Journal of Immunology Research

Immunomodulation Induced By Host Pathogen Interaction

Lead Guest Editor: Hadi M. Yassine
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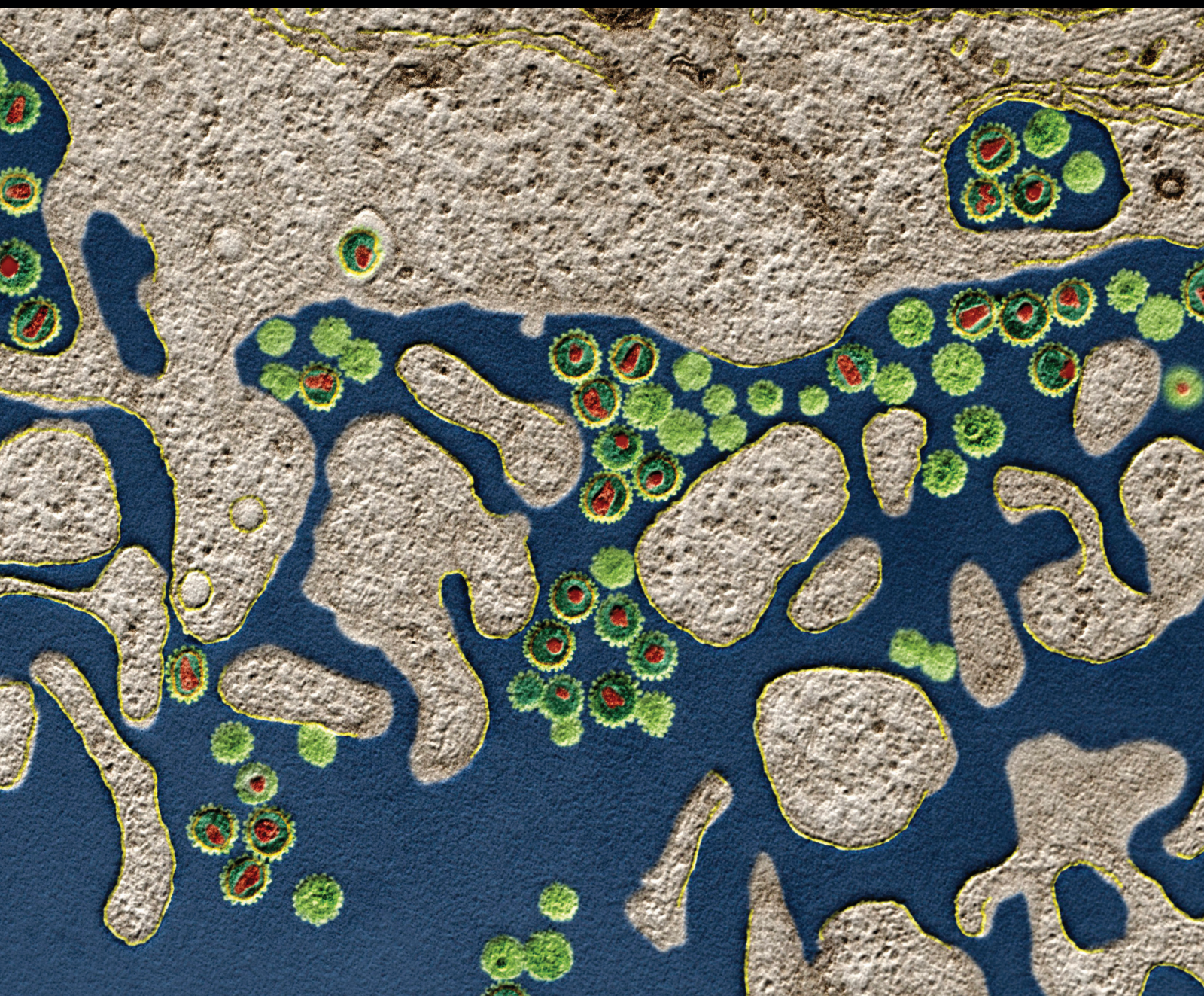

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Editorial Board

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Editorial

Immunomodulation Induced by Host Pathogen Interaction

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Controlling and preventing infections require deep understanding of the complex interplay that occurs between the host and pathogen following infection. In essence, immunomodulation is any process leading to an immune response that can be altered to a desired level. In mammals, the immune system has developed an extensive array of cells and immunomodulators to recognize, identify, and eliminate foreign invaders. On the other hand, pathogens have evolved multiple mechanisms to combat the host immune system as they establish infections. In this context and under certain circumstances, an infection may result in a subverted immune system, which may lead to an exacerbated illness. Recent advances in biotechnology have enhanced our knowledge of the complex interplay that occurs between the host and invading pathogens following infection, through understanding of the microbial virulence strategies as well as the host's approaches to combat the infection.

This special issue collects six high-quality papers, four research articles and two reviews, related to understanding immunomodulation in the context of viral and parasitic infections.

In the article titled "*Taenia crassiceps*-Excreted/Secreted Products Induce a Defined MicroRNA Profile that Modulates Inflammatory Properties of Macrophages" by D. Martínez-Saucedo et al., the authors described how *Taenia crassiceps*-excreted/secreted antigens (TcES) can modulate proinflammatory responses in macrophages by inducing regulatory posttranscriptional mechanisms and, hence, reduce detrimental outcomes in hosts running with inflammatory diseases. In summary, their study demonstrates a role for TcES in regulating the production of key inflammatory cytokines, "possibly by inducing microRNAs that target inflammatory transcripts and promoting the release of IL-10 in macrophages." This phenomenon shapes the transcriptomic profile of macrophages and consequently the outcome of the immune response. These findings increase the basic understanding of how the released molecules from helminths would regulate inflammation and, thus, may offer new approaches for the treatment of autoimmune and inflammatory diseases.

The remaining three research articles focused on viral infections of three different families, namely, the lymphocytic choriomeningitis virus (LCMV), human papillomavirus type 16, and MERS-coronavirus. The first of these studies was titled "Lymphocytic Choriomeningitis Virus Infection Demonstrates Higher Replicative Capacity and Decreased Antiviral Response in the First-Trimester Placenta" by E. A. L. Enninga and R. N. Theiler from the University of Texas Medical Branch. LCMV is a zoonotic pathogen, of which rodents are the natural reservoir. Although the majority of persons infected with LCMV show relatively minor illness, nonetheless, the virus can cross the placental barrier during pregnancy and cause congenital defects in fetuses. In this study, differences in immunomodulation between the first- and third-trimester placental explants infected with LCMV were evaluated.

Generated data suggested that the innate immune response to LCMV infection of the human placenta is more vigorous in the third trimester compared to the first trimester. The authors attributed the absence of viral replication in term placental explants to the robust innate antiviral response in this tissue. These findings are in agreement with clinical observations of decreased transplacental transmission and less severe fetal phenotypes of viral pathogens acquired in later gestation.

In the other study titled "Human Papillomavirus Type 16 Disables the Increased Natural Killer Cells in Early Lesions of the Cervix," J. Zhang et al. retrospectively analyzed the histologic pathology results of 245 women with HPV type 16 only (HPV16+) or type 18 only (HPV18+). The study was the first on emphasizing the unique immune profiles of the cervical microenvironment between two high-risk HPV types. In summary, the authors indicated that more severe lesions are found in the cervix of HPV16+ women compared with those in HPV18+ women. Briefly, their data demonstrated that the number of NK cells was increased but their cytotoxic function was abnormal in an HPV16-infected cervix. The involved mechanisms may partially explain why HPV16 is the most likely to cause cervical cancer and may provide new potential strategies for its clinical management.

Several serological studies indicated that the Middle East respiratory syndrome coronavirus (MERS-CoV) has been circulating in camels in the Arabian Peninsula for more than two decades. However, it is still intriguing why the virus was first detected in humans in 2012. It is worth noting that infection with MERS-CoV could be asymptomatic or cause mild influenza-like illness. This may suggest that the prevalence of MERS-CoV infections in the general population is underestimated. The aim of the study by R. A. Al Kahlout et al. was to evaluate the performance of various serological assays and to estimate the seroprevalence of anti-MERS-CoV antibodies in high- and low-risk groups in Qatar. The paper reported low prevalence of anti-MERS antibodies in the general population, which coincides with the low number of all reported cases by the time of their study. Importantly, serological analysis indicated high cross-reactivity between MERS-CoV and other coronaviruses, which necessities more detailed investigation of the immune responses to coronavirus infections.

One of the two reviews in this issue by W. Zeng et al. examined the transplantation of probiotics and fecal microbiota on immunomodulation. Probiotics or microbiota are commensal/nonpathogenic microbes that provide beneficial effects to the host through several mechanisms, including but not limited to competitive exclusion of pathogenic bacteria and modulation of immune responses. The aim of this review was to follow through the recent literature on immunomodulatory effects and mechanisms of probiotics and fecal microbiota transplantation (FMT) and also the efficacy and safety of probiotics and FMT in clinical trials and applications. The authors concluded that the immunomodulation induced by probiotics is a complex interplay between different hosts and microorganisms, and hence, the immunomodulatory characteristics of specific probiotics cannot be generalized. Accordingly, "personalized probiotics intervention and standardized fecal bacteria transplantation should be challenges and prospects for future research on the intervention model of intestinal flora."

Macrophages are indispensable modulators of the innate immune system because they maintain a delicate balance between immunity against pathogenic bacteria and tolerance of commensals in the intestine. In their review titled "Functions of Macrophages in the Maintenance of Intestinal Homeostasis," S. Wang et al. focused their attention on intestinal macrophages, "describing the recent insights into the role of intestinal macrophages in maintaining gut homeostasis and managing gut inflammation." They also discussed the nutritional modulation of intestinal macrophage functions and the potential of nutritional strategies to manipulate intestinal macrophages and ameliorate inflammatory bowel disorders. They concluded that "a better understanding of mechanisms employed by intestinal macrophages in maintaining intestinal homeostasis and the action of enteral nutrients in the regulation of intestinal macrophages will facilitate the development of nutritional strategies in gut health improvement as well as prevention and control of inflammatory bowel disorders."

This special issue provides original research and review articles that would be helpful to experts and broad audience alike in enhancing their understanding of the complex host-pathogen interactions.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue.

Hadi M. Yassine
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Research Article

Taenia crassiceps-Excreted/Secreted Products Induce a Defined MicroRNA Profile that Modulates Inflammatory Properties of Macrophages

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Helminth parasites modulate immune responses in their host to prevent their elimination and to establish chronic infections. Our previous studies indicate that *Taenia crassiceps*-excreted/secreted antigens (TcES) downregulate inflammatory responses in rodent models of autoimmune diseases, by promoting the generation of alternatively activated-like macrophages (M2) *in vivo*. However, the molecular mechanisms triggered by TcES that modulate macrophage polarization and inflammatory response remain unclear. Here, we found that, while TcES reduced the production of inflammatory cytokines (IL-6, IL-12, and TNFα), they increased the release of IL-10 in LPS-induced bone marrow-derived macrophages (BMDM). However, TcES alone or in combination with LPS or IL-4 failed to increase the production of the canonical M1 or M2 markers in BMDM. To further define the anti-inflammatory effect of TcES in the response of LPS-stimulated macrophages, we performed transcriptomic array analyses of mRNA and microRNA to evaluate their levels. Although the addition of TcES to LPS-stimulated BMDM induced modest changes in the inflammatory mRNA profile, it induced the production of mRNAs associated with the activation of different receptors, phagocytosis, and M2-like phenotype. Moreover, we found that TcES induced upregulation of specific microRNAs, including miR-125a-5p, miR-762, and miR-484, which are predicted to target canonical inflammatory molecules and pathways in LPS-induced BMDM. These results suggest that TcES can modulate proinflammatory responses in macrophages by inducing regulatory posttranscriptional mechanisms and hence reduce detrimental outcomes in hosts running with inflammatory diseases.

1. Introduction

Helminth infections induce polarized T_H2-type biased immune responses that play a role in parasite expulsion, tissue repair, and regulation of unrelated inflammatory and autoimmune responses in the host [1–3]. The striking ability of helminth parasites in conferring protection from diseases of immune dysregulation has increased the attention into the immunomodulatory mechanisms evoked by these pathogens. Previous studies in our laboratory, using a murine model of cysticercosis, demonstrated that chronic infection with the helminth *Taenia crassiceps* or administration of its excreted/secreted products (TcES) ameliorates the development of experimental ulcerative colitis, autoimmune encephalomyelitis (EAE), and type 1 diabetes [4–8]. The ability of *T. crassiceps* and TcES to counteract these inflammatory

responses was demonstrated to be dependent on a population of macrophages that produced markers of alternative activation (M2), such as PD-L2, IL-4Rα, MR, IL-10, ARG1, YM1, and FIZZ1 [9].

Macrophages can be activated towards an M2 phenotype after being stimulated with IL-4 produced by T_H2 lymphocytes during parasitic infections or exposure to allergens [10, 11]. In contrast, released IFN-γ and pathogen or danger-associated molecular patterns (PAMPs or DAMPs) during infections or tissue injury, respectively, promote classical (M1) activation in macrophages [1, 12]. Although a crucial role for *T. crassiceps*-induced M2 macrophages in regulating detrimental autoimmune and inflammatory responses has been demonstrated [3], the transcriptional events elicited by TcES that modulate macrophage activation have not been elucidated.

Helminth infections and/or their antigens can trigger the levels of microRNAs to modulate inflammatory responses in the host [13–15]. MicroRNAs are small noncoding RNAs that regulate cell functions posttranscriptionally through direct binding to the 3′-UTR (untranslated region) of target messenger RNAs (mRNAs), resulting in the destabilization of mRNAs and repression of translation [16]. Recently, microRNAs have been associated with helminth-induced M2 macrophages *in vitro* and *in vivo*. For instance, Rückerl et al. reported that macrophages obtained during acute (3 weeks) *Brugia malayi* infection induced microRNAs associated with M2 macrophages, such as miR-199-5p, miR-378-3p, and miR-125b-5p [15]. In addition, Guo and Zheng identified distinct microRNAs, including miR-146a-5p, miR-155-5p, miR-21a-5p, miR-146b-5p, miR-99b-3p, miR-125a-5p, and miR-378, in RAW 264.7 macrophages cocultured with metacestodes of *Echinococcus multilocularis*. In these studies, the authors suggested a role for these microRNAs in targeting important inflammatory mRNAs (*Tnf*, *Il1a*, *Il6*, *Il12a*, *Il12b*, *Ccl22*, and *Ccl18* mRNA) [14]. Thus, microRNAs may be a key mechanism elicited by helminths in the regulation of inflammatory responses in the host.

Although we have previously demonstrated a role for the TcES in preventing STAT1 phosphorylation in inflammatory macrophages [17], the influence of TcES in macrophage polarization and the transcriptional pathways regulating this process remain unknown. Here, we determined the effect of TcES alone or in combination with LPS or IL-4, in the regulation of multiple mRNA transcripts and microRNAs induced in macrophages. Our results indicate that TcES decreased the production of inflammatory cytokines (IL-12, TNFα, and IL-6) in LPS-induced macrophages but has a limited role in inducing directly the production of M1- and/or M2-associated molecules. The immune-modulatory ability of TcES was further associated with increased levels of specific microRNAs, which are predicted to target, according to our bioinformatic analysis, numerous inflammatory mRNAs involved in the TNF and NF-κB signaling pathways. These findings suggest a role for TcES in modulating the transcriptional profile of macrophages via altering their microRNA profile and, consequently, the inflammatory properties of these immune cells.

