grade, compared to WT mice (97). However, it is currently not known whether IL-33 fulfills a similar function in the human intestine, where manipulation of the microbiota may represent a therapeutic strategy for the treatment of CRC, independently of IL-33 (81).

Taken together, the ambivalent role of IL-33/ST2 signaling for CRC tumorigenesis in mouse models makes it difficult to assess this pathway as a possible target for CRC therapy in humans. It seems important to compare the different murine models presented above side-by-side. Moreover, additional experiments should be performed that involve blockade of the IL-33/ST2 pathway at different stages of CRC progression. Also, chemotherapy may differently affect ST2 and IL-33 levels in CRC patients (96), which should be considered in the design of future human studies on these molecules.

IL-33 in Myeloproliferative Neoplasms (MPNs)

Myeloproliferative neoplasms constitute a group of chronic hematologic malignancies which are characterized by an abnormal proliferation of myeloid cells (98). They are classified as BCR-ABL1-positive MPN, for chronic myeloid leukemia (CML), or BCR-ABL1-negative MPNs, which mainly comprise polycythemia vera, essential thrombocythemia, and primary myelofibrosis (98, 99). Most cases of BCR-ABL-negative MPNs present a mutation in a molecule involved in JAK/STAT signaling (100). As a consequence of these diverse genetic aberrations, cytokine signaling is dysregulated in both classes of MPNs (98).

Interleukin-33/ST2 signaling supports the development of both BCR-ABL1-positive and -negative MPNs. Increased levels of nuclear IL-33 protein are present in trephine biopsies of BCR-ABL1-negative MPN patients, and high amounts of circulating soluble ST2 were detected in the plasma from CML patients, compared to controls. Furthermore, ST2 is upregulated on the surface of $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) of BCR-ABL1-positive and -negative MPN patients, compared to healthy donors (101, 102). Addition of exogenous IL-33 increased the colony forming potential of $CD34^+$ HSPCs of MPN patients (102).

In mouse models, we found that IL-33 supports MPN-like disease and myeloproliferation in mice deficient in *Inppd5* or in irradiated recipients transfused with BM cells transgenic for human $JAK2^{V617F}$, one of the most common driver mutations in MPN patients (102). $CD34^+$ HSPCs from CML patients engrafted better in immunodeficient mice after pre-treatment with IL-33, and BCR-ABL-expressing BM cells showed reduced ability to induce CML-like disease when transplanted into *Il33*-deficient versus *Il33*-competent recipients (101).

Interleukin-33 is expressed in radio-resistant cells, including endothelial cells, in the BM. ST2 expression in the BM is mainly restricted to endothelial, mesenchymal, and early myeloid cells. Importantly, ST2 was not detected on murine HSPCs. The IL-33/ST2 pathway in the BM can be activated both in stromal/non-hematopoietic and in hematopoietic cells. Engagement of ST2 on these cells leads to secretion of cytokines known to promote the development and proliferation of myeloid cells, including IL-6, GM-CSF, G-CSF, and IL-3 (102). Since human $CD34^+$ HSPCs express ST2, they are able to directly respond to IL-33 stimulation *in vitro*, which induces cytokine production and subsequent cell proliferation (101, 102). These cytokines, in turn, activate the STAT5 pathway and can confer resistance to imatinib mesylate, a specific kinase inhibitor of the BCR-ABL1 fusion protein (101).

While these data suggest a potential therapeutic benefit in blocking IL-33/ST2 signaling in MPNs, additional experiments should first assess the role of this pathway for disease progression and drug resistance in MPN patients.

IL-33 As Immune Adjuvant for Vaccine Therapy

Except for a few particular examples presented above, IL-33 has mainly a pro-tumorigenic function for cancer development (96, 103). However, IL-33 may also function as an immune adjuvant for antitumor immune response. Indeed, IL-33 has been shown to promote potent cancer-specific effector and memory T cell immunity when used as an adjuvant for DNA vaccination in mice (104). This is in line with findings indicating that IL-33 increases the numbers of $CD8^+$ T cells and NK cell-producing IFN-γ in transgenic B16 tumors overexpressing IL-33, thereby mediating a microenvironment favoring tumor rejection. However, IL-33 also promoted the accumulation of immunosuppressive ST2-expressing T_{regs} in this model (103).