2. Materials and Methods

2.1. Ethics Statement. All experiments in this study were performed according to the guidelines for the Care and Use of Laboratory Animals adopted by the US National Institutes of Health. The Institutional Animal Care and Use Committee (IACUC) at the Research Institute at Nationwide Children's Hospital and the Ohio State University approved all protocols.

2.2. Mice. Adult 6- to 8-week female BALB/c mice were purchased from The Jackson Laboratory. All animals were maintained in a pathogen-free environment and established as breeding colonies in the Transgenic Mouse Facility at the Research Institute at Nationwide Children's Hospital or in specific pathogen-free conditions at the Ohio State University Laboratory Animal Resources. The mice were housed in sterilized polycarbonate cages with basic filter top caging containing pine wood shavings and were offered mouse ration and water *ad libitum*. The cages were held in Isolation and Containment cubicles (Britz and Co., Wheatland, WY).

2.3. Parasites and TcES. Metacestodes of *T. crassiceps* (ORF strain) were harvested under sterile conditions from the peritoneal cavity of female BALB/c mice after 8-10 weeks of intraperitoneal (i.p.) infection. The cysticerci were washed four times in physiological saline solution prior to maintaining them in culture with a sterile saline solution at 37°C for 24 h. The supernatant was recovered and centrifuged for 10 min at 1000 g. The heavy fraction of TcES was concentrated using the 50 kDa Amicon Ultra Filter (Millipore), 30 min at 1000 g. Protease inhibitors were added to the >50 kDa fraction, and samples were stored at -70°C until further use.

2.4. Bone Marrow-Derived Macrophages (BMDM). To generate bone marrow-derived macrophages (BMDM), we followed the protocol previously described [18]. Briefly, bone marrow cells were obtained by flushing femurs and tibias from BALB/c mice with a sterile saline solution. The isolated cells were plated in 100 mm Petri dishes at 1×10^6 cells/mL in Dulbecco's modified Eagle's media (DMEM, Mediatech, Herndon, VA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Grand Island, NY), 1% penicillin/streptomycin, 1% glutamine, and 20 ng/mL of macrophage colony-stimulating factor (M-CSF, BioLegend). On day 7, the cells were harvested, washed, counted, and replated in culture media (without M-CSF) at a density of 2×10^6 cells/well (12-well plate, Falcon polystyrene). BMDM were incubated with either TcES (25 μg/mL), *Escherichia coli* LPS (1 mg/mL, Sigma-Aldrich), interleukin-4 (20 ng/mL), TcES+LPS or TcES+IL-4. After 4 and 24 h poststimulation, BMDM were harvested for flow cytometric and transcriptomic analysis. The supernatants were recovered for cytokine detection by ELISA.

2.5. Flow Cytometric Analysis. Flow cytometric analysis was performed as previously described [19]. Briefly, harvested

BMDM were incubated in 1 μg/mL of anti-mouse Fc receptor antibody in 100 mL PBS containing 0.5% BSA plus 0.02% NaN_3 (FACS buffer) for 15 min on ice. Subsequently, single-cell suspensions were stained for 15 min at 4°C with blue-fluorescent reactive dye, L23105 (Life Technologies) to discriminate dead cells. After washing, $1\text{-}3 \times 10^6$ cells were surface-stained in FACS buffer for 15 min at 4°C with antibodies recognizing CD11b (Alexa Fluor 700, BioLegend), F4/80 (Brilliant Violet 785, BioLegend), CD86 (Brilliant Violet 421, BioLegend), PD-L1 (PE-Cy7, BioLegend), and PD-L2 (PE, BioLegend). Surface-stained cells were washed three times with FACS buffer and treated with Fix/Perm reagent according to the protocol of the cytofix/cytoperm kit (BD Biosciences, San Jose, CA, USA). The cells were intracellularly stained in FACS buffer containing anti-Nos2 (PE, eBiosciences) and anti-h/m arginase 1 (APC, R&D systems) for 30 min at 4°C and further collected on an LSR II cytofluorometer (BD, Franklin Lakes, NJ). Stained cells were gated according to size (SSC-A) and forward scatter (FSC-A) to eliminate debris. Doublets were excluded from the analysis by using forward scatter height (FSC-H) and FSC-A. Data analysis was performed using FlowJo Software (FlowJo, LLC).

2.6. Cytokine Assay. Supernatants from cell cultures of stimulated macrophages were recovered at 4 and 24 h poststimulation, and the levels of the cytokines IL-10, IL-6, TNFα, and IL-12 were measured by ELISA according to the manufacturer's instructions (PeproTech).

2.7. RNA Extraction and Arrays. Total RNA was extracted from BMDM stimulated with LPS (M_{LPS}), TcES (M_{TcES}), LPS+TcES ($M_{TcES+LPS}$), or culture media (M_0) using QIAzol reagent (QIAGEN), according to the manufacturer's specifications, and stored at -80°C. Next, RNA was purified following the miRNeasy kit protocol (QIAGEN). RNA concentration and integrity were determined using a NanoDrop™ spectrophotometer (Thermo Scientific, Wilmington, DE) and Agilent Bioanalyzer 2100, respectively. For transcriptomic analysis, 50 ng/μL of RNA was used for the nCounter Inflammation Panel (NanoString mRNAs) and the nCounter miRNA Assay set (microRNAs). Both mRNA and microRNA arrays were performed following the manufacturer's instructions at the Genomics Shared Resource, OSU. Data analysis for the nCounter Inflammatory Panel (mRNA) and for the nCounter miRNA Assay set was conducted using the nSolver™ Analysis Software according to the manufacturer. For the nCounter Inflammatory Panel (mRNA), we normalized using the normalization factor and subtracted the background (mean of negative controls ± 2 standard deviations). Next, we normalized using the geometric mean of housekeeping genes as reported [20]. Then, using the normalized counts, we calculated the fold change (FC) by comparing M_{TcES}, $M_{TcES+LPS}$, and M_{LPS} to M_0. For the nCounter miRNA Assay set, we first normalized using the normalization factor. The background was subtracted from the data using the mean of negative controls ± 2 standard deviations. Finally, we used the top 75 microRNAs [21]. The normalized counts were used to calculate the FC by comparing M_{TcES}, $M_{TcES+LPS}$, and M_{LPS} to M_0. Of the 566 total probes measured in the assay, 183 and 236 microRNAs for 4 h and 24 h, respectively, were identified and used for analyzing significant changes in microRNA levels among samples. MultiExperiment Viewer (MeV) was used to generate heat maps, which represent $\log_2$-transformed data.

2.8. Real-Time PCR. TaqMan gene expression assays (Applied Biosystems) were used to quantify and/or validate the levels of mRNAs and microRNA transcripts. cDNA was generated from mRNAs, using a 15 μL RT reaction consisting of 2.0 μL of Buffer (10x), 0.8 μL 100 mM dNTPs (100 mM), 1.0 μL reverse transcriptase, 2.0 μL of mRNA primer, and 1 μg of total RNA. RT reaction was incubated for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. For microRNA levels, a 15 μL reaction was prepared with 2.0 μL of buffer (10x), 0.2 μL 100 mM dNTPs (100 mM), 1.0 μL reverse transcriptase, 0.2 μL RNAse inhibitor (20 U/μL), 3.0 μL of microRNA primer, and 100 ng of total RNA. RT reaction was incubated as mentioned before. For both mRNA and microRNAs, quadruplicate real-time PCR reactions were performed in the 7500 Real-Time PCR system. The amplification reaction mix was composed of 10 μL of TaqMan Universal PCR Master Mix (2x), 1 μL of the specific mRNA or microRNA probe, and 1 μL of specific microRNA cDNA. The reactions were preincubated for 10 minutes at 95°C and amplified with 40 cycles consisting of 10 sec at 95°C, 40 sec at 60°C, and 5 sec at 72°C (fluorescence acquisition). To assess possible bias for reference RNA, we used 18S RNA, *Actb*, and *Gapdh* mRNAs. Relative quantification was calculated by $2^{-\Delta\Delta Cf}$. All mRNA and microRNA assays were tested for reproducibility and linearity (PCR efficiency was between 1.9 and 2.0 for all assays). All primers were purchased from Applied Biosystems. The primer sequences are shown in Table S1.

2.9. mRNA and MicroRNA Target Gene Prediction and Bioinformatics Analysis. Target mRNAs of differentially produced microRNAs were predicted using DIANA-TarBase database v6.0, which includes experimentally validated targets from the literature. To explore the potential biological function of the microRNAs' profile and their targets, DIANA-mirPath v2.0 (http://snf-515788.vm.okeanos.grnet.gr/) was used to perform enrichment analysis of microRNA's target mRNAs in the KEGG pathway and in GO terms [22].

2.10. Statistical Analysis. Data analyses were performed using GraphPad Prism 6 software. Statistical comparisons were performed by using Student's *t*-test. p values less than 0.05 were considered significant. Graphed data are presented as mean ± SD or SEM.

3. Results

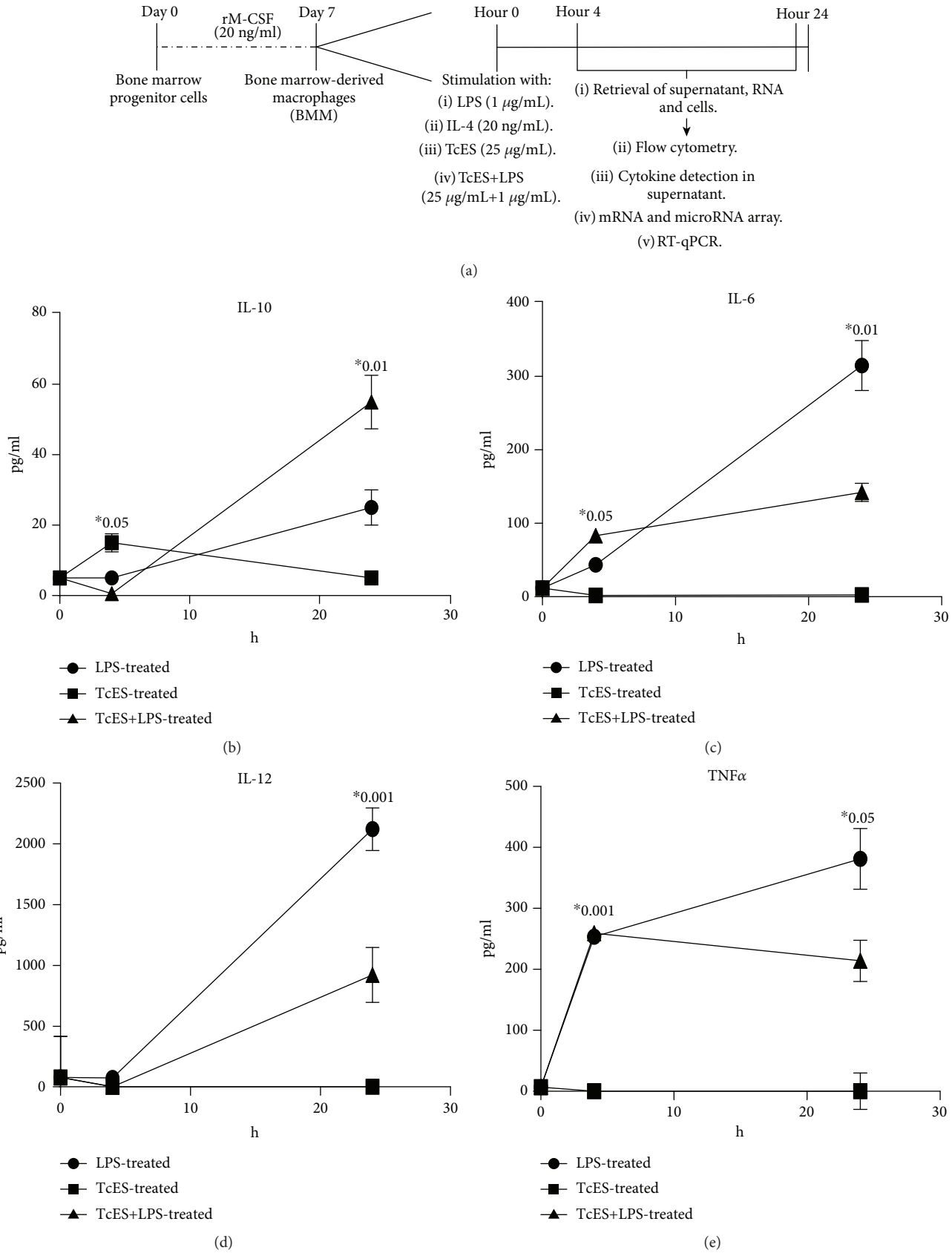
3.1. TcES Reduces the Inflammatory Response of LPS-Induced BMDM. Previously, we demonstrated the ability of TcES in reducing the development of inflammatory and autoimmune diseases in rodent models [4–8]. The effect of TcES in

counteracting detrimental inflammatory responses *in vivo* is associated with the emergence of polarized macrophages towards an M2 phenotype [4, 5, 11]. Although studies in our laboratory indicate a role for TcES in blocking the IFN-γ/STAT1 signaling pathway in macrophages [17], the effect of TcES in inducing directly M2 macrophages remains to be elucidated. To define the macrophage profile elicited by TcES, we first determined the levels of the inflammatory cytokines IL-12, IL-6, TNFα, and IL-10 in cultures from BMDM. The cells were stimulated (Figure 1(a)) for 4 h or 24 h with TcES (henceforth M_{TcES}), *E. coli* lipopolysaccharide (M_{LPS}), interleukin-4 ($M_{IL\text{-}4}$), TcES+LPS ($M_{TcES+LPS}$), TcES+IL-4 ($M_{TcES+IL\text{-}4}$), or PBS (M_0). Supernatants obtained from M_{TcES} displayed higher levels of IL-10 and deficient levels of inflammatory cytokines (IL-6 and TNFα) compared to all the groups at 4 h poststimulus (Figures 1(b) and 1(e)). However, IL-10 production by M_{TcES} did not continue at 24 h. Interestingly, we found that exposure of macrophages to TcES and stimulated with LPS ($M_{TcES+LPS}$) significantly reduced the production of IL-12, IL-6, and TNFα compared to those in M_{LPS} at 24 h (Figures 1(c) and 1(e)). Increased IL-10 levels were observed in supernatants from $M_{TcES+LPS}$ compared with all groups at 24 h (Figure 1(b)). A similar trend was identified in the levels of the mRNA for *Tnf* at 24 h poststimulus (Figure 1(f)), whereas levels of *Il10* mRNA were similar between all groups at 24 h (Figure 1(g)). Our results suggest that TcES play a role in downregulating the production of proinflammatory cytokines in LPS-induced BMDM, by increasing the production of a regulatory cytokine.

To gain insight in the phenotypic profile induced by TcES in macrophages, we used flow cytometry technique to determine the production of intracellular nitric oxide synthase (NOS2), and arginase-1 (ARG1), as the conventional markers for M1 and M2 profiles, respectively, in BMDM. Our results showed that while M_{LPS} and $M_{IL\text{-}4}$ presented increased percentages of $NOS2^+$ and $ARG1^+$ macrophages, respectively, M_{TcES} displayed limited production of these molecules (Figures 2(a) and 2(d)). Additionally, similar percentages of $NOS2^+$ BMDM were found between $M_{TcES+LPS}$ and M_{LPS}, and comparable $ARG1^+$ BMDM were observed when analyzing $M_{TcES+IL\text{-}4}$ versus $M_{IL\text{-}4}$ (Figures 2(a) and 2(d)). Levels of mRNA *Arg1* by RT-qPCR showed similar trends as the flow cytometric analysis (Figure 2(f)). While the levels of *Nos2* mRNA were upregulated in $M_{TcES+LPS}$ compared to M_0 but significantly reduced compared to M_{LPS} (Figure 2(e)). These data suggest that the stimulus with TcES, either alone or in combination with LPS or IL-4, has a limited role in inducing the production of canonical M1 or M2 markers. Nevertheless, these antigens play a role in downregulating the proinflammatory response to LPS in BMDM.

3.2. TcES Modify the Proinflammatory mRNA Profile of LPS-Induced BMDM. Because our data suggest a novel role for TcES in attenuating the proinflammatory response of LPS-induced BMDM, and the current M1/M2 paradigm scarcely describes the influence of TcES in the transcriptional profile of macrophages, we performed a proinflammatory mRNA array screen (see "mRNA array" for details) on M_0, M_{LPS}, M_{TcES}, and $M_{TcES+LPS}$, at 4 and 24 h poststimulus (Figure 3). Commonly produced mRNAs among the groups of M_{LPS}, M_{TcES}, and $M_{TcES+LPS}$ are displayed in Table S2. As expected, our results indicate increased levels of multiple proinflammatory mRNAs in M_{LPS} with respect to M_0 (Table 1 and Table S3), including *Il1a*, *Il6*, *Il12a*, *Il12b*, *Tnf*, and *Nos2*, among other mRNAs, at 4 and 24 h poststimulus. These molecules correspond to previously reported markers for LPS-stimulated macrophages [1]. In contrast, M_{TcES} downregulated the levels, with respect to M_0, of cytokines, chemokines, and transcriptional factors distinctive of M1-activated macrophages, while displaying upregulated levels mainly associated with enzymes, as MAPK pathway, at 4 and 24 h poststimulus (Table 1 and Table S4). Noticeably, although $M_{TcES+LPS}$ presented 132 and 96 upregulated mRNAs (Table S5), these macrophages only shared 6 and 3 upregulated mRNAs with M_{TcES} at 4 and 24 h poststimulus, respectively. However, $M_{TcES+LPS}$ shared 89 and 65 upregulated mRNAs with M_{LPS} at 4 and 24 h poststimulus, respectively, including transcripts for cytokines, chemokines, receptors, and transcriptional factors as *Il1a*, *Il1b*, *Il6*, *Il12a*, *Il12b*, *Ccl3*, *Ccl5*, *Ccl2*, *Ccl7*, *Cd86*, *Tlr2*, *Stat1*, *Stat3*, and *Nfkb1* mRNA. The differentially induced mRNAs between $M_{TcES+LPS}$ and M_{LPS} are shown in Table S6. Next, we validated 7 mRNAs associated with M1 (*Il1b*, *Stat1*, *Cd86*, *Il6*, and *Il12b*) and M2 (*Stat6* and *Chi3l3*) macrophages by RT-qPCR. The levels of these mRNAs were comparable to those observed in the mRNA array (Figure 4), which attest for the high quality of our array, supporting that a posttranscriptional mechanism induced by TcES may have a role in macrophage's response to LPS. Interestingly, although the levels of IL-6 and IL-12 in supernatants from $M_{TcES+LPS}$ were significantly reduced respect to M_{LPS} (Figures 1(a) and 1(b)), the levels of their mRNAs of these cytokines were comparable between $M_{TcES+LPS}$ and M_{LPS}. These data suggest that posttranscriptional mechanisms triggered by TcES may have a role in modulating the production of specific inflammatory cytokines.

3.3. TcES Modulate the Profile of MicroRNAs in LPS-Stimulated BMDM. MicroRNAs participate in diverse biological processes at the posttranscriptional regulatory level. The complementary binding of microRNAs to mRNAs reduces either transcription or translation of mRNA transcripts [16]. Recently, a handful of studies indicate a role for helminth parasites and their antigens in inducing microRNAs to modulate host immune responses [14, 15, 23]. To determine whether the ability of TcES in attenuating the inflammatory response of BMDM is associated with the production of specific microRNAs, we performed a microRNA array (see "microRNA array" in Materials and Methods for details) in M_0, M_{LPS}, M_{TcES}, and $M_{TcES+LPS}$, at 4 and 24 h poststimulus. As a result, we identified 7 and 89 upregulated microRNAs in M_{LPS} at 4 h and 24 h, respectively. M_{TcES} displayed 13 (4 h) and 3 (24 h), and $M_{TcES+LPS}$ showed 19 (4 h) and 28 (24 h) upregulated microRNAs (Figure 5). The top 10 up- and downregulated microRNAs in M_{LPS}, M_{TcES}, and



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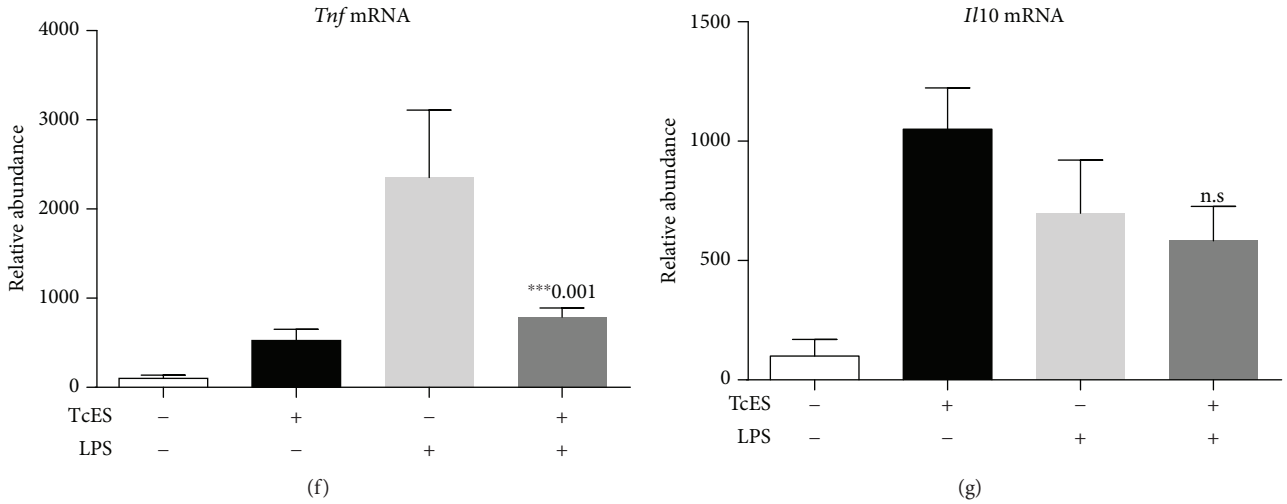
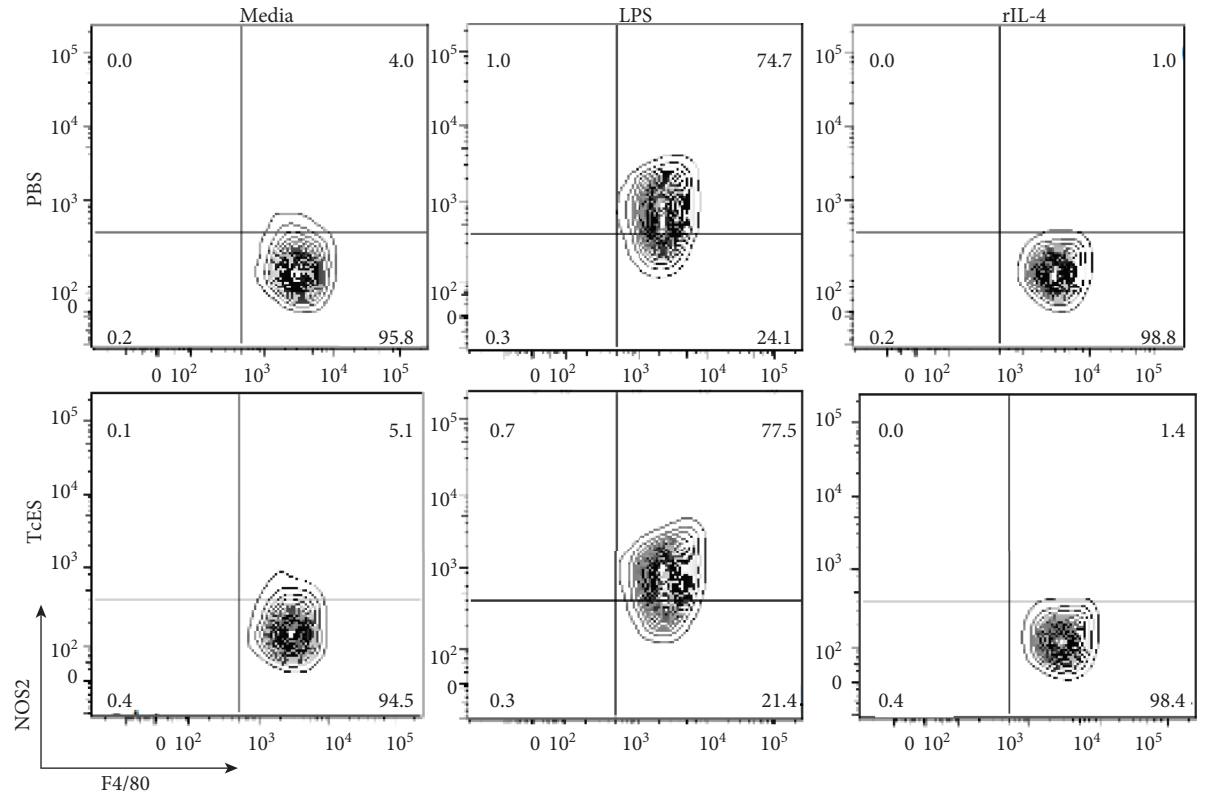


FIGURE 1: TcES regulates the production of inflammatory cytokines in LPS-induced macrophages. (a) Flow diagram of general experimental design. For differentiation of macrophages, bone marrow progenitor cells were cultured with rM-CSF at 37°C 5% CO_2 for 7 days. On completion day, BMDM were washed and stimulated with the following stimuli: LPS (1 μg/mL), TcES (25 ng/mL), or both for 4 or 24 hours. The supernatants, total RNA or cells were harvested for later procedures. (b) Kinetics levels of IL-10, (c) IL-6, (d) IL-12, and (e) TNFα in supernatants from stimulated BMDM. Evaluation of the levels of (f) *Tnf* and (g) *Il10* mRNA by RT-qPCR in groups of stimulated macrophages mentioned above ($n = 6$, 3 replicates condition). mRNA levels are represented as mean relative (±SD). Data are representative of 3 independent experiments using 3 replicates per stimuli. Significance was calculated using t-test. $^{*}p < 0.01$, $^{**}p < 0.05$, and $^{***}p < 0.001$.

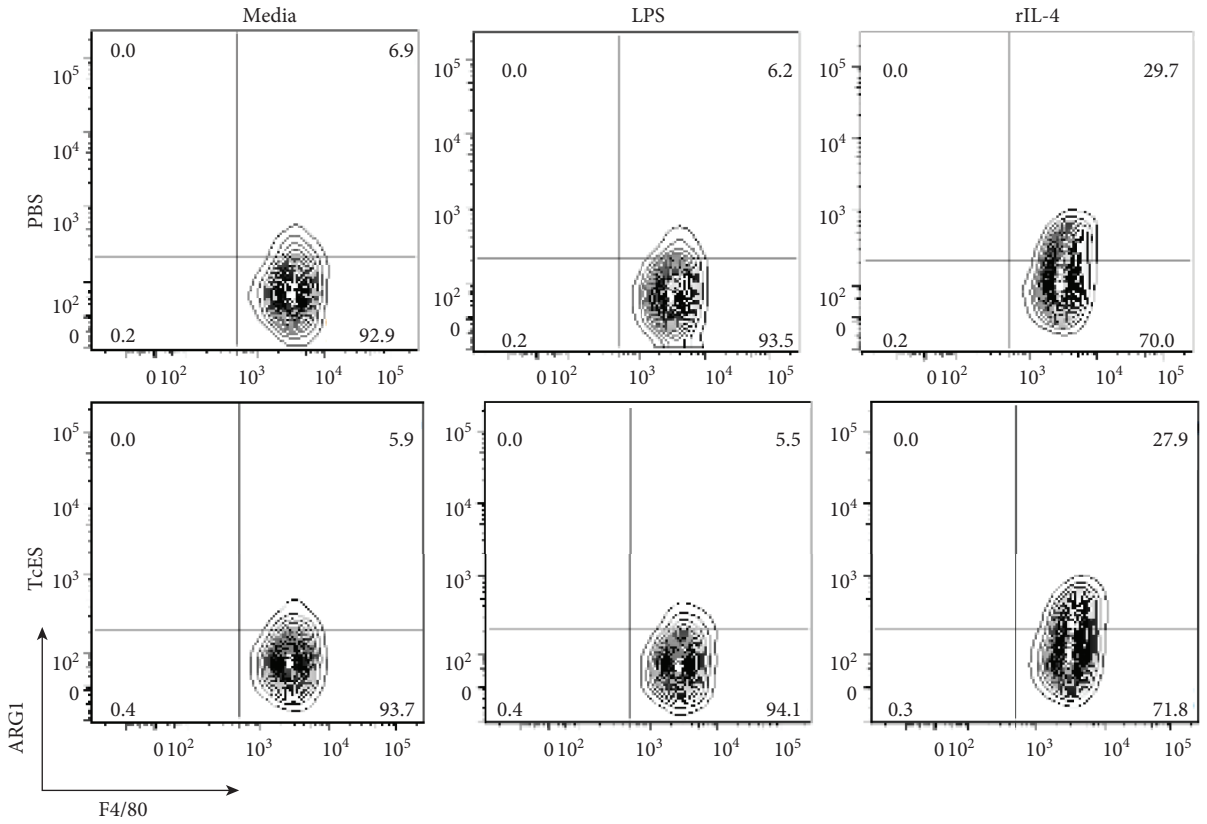
$M_{TcES+LPS}$ are shown in Table 2. The complete lists of microRNAs are shown in Table S7–S9. Additionally, we found 4 and 2 microRNAs shared among the groups of stimulated BMDM at 4 and 24 h, respectively (Table S10). Interestingly, $M_{TcES+LPS}$ shared 6 upregulated microRNAs with M_{TcES} and only 3 with M_{LPS} at 4 h poststimulus. However, $M_{TcES+LPS}$ did not share microRNAs with M_{TcES} and shared 22 with M_{LPS} at 24 h poststimulus. Finally, $M_{TcES+LPS}$ differentially induced 3 and 20 microRNAs compared to M_{LPS} at 4 and 24 h poststimulation, respectively (Table S11). These data suggest that TcES induce the early production (4 h) of microRNAs, followed by the stimulus with LPS (24 h), in $M_{TcES+LPS}$. This phenomenon is associated with an increased number of upregulated microRNAs in M_{TcES} compared to M_{LPS} (13 vs. 7 microRNAs, Figure 5) at 4 h poststimulation.