Interestingly, aluminum-based adjuvants (alum) induce cell necrosis that leads to extracellular release of IL-33 and the subsequent induction of several IL-33-dependent inflammatory cytokines. In addition, IL-33 injected together with the NP-CGG model antigen increased NP-specific IgM titers in the primary response and T cell-dependent NP-specific IgG1 titers after antigen boost. This indicates that IL-33 can induce robust antigen-specific antibody responses (105). IL-33 was also reported to reduce the accumulation of MDSCs in the spleen and tumor microenvironment, and to decrease the immunosuppressive activity of these cells, which limited tumor growth (106).

Together, these findings indicate the possible use of IL-33 as an immune adjuvant. Yet, there may be some challenges in applying IL-33 for immunomodulation or vaccination, as high levels of IL-33 may lead to lethal inflammatory disease in mice (107, 108). Therefore, it might be difficult to find the right dose of IL-33 to support the desired antitumor-specific immune response while

avoiding exacerbated inflammation. This may restrict the possibility of using IL-33 in a vaccine setting.

IL-33 As Tumor Biomarker or Therapeutic Target

As presented above, IL-33 contributes to the modulation of the tumor environment by promoting the recruitment of pro-tumorigenic immune cells or the secretion of tumorigenic cytokines. However, IL-33 can be detected not only in the tumor environment, but also in the serum of cancer patients. For instance, IL-33 levels are increased in the serum of lung and gastric cancer patients and correlate with disease stage, suggesting that IL-33 may be a negative prognostic marker for these types of cancer (109, 110). Moreover, expression levels of *IL33* and *ST2* correlate with tumor grade and inferior survival of glioblastoma patients (111). However, IL-33 expression indicates favorable prognosis in patients with malignant salivary gland tumors (112). In addition, loss of IL33 expression may also promote tumor escape in patients with metastatic prostate carcinomas or kidney renal clear cell carcinomas, *via* down-modulation of genes involved in antigen processing and of major histocompatibility complex (MHC-I/HLA)-genes (113). Therefore, IL-33 and ST2 differently contribute to tumorigenesis depending on the nature of the malignant tissue.

For breast cancer, IL-33 and sST2 may also serve as non-invasive diagnostic marker, as these two proteins are upregulated in the serum of patients [(52) and see also the paragraph on breast cancer above]. However, IL-33 is upregulated in different types of inflammatory diseases (114–116) and cancers (111, 112), and this lack of specificity may prevent its use as a biomarker in the daily clinic diagnostic. Furthermore, while high levels of IL-33 in absence of common markers of inflammation may indicate the presence of cancer, it would not indicate the identity of the cancerous tissue, and more specific markers would be additionally required for diagnostics. Another level of complexity comes from the observation that the IL-33/ST2 pathway appears to be differently regulated during the progression of distinct types of cancers, as mentioned just above.

While cytokine blockade is currently applied for the treatment of inflammatory disorders, including for instance the use of antitumor necrosis factor-α monoclonal antibodies to treat ulcerative colitis and Crohn's disease, it remains to be investigated whether a blockade of the IL-33/ST2 pathway may represent a valid approach for the therapy of established IL-33-dependent tumors. Experimental evidences are currently lacking that clearly indicate the suitability of such an approach. Alternatively, IL-33 blockade might be applied in combination with other (immuno) therapies. As IL-33 mainly acts as an amplifier of inflammation (117), targeting IL-33 may suppress the pro-tumorigenic inflammation in the tumor stroma, therefore improving the treatment with conventional therapies. For instance, combination of imatinib and IL-33 blockade in CML may allow elimination of cytokine-dependent malignant stem cells (118) or prevent the emergence of drug resistance (101). Along these lines, combined administration of IL-33 and PD-1 blockade was recently shown to improve the survival of mice suffering from acute myeloid leukemia (119).