To assess the potential biological relevance of the upregulated microRNAs in the transcriptional profile of activated macrophages, we conducted bioinformatic analysis as GO terms and KEGG pathway analysis by comparing M_{TcES}, $M_{TcES+LPS}$, or M_{LPS} vs. M_0 at both 4 and 24 h poststimulus. The GO terms in M_{TcES} and $M_{TcES+LPS}$ were mainly enriched for the biological process associated with anatomical structure development, cell differentiation, and cellular protein differentiation process at 4 h poststimulus (Figures 6(a) and 6(b)). Anatomical structure development, cell differentiation, and chromosome organization were predicted to be a target by microRNAs in M_{TcES}, while organelle, anatomical structure, and cell differentiation were enriched in $M_{TcES+LPS}$ at 24 h poststimulus (Figures 6(d) and 6(e)). Lastly, GO terms enriched for M_{LPS} are chromosome organization, biosynthetic process, and protein complex as well as organelle, anatomical structure, and cell differentiation at 4 and 24 h poststimulus, respectively (Figures 6(c) and 6(f)). The KEGG pathway enrichment analysis revealed that at 4 h stimulus, upregulated microRNAs were regulating glioma, chronic myeloid leukemia, and TGF-β signaling pathway in M_{TcES} (Figure 6(a)); ubiquitin-mediated proteolysis, p53, and GhRH signaling pathway in $M_{TcES+LPS}$ (Figure 6(b)); and prostate cancer, steroid biosynthesis, and FoxO signaling pathway in M_{LPS} (Figure 6(c)). In contrast, the KEGG enrichment pathways at 24 h poststimulus were axon guidance, insulin signaling pathway, and HTLV-I infection in $M_{TcES+LPS}$ (Figure 6(e)) and inositol phosphate metabolism, pathways in cancer, and insulin signaling pathway in M_{LPS} (Figure 6(f)). For more details of GO enrichment analysis and KEGG pathways, refer to Table S12 and Table S13. These data suggest a role for TcES in inducing microRNAs that regulate important metabolic, cell signaling, and inflammatory pathways in LPS-stimulated BMDM.

Next, we selected and validated by RT-qPCR four microRNAs (miR-125a-5p, miR-762, miR-155-5p, and miR-484), which are potentially involved in the regulation of inflammatory mRNAs, as indicated by previous studies and our bioinformatics analysis. We found that both M_{LPS} and M_{TcES} showed increased levels of miR-125a-5p (Figure 7(a)), a microRNA reported to reduce the production of inflammatory cytokines (IL-6, IL-12, and TNFα) [24]. The levels of miR-125a-5p were sustained in M_{LPS} and $M_{TcES+LPS}$ until 24 h poststimulus (Figure 7(b)). The combined stimuli of TcES and LPS induced an additive effect in the levels of this microRNA at 4 h poststimulation (Figure 7(a)). Furthermore, M_{TcES} and $M_{TcES+LPS}$ showed increased levels of miR-762, a microRNA known to directly target the mRNA of



(a)



(b)

Figure 2: Continued.

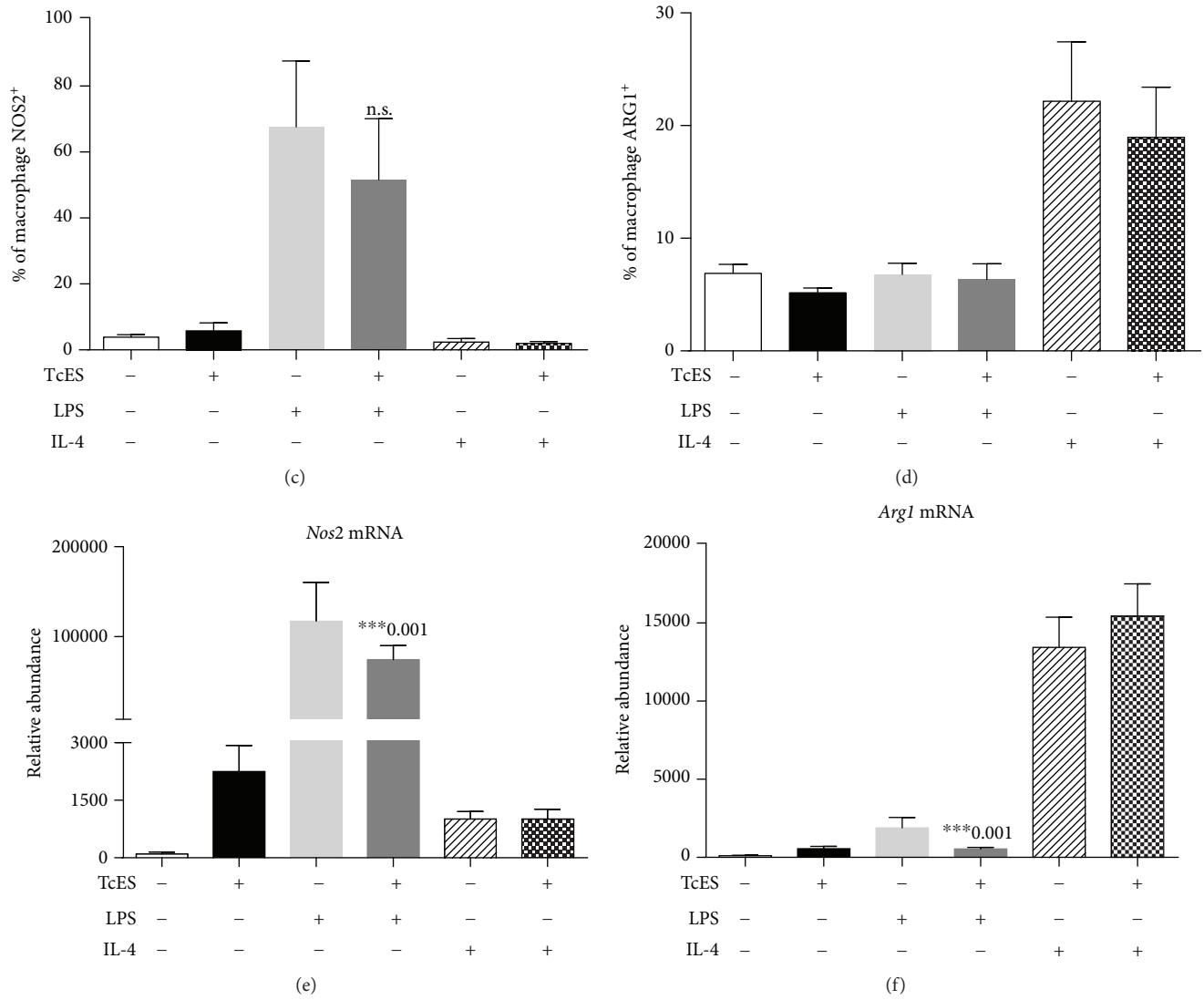


Figure 2: TcES do not modify the production of canonical M1/M2 macrophage markers. Representative dot plots, obtained by flow cytometry, of (a) F4/80$^+$NOS2$^+$ and (b) F4/80$^+$ARG1$^+$ BMDM, after 24 h poststimulus with one of the following stimuli: LPS (1 μg/mL), TcES (25 ng/mL), IL-4 (20 ng/mL), TcES+LPS, TcES+IL-4, or PBS. Bar graphs representing the percentage of (c) F4/80$^+$NOS2$^+$ and (d) F4/80$^+$ARG1$^+$ BMDM at 24 h poststimulus. (e) Evaluation of the levels of *Nos2* and (f) *Arg1* mRNA by RT-qPCR in BMDM stimulated for 24 h ($n = 6$, 3 replicates condition). mRNA levels are represented as mean relative (±SD). Data are shown as a representative of two independent experiments. Significance was calculated using t-test. $^*p < 0.01$, $^{**}p < 0.05$, and $^{***}p < 0.001$.

inflammatory transcription factor *Irf7* [25], at 4 h poststimulus (Figure 7(c)). This microRNA was later produced in M_{LPS} at 24 h poststimulation (Figure 7(d)). In addition, miR-484 was highly produced in M_{TcES} compared to both $M_{TcES+LPS}$ and M_{LPS} at 4 and 24 h poststimulus (Figures 7(e)–7(f)). Our bioinformatic analysis suggests that miR-484 can potentially target *Nfkb*, *Stat5a*, *Irf1*, *Myd88*, *Stat1*, and *Il12a* mRNAs. Finally, miR-155-5p, a well-defined microRNA in M1 macrophages, was upregulated in M_{LPS} and $M_{TcES+PS}$ compared to M_{TcES} at 4 and 24 h poststimulation (Figures 7(g)–7(h)). The profile of these miRNAs was comparable to those observed in the microRNA array. Altogether, our findings suggest a role for miR-125a-5p, miR-762, and miR-484 in the immunomodulatory effect of TcES in BMDM.

4. Discussion

Helminth parasites and their antigens can counteract proinflammatory responses generated during autoimmune diseases [3]. In our laboratory, we have previously demonstrated that infection with the helminth parasite *T. crassiceps* or the administration of TcES reduced the symptoms of EAE, type I diabetes, and ulcerative colitis, in part due to the polarization of macrophages *in vivo* towards an M2 phenotype [4–8, 26]. However, the functional role of TcES in regulating the activation and inflammatory response of macrophages remains unknown. In this study, we evaluated the effect of TcES on the polarization towards an M2 profile, inflammatory immune response, and transcriptomic profile of macrophages *in vitro*.

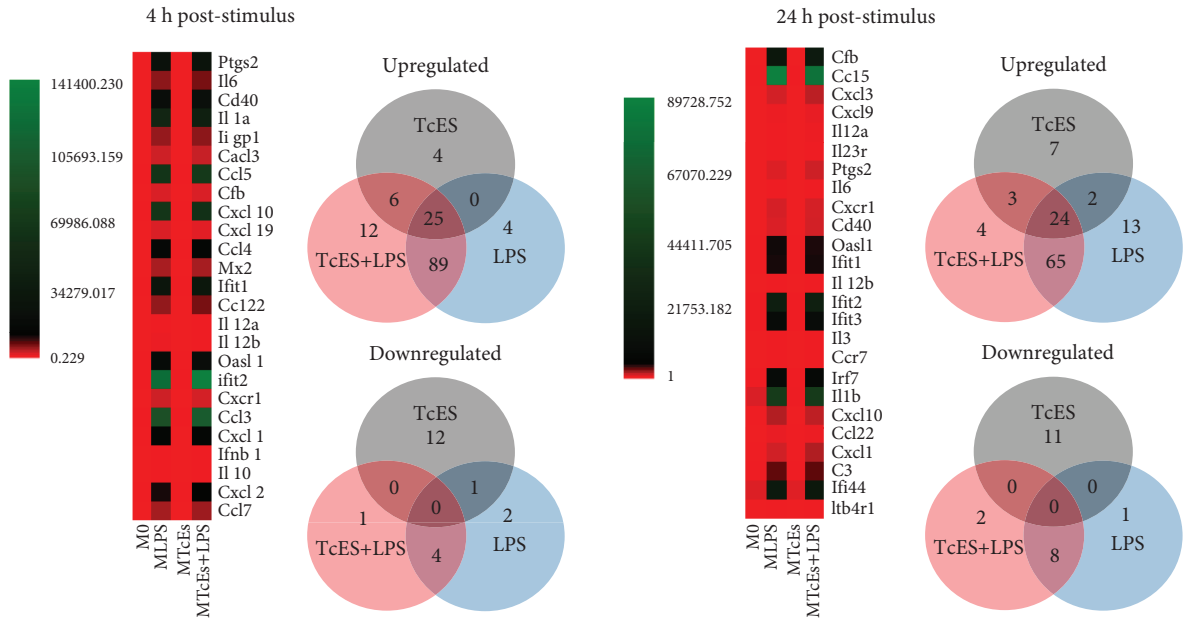


FIGURE 3: Top 25 upregulated mRNAs in stimulated BMDM. Heat maps show mRNA levels among M_{TcES}, $M_{TcES+LPS}$, and M_{LPS} at 4 and 24 h poststimulus. Each row represents mRNA levels, and each column represents a specific sample. The color scale illustrates the relative levels of mRNA: green, increased production; red, decreased production; and black, mean value. Venn diagrams show the unique and overlapping mRNA transcripts among the samples.

We first measure the production of the cytokines IL-6, IL-10, IL-12, and TNFα in BMDM-stimulated with TcES alone or in combination with LPS and observed that TcES increased the levels of the regulatory cytokine IL-10 and reduced the release of the inflammatory cytokines IL-6, IL-12, and TNFα in supernatants from LPS-stimulated BMDM. TcES alone did not increase the production of inflammatory cytokines but induced the release of IL-10 in BMDM. The levels of both mRNAs of *Il10* and *Tnf* measured by RT-qPCR showed similar trends when compared to the levels of cytokines obtained by ELISA assay, suggesting a consistent role for TcES in regulating cytokine production by inhibition of their transcripts.

Here, we evaluated the production of NOS2 and ARG1 in BMDM stimulated with TcES alone or in combination with IL-4 or LPS. M1 macrophages normally produce NOS2, which metabolizes L-arginine to nitric oxide (NO), while M2 macrophages produce ARG1, which metabolizes L-arginine to produce prolines and polyamines [2, 27]. We found that whereas BMDM stimulated with IL-4 or LPS alone showed increased levels of ARG1 and NOS2, respectively, TcES did not alter the production of both NOS2 and ARG1, after 24 h poststimulation. Our data are in agreement with previous studies using *Fasciola hepatica* tegumental antigens, which also failed to directly induce the production of molecules associated with M2 macrophages *in vitro* but not *in vivo* [28]. The production of M2 canonical molecules such as ARG1 has been reported to be IL-4-dependent, which is produced by T_H2 T cells, natural killer T cells, and basophils but not macrophages [29–31]. Therefore, helminth antigen stimulation alone is not enough to induce functional polarization of BMDM towards M2; however, they influence the inflammatory properties of these cells. Therefore, TcES do not induce the production of M2-associated molecules but counteract inflammatory response in macrophages *in vitro*.

Recent studies indicate a regulatory role for helminth antigens obtained from *Trichinella spiralis*, *Spirometra erinaceieuropaei*, *Schistosoma mansoni*, and *Hymenolepis diminuta* in reducing cytokine production and subsequent inflammation [32–41]. However, the analysis of a small number of inflammatory products and/or conventional M1 and M2 markers poorly describes the effect of these antigens in the proinflammatory profile of macrophages. Therefore, using array approaches (nCounter Inflammation Panel, NanoString mRNAs), we determined the levels of multiple mRNAs involved in macrophage inflammatory response. As expected, M_{TcES} displayed a lower number of upregulated inflammatory mRNAs, when compared to M_{LPS} at 4 (42 vs. 120 mRNAs) and 24 h poststimulus (36 vs. 104 mRNAs). M_{TcES} induced mRNAs associated with phagocytosis, M2 macrophage, and anti-inflammatory response. For instance, M_{TcES} showed increased levels of *Pkca* mRNA necessary for the biogenesis of phagolysosomes [42]. In addition, M_{TcES} increased levels of *Irf3*, *C1s*, and *Ptgs* mRNAs which have been previously associated with anti-inflammatory microenvironments and identified in M2 macrophages [43–53]. Although our results suggest that TcES induce mRNAs associated with M2 macrophages, the stimulus with these helminth-derived molecules is not enough to induce a full expression of all M2 markers in macrophages as observed in previously reported studies [28].

In contrast, $M_{TcES+LPS}$ and M_{LPS} shared more than 60 proinflammatory mRNAs at both 4 and 24 h

Table 1: Top 10 of mRNA.

(a)

4 h poststimulus											
MTcES vs. M0				MTcES+LPS vs. M0				MLPS vs. M0			
Upregulated		Downregulated		Upregulated		Downregulated		Upregulated		Downregulated	
mRNAs	FC	mRNAs	FC	mRNAs	FC	mRNAs	FC	mRNAs	FC	mRNAs	FC
Cytokines		Cytokines		Cytokines		Receptors		Cytokines		Cytokines	
Ifna1	2.8	*Il12b*	0.2	*Il6*	5974.7	*Ccr2*	0.2	*Il6*	5140.9	*Ifna1*	0.2
Chemokines		*Il23a*	0.2	*Il1a*	3556.5	*Cd163*	0.1	*Il1a*	4198.0	Transcriptional factors	
Ccl21a	6.3	*Il1b*	0.1	*Il12a*	626.2	*Ptger3*	0.1	*Il12a*	739.4	*Mef2c*	0.2
Transcriptional factors		Complement proteins		*Il12b*	582.3	*Ccr3*	0.1	*Il12b*	957.3	Receptors	
Irf3	8.8	*C2*	0.2	Chemokines		Transcriptional factors		Chemokines		*Ptger3*	0.1
Mafg	3.1	Receptors		*Cxcl3*	2527.8	*Mef2c*	0.2	*Cxcl3*	2285.7	*Ccr2*	0.2
Complement proteins		*Cxcr1*	0.2	*Cxcl10*	2060.0	Enzymes		*Cxcl10*	2215.6	*Cd163*	0.1
C1s	6.3	Transcriptional factors		*Cxcl9*	1285.0	*Limk1*	0.4	*Cxcl9*	1699.8	Enzymes	
Antiviral proteins		*Elk1*	0.2	*Ccl22*	666.9			*Ccl22*	540.2	*Plcb1*	0.5
Hspb1	4.5	Inflammatory-related proteins		Inflammatory-related proteins				Inflammatory-related proteins		*Ptgs1*	0.4
Enzymes		*Lta*	0.2	*Ptgs2*	6106.5			*Ptgs2*	5432.4	*Limk1*	0.3
Defa-rs1	8.0	Enzymes		*Oasl1*	570.6			*Oasl1*	504.8		
Prkca	8.0	*Flt1*	0.2								
Ptgs1	2.1	*Plcb1*	0.1								
Others		Others									
Mbl2	4.5	*Kng1*	0.4								

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(b)

24 h poststimulus												
MTcES vs. M0				MTcES+LPS vs. M0				MLPS vs. M0				
Upregulated		Downregulated		Upregulated		Downregulated		Upregulated		Downregulated		
mRNAs	FC	mRNAs	FC	mRNAs	FC	mRNAs	FC	mRNAs	FC	mRNAs	FC	
Cytokines		Cytokines		Cytokines		Cytokines		Cytokines		Cytokines		
Ifna1	3.7	*Il1b*	0.4	*Il12a*	338.8	*Tgfb3*	0.2	*Il12a*	252.8	*Tgfb3*	0.2	
Chemokines		Chemokines		*Il6*	194.2	Receptors		*Il6*	165.3	Receptors		
Ccl21a	6.4	*Cxcl10*	0.0	Chemokines		*Ccr3*	0.1	Chemokines		*Mrc1*	0.0	
Antiviral proteins		*Ccl24*	0.3	*Ccl5*	2687.8	*Cd163*	0.2	*Ccl5*	3007.4	*Cd163*	0.1	
Hspb1	4.8	*Cxcl2*	0.4	*Cxcl3*	1206.0	*Mrc1*	0.0	*Cxcl3*	823.1	*Trem2*	0.2	
Enzymes		Receptors		*Cxcl9*	410.0	*Trem2*	0.2	*Cxcl9*	332.7	*Ccr3*	0.4	
Alox5	8.9	*Tlr2*	0.2	Enzymes		Enzymes		Complement-related proteins		Enzymes		
Plcb1	5.2	*Cd86*	0.3	*Ptgs2*	206.4	*Ptgs1*	0.0	*Cfb*	17948.5	*Prgs1*	0.1	
Prkca	12.1	Transcriptional factors		Receptors		Transcriptional factors		Receptors		Transcriptional factors		
Map2k6	5.3	*Cebpb*	0.4	*Il23r*	206.8	*Myc*	0.2	*Cxcr1*	175.2	*Maff*	0.2	
Ppp1r12b	4.0	*Relb*	0.5	*Cxcr1*	175.7	*Mef2c*	0.4	*Il23r*	215.8	*Myc*	0.1	
Defa-s1	3.1	Enzymes		*Cd40*	155.6			*Cd40*	128.3	*Mef2c*	0.4	
Map3k9	3.1	*Nos2*	0.3	Complement-related proteins				Enzymes				
		Antiviral proteins		*Cfb*	19872.2			*Ptgs2*	144.5			
		Ifit1	0.3	Inflammatory-related proteins								
				Areg	2.5							

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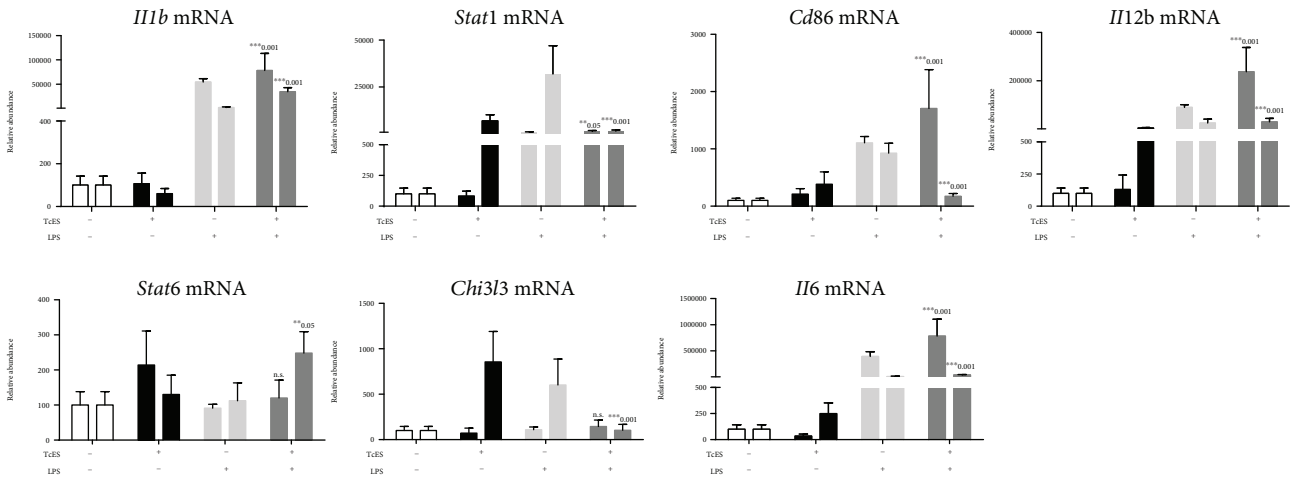

FIGURE 4: Validation and identification of mRNAs in stimulated BMDM. Macrophages were cultured in the presence of TcES (25 μg/mL), LPS (1 μg/mL), or both TcES+LPS for 4 (left bar) and 24 h (right bar) poststimulation. Relative levels of selected mRNA were determined by TaqMan mRNA assays after normalization with 18S RNA. The levels of mRNA are represented as fold change relative to the PBS-treated group (FC ± SD). Data shown are representative of two independent experiments ($n = 6$, 3 replicates condition). Significance was calculated using t-test. $^{*}p < 0.01$, $^{**}p < 0.05$, and $^{***}p < 0.001$.

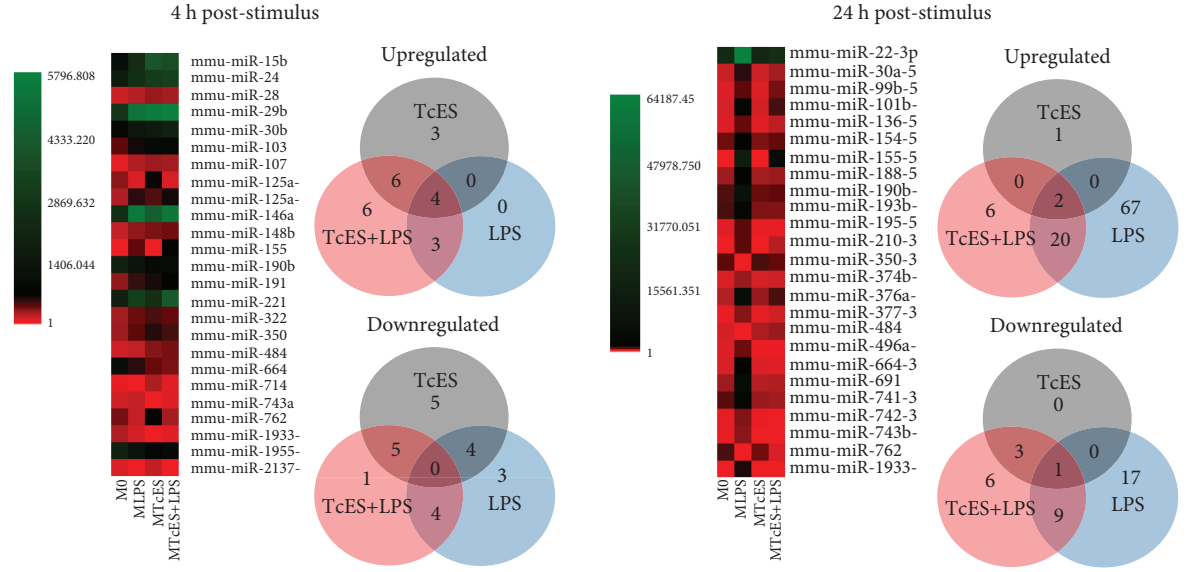

FIGURE 5: Differentially and commonly produced microRNAs in stimulated BMDM. The heat map shows the top 25 of microRNAs produced among M_{LPS}, M_{TcES}, and $M_{TcES+LPS}$ 4 h and 24 h poststimulus. Each row represents a microRNA, and each column represents a specific sample. The color scale illustrates the relative level of microRNAs: green, increased production; red, decreased production; and black, mean value. Venn diagram showing the unique and overlapping microRNAs. A total of 22 and 96 modified microRNAs were found at 4 and 24 h poststimulation.

poststimulation. Interestingly, we observed reduced levels of different inflammatory mRNAs, e.g., *Nox1*, *Ccl21a*, *Ccr4*, and *Cxcr2*, in $M_{TcES+LPS}$ with respect to M_{LPS} at 24 h poststimulus. Noteworthily, although the levels of *Il6*, *Il12a*, and *Il12b* mRNAs were similar between $M_{TcES+LPS}$ and M_{LPS}, reduced levels of these cytokines were detected in supernatants from $M_{TcES+LPS}$ with respect to M_{LPS}. A similar phenomenon has been reported for *Acanthocheilonema viteae* antigens, in decreasing TNFα production in macrophages without altering *Tnf* transcripts [35], suggesting the participation of other posttranscriptional mechanisms. Additionally, M_{TcES} and $M_{TcES+LPS}$ shared levels of the mRNAs for *Irf3*, *Defa1*, *C1s1*, and *Ifna1* at 4 h, and Hspb1, Maff, and Map2k6 at 24 h post stimulus. While levels of *Irf3* and *C1s1* mRNAs suggest an M2-like profile, levels of *Ifna1* mRNA suggest that TcES could be recognized through TLR3, TLR7/8, or TLR9 [54, 55]. To note, *Defa1* mRNA codifies protein HNP1 (human neutrophil-α defensin), which inhibits macrophage-driven inflammation through targeting proinflammatory cytokines and NO [56, 57]. Lastly, we noted that *Tlr2* mRNA was upregulated in $M_{TcES+LPS}$

Table 2: Top 10 of microRNAs.

(a)

4 h poststimulus											
MTcES vs. M0				MTcES+LPS vs. M0				MLPS vs. M0			
Upregulated		Downregulated		Upregulated		Downregulated		Upregulated		Downregulated	
MicroRNA	FC	MicroRNA	FC	MicroRNA	FC	MicroRNA	FC	MicroRNA	FC	MicroRNA	FC
mmu-miR-421-3p	102.60	mmu-miR-539-5p	0.02	mmu-miR-155-5p	676.18	mmu-miR-190b-5p	0.49	mmu-miR-155-5p	402.62	mmu-miR-125a-3p	0.33
mmu-miR-484	2.35	mmu-miR-467c-5p	0.02	mmu-miR-546	112.84	mmu-miR-1955-5p	0.47	mmu-miR-546	76.67	mmu-miR-1224-5p	0.31
mmu-miR-350-3p	2.22	mmu-miR-1193-3p	0.02	mmu-miR-128-3p	88.90	mmu-miR-1933-5p	0.41	mmu-miR-146a-5p	2.09	mmu-miR-467f	0.02
mmu-miR-148b-3p	2.14	mmu-miR-199a-3p	0.01	mmu-miR-421-3p	84.34	mmu-miR-125a-3p	0.36			mmu-miR-539-5p	0.02
mmu-miR-125a-3p	2.13	mmu-miR-383-5p	0.01	mmu-miR-331-3p	74.08	mmu-miR-664-3p	0.35			mmu-miR-380-3p	0.02
mmu-miR-191-5p	2.12	mmu-miR-489-3p	0.01	mmu-miR-484	2.62	mmu-miR-1224-5p	0.35			mmu-miR-467c-5p	0.02
mmu-miR-30b-5p	2.09	mmu-miR-1953	0.01	mmu-miR-191-5p	2.46	mmu-miR-1193-3p	0.02			mmu-miR-714	0.02
mmu-miR-103-3p	2.07	mmu-miR-743a-3p	0.01	mmu-miR-30b-5p	2.44	mmu-miR-2137	0.01			mmu-miR-489-3p	0.01
mmu-miR-29b-3p	1.99	mmu-miR-410-3p	0.01	mmu-miR-99b-5p	2.40	mmu-miR-210-3p	0.01			mmu-miR-2137	0.01
		mmu-miR-1933-5p	0.00	mmu-miR-148b-3p	2.32	mmu-miR-804	0.01			mmu-miR-804	0.01

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(b)

24 h poststimulus											
MTcES vs. M0				MTcES+LPS vs. M0				MLPS vs. M0			
Upregulated		Downregulated		Upregulated		Downregulated		Upregulated		Downregulated	
MicroRNA	FC	MicroRNA	FC	MicroRNA	FC	MicroRNA	FC	MicroRNA	FC	MicroRNA	FC
mmu-miR-362-5p	77.49	mmu-miR-743b-5p	0.01	mmu-miR-155-5p	4272.7	mmu-miR-326-3p	0.48	mmu-miR-155-5p	14139.0	mmu-miR-2137	0.01
mmu-miR-421-3p	93.67	mmu-miR-1949	0.01	mmu-miR-210-3p	285.39	mmu-miR-361-5p	0.34	mmu-miR-1933-5p	901.35	mmu-miR-362-3p	0.01
mmu-miR-1929-5p	74.93	mmu-miR-2134	0.01	mmu-miR-674-5p	171.63	mmu-miR-27a-3p	0.32	mmu-miR-210-3p	639.8	mmu-miR-484	0.01
		mmu-miR-2137	0.01	mmu-miR-331-3p	127.7	mmu-miR-221-3p	0.29	mmu-miR-574-3p	579.11	mmu-miR-152-3p	0.01
				mmu-miR-7a-5p	109.76	mmu-miR-23a-3p	0.26	mmu-miR-673-3p	518.4	mmu-miR-714	0.005
				mmu-miR-574-3p	96.8	mmu-miR-27b-3p	0.26	mmu-miR-674-5p	331.59	mmu-miR-107-3p	0.004
				mmu-miR-467a-5p	92.80	mmu-miR-762	0.23	mmu-miR-466a-5p	308.23	mmu-miR-324-5p	0.004
				mmu-miR-1929-5p	88.81	mmu-miR-1224-5p	0.22	mmu-miR-489-3p	284.88	mmu-miR-148b-3p	0.003
				mmu-miR-489-3p	87.81	mmu-miR-199a-3p	0.01	mmu-miR-1900	261.532095	mmu-miR-350-3p	0.002
				mmu-miR-139-5p	83.82	mmu-miR-145-5p	0.01	mmu-miR-1953	242.8512311	mmu-miR-762	0.001