CONCLUSION AND DISCUSSION

The tumor microenvironment represents a critical incubator for tumor development, local invasion, and metastasis (3). This environment can be modulated by different factors including pro- or anti-tumorigenic cytokines (15). The alarmin IL-33, an amplifier of innate immune response (117), has been shown to contribute to different types of inflammatory diseases and more recently to modulate tumorigenesis (**Figure 2**) (35, 42). Several investigations using patient-derived material, *in vitro* approaches, or *in vivo* models have uncovered a differential role of the IL-33/ST2 pathway in the tumor microenvironment, for tumor initiation, development, and resistance to therapy (**Table 1**). While IL-33 has generally a pro-tumorigenic function in various cancers, for some cancer types the findings generated so far are controversial. We discuss below possible reasons for these discrepancies and how they may be possibly addressed or resolved.

Different IL-33- and ST2-Expressing Cell Types in the Tumor Environment

Several of the expression studies presented above analyzed whole tumor tissues, without distinction of the different IL-33- and ST2-expressing cell types in the tumor microenvironment. However, not only transformed cells, but also infiltrating immune cells, endothelial cells, or myofibroblasts may express IL-33 or ST2 in the tumor stroma (54, 83, 86, 102, 120). Therefore, the presence or contribution of these non-cancerous cells should be separately evaluated for future correlative or functional studies.

The relevance of IL-33- and ST2-expressing non-cancerous cells to tumorigenesis may also explain the contradictory outcomes from distinct animal models. Indeed, xenograft studies are not suitable to address the contribution of stromal cell-specific IL-33/ST2 signaling to tumorigenesis. While there is a 55% homology (19) and a 66% identity at the amino-acid level between human and murine IL-33 and ST2, respectively, it is not known whether these molecules can cross-react between the two species. Moreover, immune cells can engage ST2 upon IL-33 binding, whose contribution cannot be assessed using immunocompromised animals as recipients of xenografts. Finally, for certain types of cancers, heterotopic models may not properly address the role of the physiologic tumor microenvironment. This holds true in particular for CRC, since IL-33 can control dysbiosis in the intestine, which in turn impacts on colon tumorigenesis (97)—a parameter likely omitted by studies involving subcutaneous application of CRC cells. Therefore, these limitations shall be taken into account, especially when evaluating the clinical relevance of the different findings.

Spatiotemporal Contribution of IL-33 and ST2 to Tumorigenesis

Interleukin-33 may differently contribute to tumorigenesis in dependence on where it is available. As presented above, local IL-33 in the tumor microenvironment can directly trigger cancer or stromal cells. However, IL-33, in particular when given exogenously, may drain to the lymph nodes, where it can promote antitumor responses—as shown in several immune adjuvant vaccine studies (see the above paragraph on IL-33 as immune