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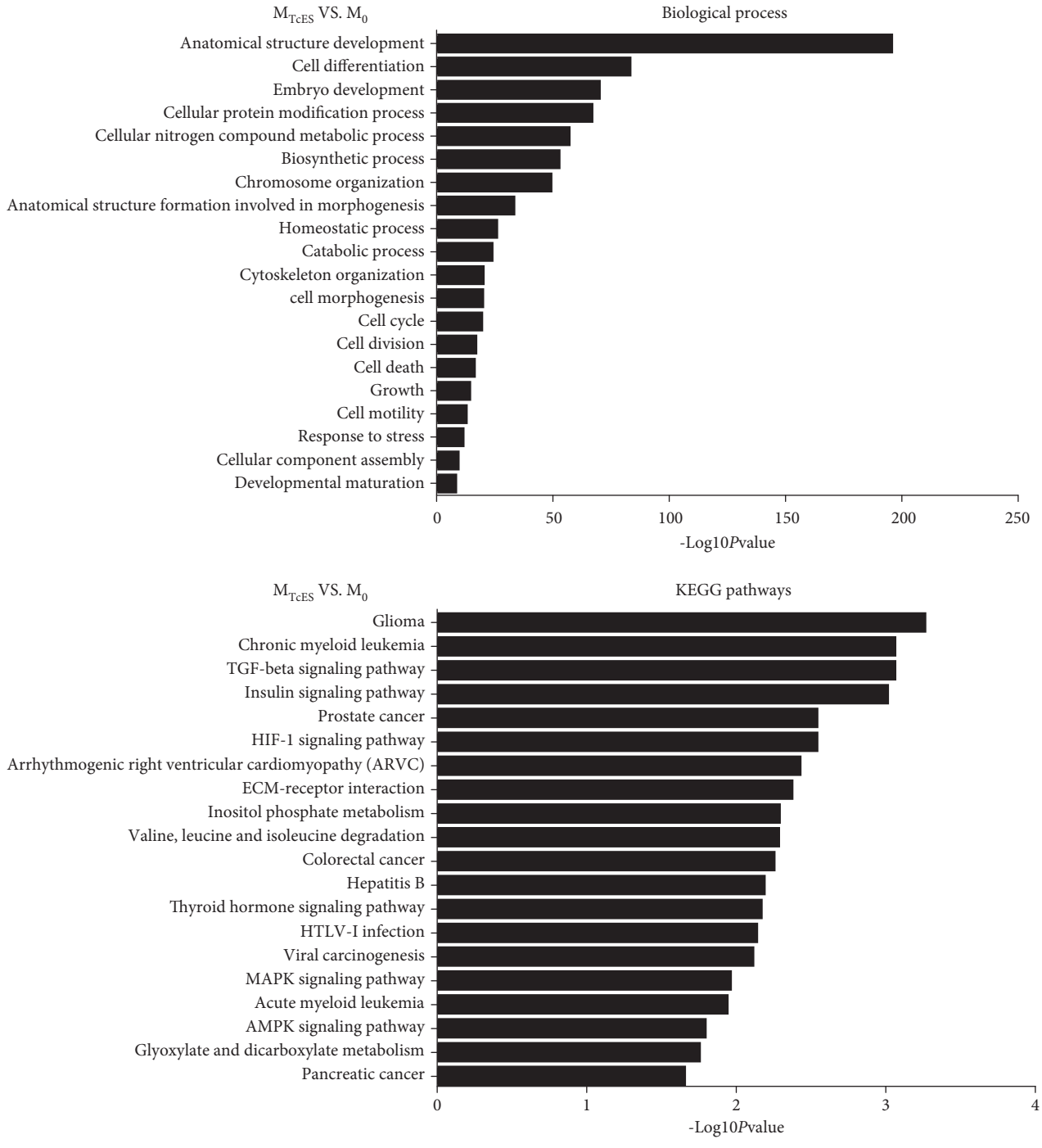
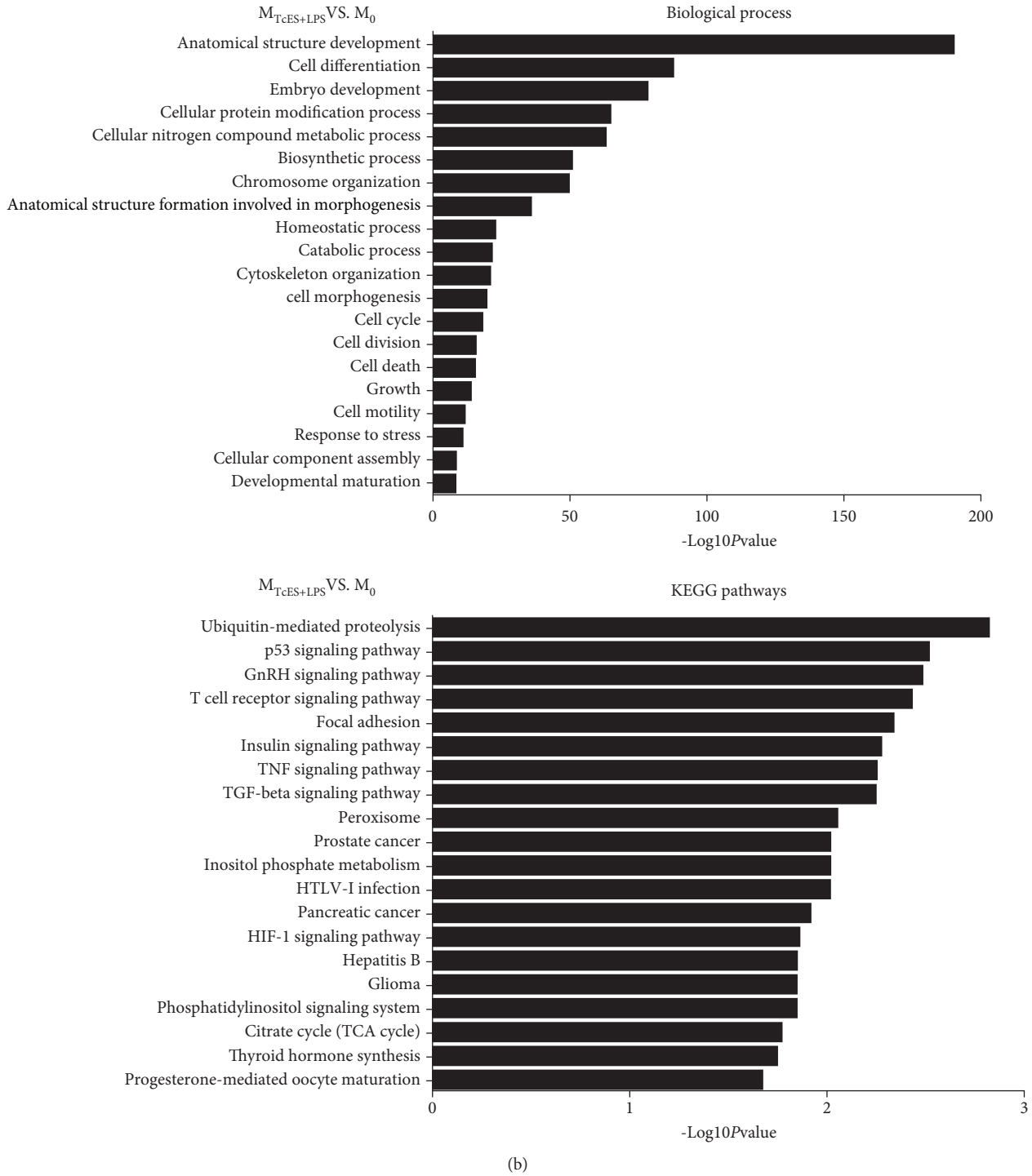


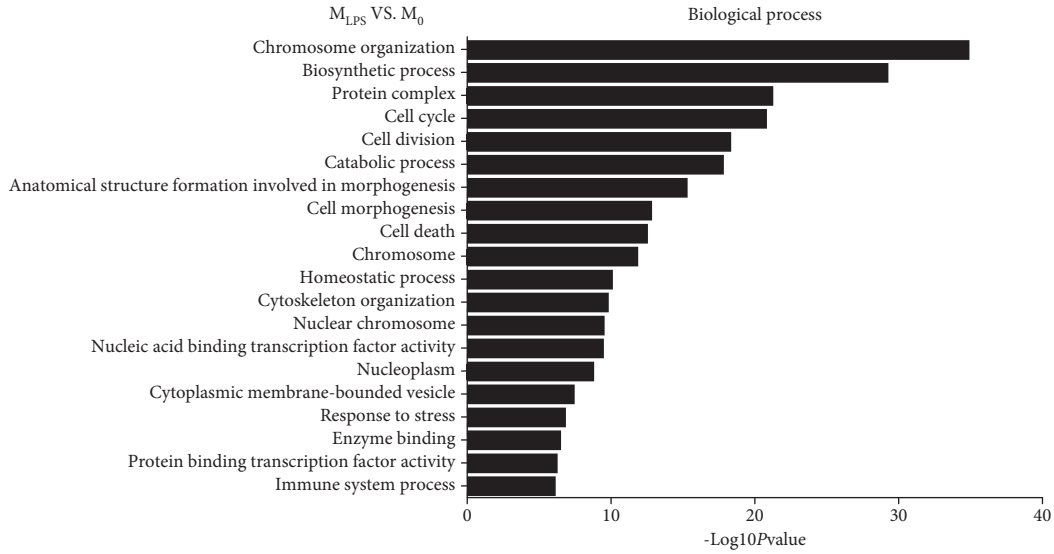
(a)

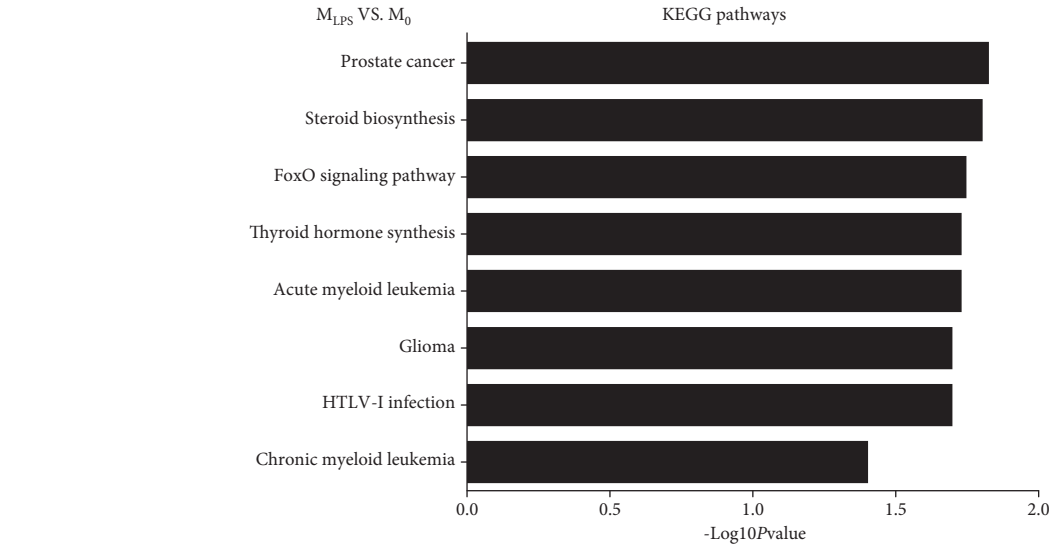
Figure 6: Continued.



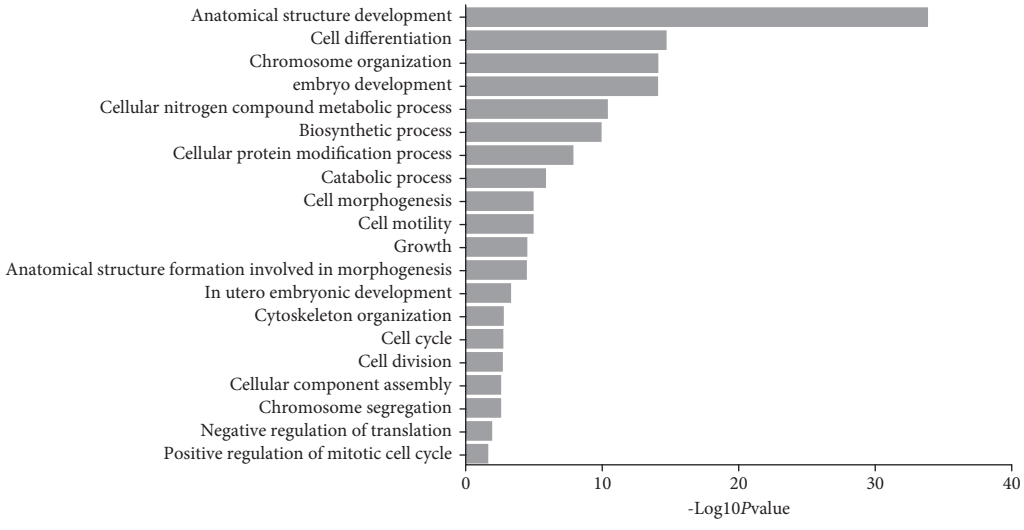
(b)

Figure 6: Continued.



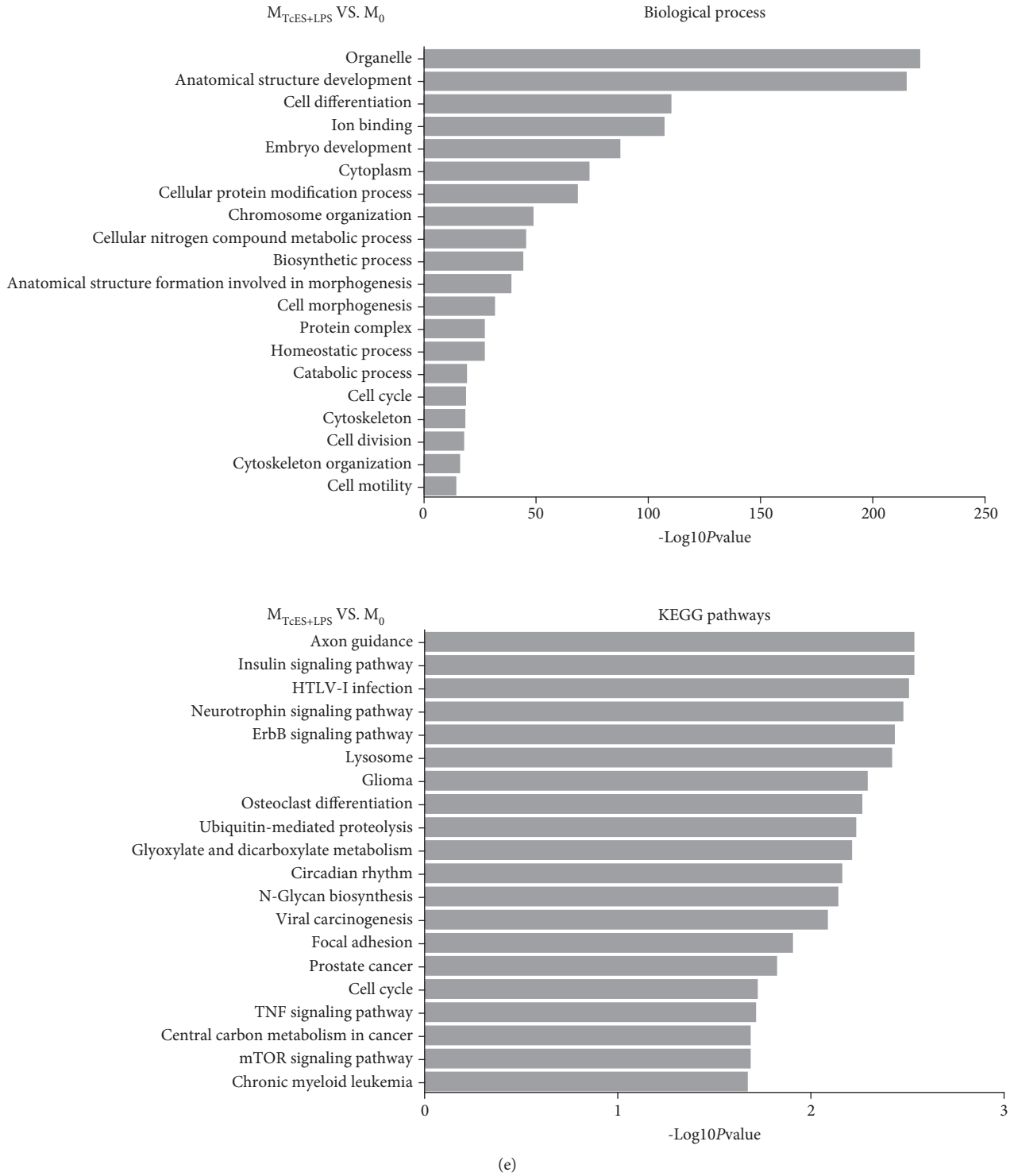


(c)



(d)

Figure 6: Continued.