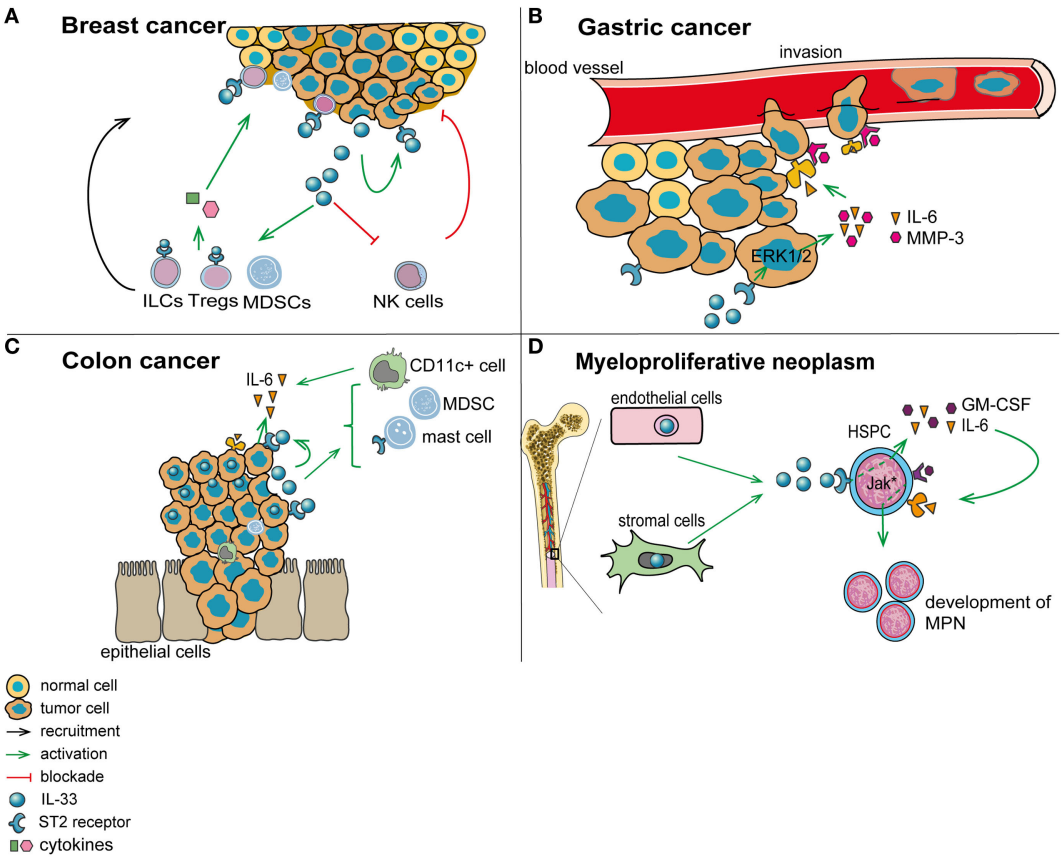

FIGURE 2 | Role of interleukin-33 (IL-33) in the tumor microenvironment. (A) In breast cancer (mouse 4T1 breast cancer model), IL-33 is released by tumor cells and acts in an autocrine/paracrine manner. In addition, IL-33 promotes the recruitment of immunosuppressive cells and inhibits the function of antitumor cytotoxic natural killer cells (NK). **(B)** In gastric cancer, IL-33 may promote vessel invasion of tumor cells by stimulating the secretion of IL-6 and MMP-3 through activation of the ERK pathway. **(C)** In colorectal cancer, IL-33 supports the recruitment to the tumor environment of pro-tumorigenic immune cells, including mast cells and myeloid-derived suppressor cells (MDSCs). In addition, IL-33 promotes the secretion of pro-tumorigenic IL-6 by leukocytes in the tumor vicinity, which are possibly $CD11c^+$ dendritic cells. **(D)** In patients with myeloproliferative neoplasms, IL-33 is released by bone marrow stromal and endothelial cells, and it engages ST2 on $CD34^+$ hematopoietic stem/progenitor cells, thereby promoting their secretion of IL-6 and GM-CSF. These cytokines, in turn, stimulate in an auto/paracrine manner the uncontrolled proliferation of the malignant clone (due to its defect in JAK/STAT signaling).

adjuvant). While this has still to be demonstrated, systemic IL-33 may also possibly keep ST2-expressing immunosuppressive cells at sites distant from (IL-33-expressing) tumors, thereby "distracting" them from their pro-tumorigenic role. Indeed, *in vivo* IL-33 administration can restrict the accumulation of MDSCs in the tumor microenvironment (106).

Finally, IL-33/ST2 signaling may contribute to different stages of tumorigenesis, including tumor growth or metastasis, and additional genetic tools are needed to systematically dissect the role of this pathway at specific time points of cancer development.

Regulation of IL-33 Release—Role of IL-33 As a Cytokine

As IL-33 may become upregulated in different types of cells upon malignant transformation, its mere presence in tumors should not always imply engagement of ST2-dependent signaling, which may also explain conflicting findings across studies. IL-33 first needs to be released extracellularly before binding to its receptor. However, the exact mechanisms for the regulation and release of IL-33 are still elusive, and they may vary between distinct experimental settings or models. Indeed, in addition to cell damage/necrosis-dependent release of IL-33, IL-33 may also become actively released. Stress conditions associated with increased extracellular adenosine triphosphate (ATP) (a DAMP) may trigger IL-33 secretion after activation of purinergic receptor P2Y2R signaling in live cells (40, 121). Moreover, alternative splicing-mediated deletion of exon 3 and 4 of *IL33* transcripts confers cytoplasmic localization of IL-33 proteins, which facilitates its extracellular secretion from airway epithelial cells of asthmatic patients. Importantly, this isoform of IL-33 was shown to retain its ability to signal through ST2 (122). Thus, it is conceivable that such IL-33 isoforms are also preferentially produced by tumor cells (or cells in the tumor microenvironment), a hypothesis that remains to be addressed. Detectable amounts of IL-33 can be measured in the serum of healthy patients (123) and naïve mice (102) suggesting that IL-33 is released and circulates even under