(e)

Figure 6: Continued.

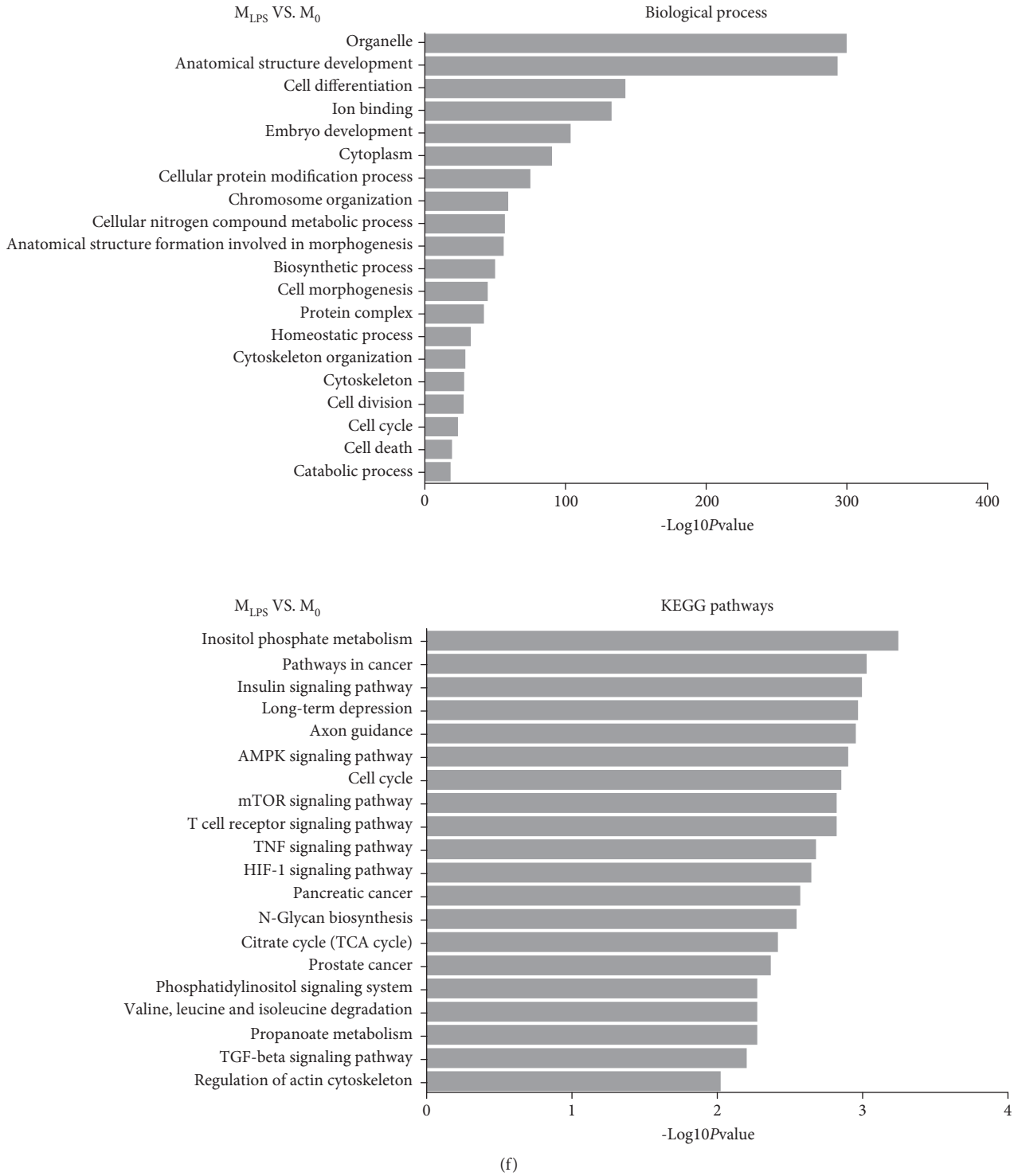


Figure 6: Top 20 of GO biological processes and KEGG pathways in M_{TcES}, $M_{TcES+LPS}$, and M_{LPS} at 4 (black) and 24 h poststimulus (gray). GO biological processes and KEGG pathways enriched by the upregulated differentially produced microRNAs between M_{TcES} vs. M_0 (a and d), $M_{TcES+LPS}$ vs. M_0 (b and e), and M_{LPS} vs. M_0 (c and f) at 4 h and 24 h poststimulus, respectively.

at 24 h post stimulus, which could be attributed to TcES's own recognition, as previously have been reported to recognize TcES [58]. These data suggest that posttranscriptional events may be involved in the regulatory mechanism triggered by TcES in regulating macrophage inflammatory responses.

microRNAs, small noncoding RNA molecules, have emerged as a key component of macrophage posttranscriptional regulation [59]. These molecules can silence the translation of mRNAs via base-pairing with complementary sequences within the RNA molecules. Hence, we further analyzed the microRNA profile in BMDM stimulated with

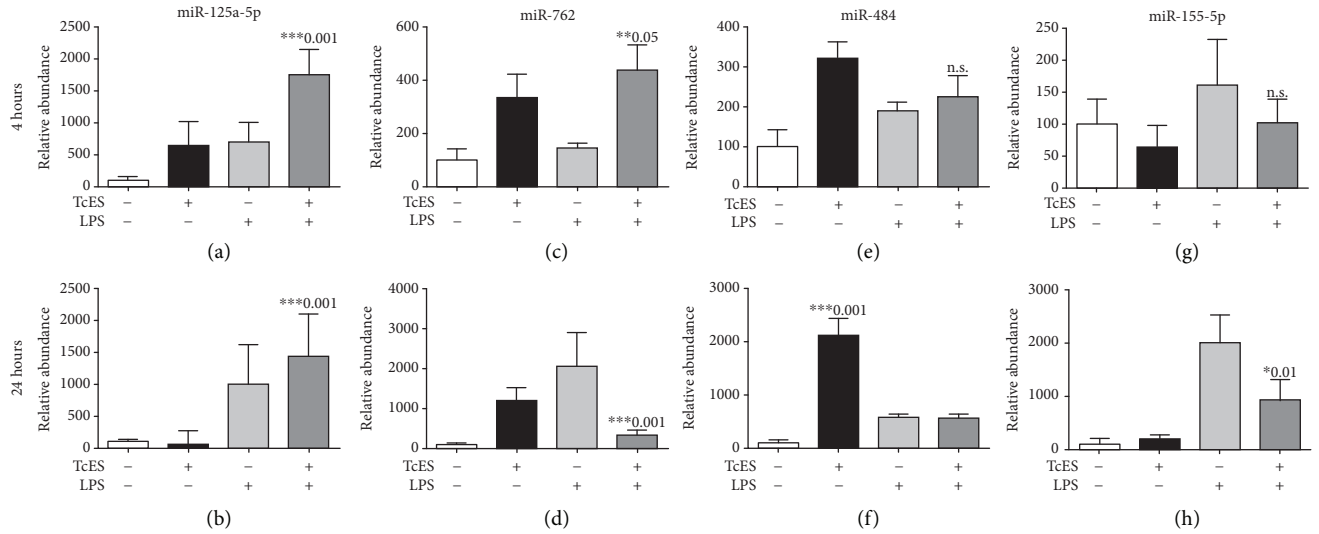


FIGURE 7: Validation and identification of microRNAs in stimulated BMDM. Macrophages were cultured in the presence of TcES (25 μg/mL), LPS (1 μg/mL), or a combination of TcES+LPS for 4 (left bar) and 24 h poststimulus (right bar). Relative levels of selected microRNAs were determined by TaqMan miRNA assays after normalization with 18S RNA. MicroRNA levels are represented as fold change relative to PBS-treated BMDM (FC ± SD). Data shown are representative of two independent experiments. Significance was calculated using t-test. $^{*}p < 0.01$, $^{**}p < 0.05$, and $^{***}p < 0.001$.

TcES alone or in combination with LPS. Our analysis demonstrated that $M_{TcES+LPS}$ shared regulatory microRNAs with M_{LPS}. For example, miR-146a-5p was upregulated in $M_{TcES+LPS}$ and M_{LPS} at 4 h and only in $M_{TcES+LPS}$ at 24 h poststimulus. This microRNA has been reported to dampen proinflammatory responses in macrophages through the inhibition of TLRs, NF-κB, and STAT signaling pathways by targeting the mRNAs of *Traf6*, *Irak1*, *Irak2*, *Nfκb*, *Stat1*, and *Ap1* [60–63]. This evidence is supported by our KEGG enrichment analysis, which indicates that overproduced microRNAs in $M_{TcES+LPS}$ target mRNAs involved in NF-κB, TNF, and MAPK signaling pathways. Of note, these data also confirm our hypothesis that TcES target proinflammatory pathways and support our previous findings indicating a role for TcES in blocking the IFN-γ/STAT1 signaling pathway in macrophages *in vitro* [17].

$M_{TcES+LPS}$ also overproduced microRNAs previously reported to target inflammatory mRNAs; for instance, let-7i and let-7e target *Tlr4* mRNA, which causes a drop in the recognition of proinflammatory antigens [64–66]. Moreover, miR-24-3p production in macrophages has been reported to significantly decrease the production of IL-6 and TNFα [67]. Furthermore, $M_{TcES+LPS}$ and M_{TcES} shared upregulated microRNAs previously reported to be elicited in macrophages exposed to *E. multilocularis* antigens (e.g., miR-146a-5p) and *S. japonicum* (miR-365 and miR-24) [14, 68]. These data suggest the presence of conserved antigens among helminths that could trigger similar posttranscriptional mechanisms to modulate immune responses in the host.

Finally, we selected four upregulated microRNAs to validate their levels by RT-qPCR and confirm the high quality of our array. We observed increased levels of miR-125a-5p in M_{TcES} and M_{LPS}, as early as 4 h poststimulus. The combined stimulus with TcES and LPS induced an additive effect in the levels of this microRNA. miR-125a-5p has been reported to increase after TLR2/4 signaling and has a key role in reducing the production of inflammatory cytokines (IL-6, IL-12, and TNFα) by targeting NF-κB and KFL4 signaling pathways [24, 69–71]. These data are associated with our previous studies suggesting that TcES is a ligand of TLR2 in phagocytic cells [58, 72]. In addition, miR-762 was selectively induced in M_{TcES} and $M_{TcES+LPS}$ at 4 h poststimulus. miR-762 has been demonstrated to increase in ovarian and breast cancer and ocular tissue [73–75] where macrophages normally acquire an M2-like phenotype [76, 77]. Furthermore, by using bioinformatic tools, we found *Il12b*, *Il6, Tnf*, *Nfkb*, and *Cd86* mRNAs as possible targets of miR-762 in M_{TcES} and $M_{TcES+LPS}$. The microRNA miR-484 was found to be upregulated in all the groups of stimulated BMDM at 4 h; however, its levels were only sustained in M_{TcES} at 24 h poststimulation. miR-484 has been previously identified in multiple types of cancers [78–82] and the cerebral cortex [83]; such microenvironments are known to promote an anti-inflammatory phenotype in macrophages. Our bioinformatic analysis shows that *Il1b*, *Nfkb*, *Stat5a*, *Irf1*, *Myd88*, *Stat1*, and *IL-12a* mRNAs are possible targets for miR-484, which suggest a possible role for miR-484 in immune tolerance.

Lastly, we observed that miR-155-5p was upregulated in M_{LPS} at 4 h and $M_{TcES+LPS}$ and M_{LPS} at 24 h poststimulus. miR-155-5p is a well-defined microRNA induced by LPS in macrophages, which enhances the proinflammatory response by targeting the immunomodulatory mRNAs *Ship1*, *Socs1*, *Il13rα*, and *C/ebpβ* and increasing the half-life of *Tnf* [64, 84–88]. However, antigens of another helminth, *Angiostrongylus cantonensis*, also upregulated miR-155-5p [89]. Therefore, it would be of interest to further study the role of miR-155-5p during exposure to helminth antigens.

In summary, our study demonstrates a role for TcES in regulating the production of key inflammatory cytokines, possibly by inducing microRNAs that target inflammatory

transcripts and promoting the release of IL-10 in macrophages. This phenomenon shapes the transcriptomic profile of macrophages and consequently the outcome of the immune response. Although we found clear associations between TcES-induced microRNAs and mRNAs involved in multiple inflammatory pathways as their targets, our study has the limitation that we did not prove a direct interaction between microRNAs and mRNAs. Therefore, future studies in our laboratory will focus on elucidating the functional roles and significance of the different microRNAs described here. These findings increase our understanding of how released molecules from helminths regulate inflammation and may offer new approaches for the treatment of autoimmune and inflammatory diseases.

Abbreviations

M2: Alternatively activated-like macrophages
Arg1: Arginase 1
EAE: Autoimmune encephalomyelitis
BMDM: Bone marrow-derived macrophages
M1: Classical activation in macrophages
DAMPs: Danger-associated molecular patterns
DMEM: Dulbecco's modified Eagle's media
FBS: Fetal bovine serum
FC: Fold change (FC)
Fizz1: Found in inflammatory zone
GO: Gene ontology
HNP1: Human neutrophil-α defensin
IL-4Rα: IL-4 receptor α
IGFBP5: Insulin-like growth factor binding protein 5
IL-10: Interleukin-10
IL-12: Interleukin-12
IL-4: Interleukin-4
IL-6: Interleukin-6
KEGG: Kyoto Encyclopedia of Genes and Genomes
LPS: Lipopolysaccharide
M-CSF: Macrophage colony-stimulating factor
MHC: Major histocompatibility complex
MR: Mannose receptor
mRNAs: Messenger RNAs
NO: Nitric oxide
Nos2: Nitric oxide synthase
PAMPs: Pathogen-associated molecular patterns
PD-L2: Programmed death ligand 2
TcES: *Taenia crassiceps*-excreted/secreted antigens
TLR: Toll-like receptor
TNFα: Tumor necrosis factor α
UTR: Untranslated region.

Data Availability

The array data used to support the findings of this study have been deposited in the GEO (Gene Expression Omnibus) database of the NCBI with the accession numbers GSE125170 for RNAm and GSE125171 for microRNA as part of the SuperSerie GSE125172 which are public once this article is published.

Conflicts of Interest

The authors have no financial or other conflicts to declare.