TABLE 1 | Interleukin-33 (IL-33) and ST2 levels and contribution in different cancers.

Type of cancer		Net effect of						Major references
		IL-33			ST2			
		Expression	Tumor progression	Metastasis	Expression	Tumor progression	Metastasis	
Head and neck squamous cancer	Human	+	+					(37, 38)
Non-small lung cancer	Human	+	+	+	+	+	+	(43)
		–	–		–	–		(44)
Breast cancer	Human	+			+			(50, 51)
	Mouse		+			+	+	(53, 54)
Hepatocellular carcinoma	Human	+	+		+			(58, 59)
Cholangiocarcinoma	Mouse		+	+	+			(63, 66)
Gastric cancer	Human		+	+		+	+	(71)
Colorectal cancer	Human	+	+	+	+	+		(82, 83, 85)
		–			–			(96)
	Mouse	+	+	+	+	+		(83, 84, 86)
			–			–		(96, 97)
Myeloproliferative neoplasms	Human	+	+		+			(101, 102)
	Mouse		+			+		(102)

+, promoting effect; –, suppressing effect.

steady-state conditions (**Figure 1**). Thus, such basal level of IL-33 may also help amplify the inflammatory milieu in chronic inflammatory disorders, which have been shown to provide favorable conditions for the development of tumors (4). Therefore, cancer studies aiming at evaluating the role of IL-33 as a cytokine should systematically assess the function of ST2 in a particular experimental model.

Role of IL-33 As a Transcriptional Regulator

In addition to its extracellular role as an alarmin triggering ST2, signaling, IL-33 also has a nuclear function. Indeed, IL-33 has been early reported to be expressed in the nucleus of epithelial barrier tissues and lymphoid organs (20, 124). Nuclear IL-33 binds to the p65 subunit of NF-κB, thereby reducing NF-κB-dependent gene expression (22). A study using fibroblast-like synoviocytes from patients with rheumatoid arthritis indicated that downregulation of IL-33 results in increased NF-κB activity and in the production of pro-inflammatory molecules. These results suggest that nuclear IL-33 transcriptionally represses NF-κB and down-modulates inflammatory responses (125). Importantly, NF-κB signaling is not only central for innate immunity and inflammatory processes; it also plays an important role for cancer initiation and progression (4, 126). Therefore, it may be particularly relevant to address the contribution of intracellular IL-33 to NF-κB signaling in tumorigenesis, which has so far not been investigated. As nuclear IL-33 generally becomes upregulated in response to inflammation (40, 127), this may provide a possible control mechanism *via* a negative feedback loop. However, the physiological relevance of this mechanism should be assessed experimentally, as nuclear IL-33 has only limited potential for binding active NF-κB and thus preventing it from binding to DNA (22).

Therefore, this nuclear function of IL-33 may explain why certain investigations using *St2*-deficient mice may not fully recapitulate the findings from *Il33*$^{-/-}$ animals. While the role of nuclear IL-33 needs to be further addressed, e.g., for possible interaction with cancer-relevant transcription factors additional to NF-κB, modulation of nuclear IL-33 expression likely represents a technical challenge for therapy.

Taken together, IL-33/ST2 signaling appears to mainly have a pro-tumorigenic role in cancer. However, several controversial reports provide caveats to a precipitated development of therapeutic strategies to block this pathway, and additional studies are required to unambiguously assess the contribution of IL-33 and ST2 to different cancer types.

AUTHOR CONTRIBUTIONS

PK: conception and writing of the manuscript; MHW: writing of the manuscript.

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