Acknowledgments

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Supplementary Materials

Table S1: sequence of primers used in RT-qPCRs to amplify the specified mRNAs (A) or microRNAs (B). Table S2: mRNAs commonly upregulated (≥2-fold change) in BMDM with TcES (MTcES), LPS (MLPS), or TcES+LPS (MTcES +LPS) at both 4 (left) or 24 h (right) poststimulus. Table S3: transcripts up (A) or downregulated (B) at 4 or 24 h poststimulus with LPS (MLPS). Table S4: transcripts up (A) or downregulated (B) at 4 or 24 h poststimulus with TcES (MTcES). Table S5: transcripts up- (A) or downregulated (B) at 4 or 24 h poststimulus with TcES+LPS (MTcES +LPS). Table S6: mRNAs expressed differently (≥2 FC) between BMDM stimulated with TcES+LPS (MTcES+LPS) and with LPS (MLPS) at 4 (left) or 24 h (right). Table S7: microRNAs up- (A) or downregulated (B) at 4 or 24 h poststimulus with LPS (MLPS). Table S8: microRNAs up- (A) or downregulated (B) at 4 or 24 h poststimulus with TcES (MTcES). Table S9: microRNAs up- (A) or downregulated (B) at 4 (left) or 24 h (right) poststimulus with TcES+LPS (MTcES+LPS). Table S10: microRNAs upregulated (≥2-fold change) in BMDM with TcES (MTcES), LPS (MLPS), and TcES+LPS (MTcES+LPS) at both 4 (left) or 24 h (right) poststimulus. Table S11: microRNAs expressed differently (≥2 FC) between BMDM with TcES+LPS (MTcES+LPS) and with LPS (MLPS) at 4 (left) or 24 h (right) poststimulus. Table S12: KEGG pathway analysis. Significantly enriched KEGG pathways ($p < 0.05$) are presented for BMDM with TcES (MTcES), TcES+LPS (MTcES+LPS), or LPS (MLPS) at 4 (left) or 24 h (right) poststimulus. Table S13: GO biological process annotations of the target genes of differentially expressed microRNAs. According to p value, GO terms of biological process are shown in BMDM with TcES (MTcES), TcES+LPS (MTcES+LPS), or LPS (MLPS) at 4 (left) or 24 h (right) poststimulus. *(Supplementary Materials)*

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Research Article

Human Papillomavirus Type 16 Disables the Increased Natural Killer Cells in Early Lesions of the Cervix

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The mechanism for pathogenesis of human papillomavirus (HPV) in the cervix has been investigated intensively. However, detailed differences in the distribution and function of innate immune cells between high-risk HPV types, especially during the chronic inflammation phase, have not been described fully. In this study, histologic pathology results of 245 women with HPV type 16 only ($HPV16^+$) or type 18 only ($HPV18^+$) were analyzed retrospectively from January 2015 to November 2016. More severe lesions of the cervix were observed in $HPV16^+$ women compared with those in $HPV18^+$ women. In total, 212 cervical brush specimens were collected from women suffering from chronic inflammation, $HPV16^+$, or $HPV18^+$ from December 2016 to December 2018. Flow cytometry analysis showed that abundant NK cells along with aberrant Treg cells were found in the HPV16-infected cervix. Quantitative real-time PCR demonstrated that higher expression levels of IFN-γ but muted IL-2 and KLRG-1 expression was detected in the cervix of patients with $HPV16^+$ compared to $HPV18^+$, which were further confirmed using 20 paraffin sections of cervical conization tissue. The ex vivo cytotoxicity experiment showed that the cytotoxicity of NK cells was significantly decreased in the cervix of $HPV16^+$ patients compared with that of $HPV18^+$ patients. Collectively, our results suggested that HPV16 disables the increased NK cells in the early lesion of the cervix, indicating that the local immune system of the cervix is hyporesponsive to HPV16 infection and this may explain its bias for malignant transformation.

1. Introduction

Based on the most recent data, the immune system of most people infected with HPV needs at least three years in order to eliminate the virus. Apart from an inadequate humoral immune response, suppression of immune cells in the local cervical environment is another major reason for these statistics [1]. HPV invades vaginal squamous epithelium as well as undifferentiated basal cells within the cervical canal epithelium transitional zone [2]. So far, more than 100 HPV types have been identified, among which 15 types (such as HPV types 16, 18, 31, and 45) are considered high-risk types which are more likely to be carcinogenic [3]. A recent worldwide comprehensive survey indicated that up to half of cervical cases is caused by HPV type 16 (HPV16), and HPV16 and HPV type 18 (HPV18) together lead to up to 80% [4, 5].

Studies have provided solid evidence on the molecular mechanisms by which HPV infiltrates local immune cells. NK cells are important biological barriers which are resident in the cervix. They can identify virus-infected or virus-transformed cells rapidly through pathways that do not require presensitization and kill them. It has been reported that NK cells emerge at an early stage in HPV-infected lesions [6]. However, HPV can force activate NK cells to lose their membrane receptors thus leading to their malfunction and this can lead to carcinogenesis [7]. A reversal of the $CD4^+/CD8^+$ ratio and a marker increase in the number of Treg cells, which are the representative immunosuppressive

cells, have also been shown in the crosstalk between cervical cancer cells and the virus [8, 9]. Nevertheless, there is no report so far focusing on the profiles of these infiltrated immune cells in tissues infected with different HPV types. The purpose of this study was to compare the distribution and functional status of innate NK cells in tissue infected with two high-risk HPV types (16 and 18) and to discuss the possible mechanisms of HPV-original disease from a different perspective.

2. Materials and Methods

2.1. Patient Characteristics. Eligible patients were married women aged between 25 and 65 without histologically or cytologically confirmed other diseases originating from the reproductive system (including the vagina, uterus, and ovary) but with cervical chronic inflammation only. This research was authorized by the Ethical Committee of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China).

2.2. Sample Collection. We used a speculum to expose the cervical canal, and the coherent mass of tissue was cleared with a cotton swab. A cervical brush was inserted into the cervical canal to approximately 1-1.5 cm and was gently rotated in the same direction for 5 times. Then, the cervical brush was slowly extracted, and the exfoliated cells were preserved in a Roche® sample collection tube for type detection or in 1640 cell culture medium containing 10% FBS. In total, 212 cervical brush specimens were collected for flow cytometry analysis, ex vivo cytotoxicity detection, and quantitative real-time PCR from December 2016 to December 2018.

2.3. HPV DNA Detection and Type Identification. HPV DNA detection and type identification were performed using a cobas® x480 nuclear acid extraction system and a cobas® z480 analyzer via a cobas® 4800 system liquid cytology preparation kit according to the manufacturer's instructions (Roche, USA). Specimens with HPV16 or HPV18 were, respectively, preserved as the $HPV16^+$ group or $HPV18^+$ group. Specimens derived from women with chronic inflammation and HPV negative were used as controls.

2.4. Fluorescence-Activated Cell Sorting (FACS). Preserved samples were washed three times with PBS before passing through a 40 μm strainer filter twice (STEMCELL Technologies, Canada). Remaining cells were conjugated to CD3, CD4, CD56/CD16 (Beckman Coulter), and either CD16 or CD25 antibodies in combination (Biolegend, USA) and subjected to flow cytometry (CD56/16 with Beckman, others with BD FACSCalibur, USA). The FACS results show the percentages of each indicated immune cell type.

2.5. RNA Extraction and Quantitative Real-Time PCR. The total RNA of cervical brush specimens was extracted using a TRIzol Reagent (Grand Island, USA) according to the manufacturer's instructions. Twenty paraffin sections of cervical conization tissue were subjected to quantitative real-time PCR. The unstained sections were deparaffinized using a standard xylene and ethanol procedure. RNA was extracted using RNeasy FFPE Kits (QIAGEN) as per the manufacturer's instructions.

The reverse transcription reaction from 1 μg of RNA template was carried out using a First Strand cDNA Synthesis Kit (TOYOBO, Japan). Quantitative real-time PCR was performed using SYBR Green Real-time PCR Master Mix (TOYOBO, Japan) and detected by the LightCycler 480 II real-time PCR system (Roche, USA). The expression level was analyzed and normalized to β-actin in the cDNA samples. The fold change of gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences are listed in Table 1.

2.6. Ex Vivo Immune Cell Cytotoxicity Experiments. The cervical cancer cell line HeLa cells were labeled with CFSE (0.5 μM, Invitrogen). $CD56^+CD16^+$ NK cells were isolated from cervical brush specimens of women infected with HPV16 or HPV18 via FACS (BD FACSAria II, USA). NK cells were cocultured with CFSE-HeLa in designated ratios in 24-well plates and incubated at a 37°C incubator for 4-6 hours. Cells were harvested, stained with 7-AAD (BD Pharmingen, USA), and subjected to flow cytometry (BD FACSCalibur, USA). Data were analyzed using the FlowJo 6.0 software (Tree Star).

2.7. Statistical Analysis. Statistical analyses were performed using PRISM 7.01 (GraphPad Software Inc., San Diego, CA). Data are presented as the means ± SD. All data were analyzed using Student's t-test, where $p < 0.05$ was considered statistically significant. The Bonferroni correction was used for multiple comparisons.

3. Results

3.1. Overall Histologic Pathology Profiles of HPV Type-Infected Women. We retrospectively analyzed the histologic pathology results of 245 HPV16- or HPV18-infected women from January 2015 to November 2016. As shown in Table 2, more severe lesions (≥CIN 2) were statistically presented in women with HPV16 infection when compared with $HPV18^+$ women.

3.2. NK Cell Infiltration in a HPV16-Infected Cervix. Both HPV16 and HPV18 are categorized into high-risk HPV types that have a strong probability to cause cervical invasive carcinoma. We wondered whether the distribution patterns of $CD56^+CD16^+$ representing NK cells were similar between these two types of virus. In total, 48 cervical brush specimens including 16 chronic inflammation controls, 16 $HPV16^+$ cases, and 16 $HPV18^+$ cases were collected. As shown in Figure 1(a), compared to chronic inflammation control, $CD56^+CD16^+$ NK cells were upregulated in both $HPV16^+$ and $HPV18^+$ cervical surfaces. Interestingly, the ratio was even more abundant in the $HPV16^+$ group than that in the $HPV18^+$ group ($CD56^+CD16^+$ NK cells 2.13 ± 0.38% vs. 0.53 ± 0.23%; $p < 0.01$). Cell smear inspection also showed a relatively high density of fried egg-like NK cells in the $HPV16^+$ group.

The second batch of cervical brush specimens consisted of 25 chronic inflammation controls, 22 $HPV16^+$ cases, and

Table 1: Quantitative real-time PCR primers used in this study.

Gene name	Primer
IFN-γ	F: gagtgtggagaccatcaagg
	R: cgacagttcagccatcactt
IL-2	F: gccacagaactgaaacatct
	R: gccttacctttagttccagaa
IL-6	F: agatttgagagtagtgaggaa
	R: actgtctttgagcctgtctt
GM-CSF	F: gccactacaagcagcactg
	R: tgtctgcctcctctctgga
CCL-3	F: taactcttcctcccttctcc
	R: tggacccctcaggcactca
CCL-5	F: atcctccctcttcttctcct
	R: ttcaggttcaaggactctcc
KLRG-1	F: tcaactccttttctgtgcatg
	R: catctatcaaagtctgacctt
CD56	F: atgatgggtgaagagaaccg
	R: aatgagatgtgtgtgtgtgc
CD27	F: gccttcagatgtgccctat
	R: cagtgggtagagagagtcc
DNAM-1	F: gaagtcccatctctaccagt
	R: agcttaaactctagtctttgg
β-Actin	F: tcatgaagtgtgacgtggacat
	R: ctcaggaggagcaatgatcttg

Table 2: Characteristics of HPV16 or HPV18 infections correlated to the severity cervical lesions.

Characteristics	HPV16$^+$	HPV18$^+$	P value
Average age (years)	40.0 ± 9.7	40.8 ± 9.6	0.8055
Histological diagnosis			<0.0001
Inflammation	54 (26.6)	27 (64.3)	
CIN 1	22 (10.8)	8 (19.0)	
CIN 2	29 (14.3)	3 (7.1)	
CIN 3	98 (48.3)	4 (9.5)	
Total	203	42	

CIN: cervical intraepithelial neoplasia; HPV: human papillomavirus.

15 HPV18$^+$ cases that were collected to evaluate six soluble cytokines that represented the NK cell-involved immune status in the cervical microenvironment. As shown in Figure 1(b), IFN-γ transcription level was relatively higher in the HPV16$^+$ group than that in the HPV18$^+$ group, which was consistent with elevated numbers of local NK cells. But despite the viral types, HPV infection induced a decreasing trend of IFN-γ transcription at the cervical surface. IL-2 is an important cytokine in microbial infection and bridges the body's innate and adaptive immune systems. We found that IL-2 levels were significantly lower in the HPV16$^+$ group indicating that the local immune response failed to be further stimulated by virus infection. On the contrary, HPV18 infection could. Other tested cytokines such as IL-4, GM-CSF, CCL-3, and CCL-5 showed no significant difference between infections with the two HPV types. To verify these changes in cervical brush specimens, we again examined women who were pathologically diagnosed with cervical chronic inflammation and collected paraffin sections of cervical conization tissue from those infected with HPV16 (10 cases) or HPV18 (10 cases). As shown in Figure 1(c), the trends of the IFN-γ and IL-2 expression difference between the two groups were identical. Taken together, the HPV16$^+$ cervix is characterized by the increased population of NK cell proportion and a distinguished cervical microenvironment.

3.3. NK Cells Exert a Discorded Effect in a HPV16$^+$ Cervix. We also tested four typical cell membrane markers of NK cells in the second batch of specimens. KLRG-1 is upregulated in cells designated as vibrant NK cells possessing a cytotoxicity effect. But its expression was dull in the HPV16$^+$ group (Figure 2(a)), indicating that it fails to respond when the virus is mature and proceed to the next biological cycle at the cervical surface. This result was also confirmed in the paraffin sections (Figure 2(b)). CD56 transcription levels of both HPV groups were similar or slightly higher than control (Figure 2(a)). This does not agree with the result from flow cytometry studies (Figure 1(a)). Considering that the combined CD56/CD16 antibody used in Figure 1 was designed for clinical use only, we chose another different commercial CD16 antibody. The flow cytometry analysis of the third batch of cervical brush specimens (16 cases in each group) showed that CD16$^+$ cells were markedly increased in HPV16$^+$ group (HPV16$^+$0.693 ± 0.507% vs. control 0.043 ± 0.037% and HPV18$^+$0.077 ± 0.083%; Figure 2(c)).

Treg cells play an important role in "self-checking" the regulatory immune response, and there is a trade-off between them and NK cells which is believed to have a great contribution towards HPV-induced carcinogenesis [10]. To investigate whether increased infiltration of NK cells implied Treg recession, 48 cervical brush specimens were analyzed by flow cytometry. As expected, the CD4$^+$CD25$^+$ Treg cell ratio in the HPV16$^+$ group was the lowest among the three groups (HPV16$^+$1.30 ± 0.28% vs. control 6.70 ± 0.85% and HPV18$^+$4.64 ± 0.73%; Figure 2(d)). The cytotoxicity of NK cells extracted from 3 HPV16$^+$ women and 3 HPV18$^+$ women was determined. As shown in Figure 3, the ratio of CFSE$^+$7-AAD$^+$-labeled cancer cells failed to further elevate when ten-fold ex vivo NK cells were added for coculture, indicating that infiltrated NK cells do not exert an equivalent function to eliminate transformed cells.

4. Discussion

The aim of this study was to prospectively study the distribution patterns of infiltrated immune cells, in particular NK cells, and HPV16- and HPV18-infected cervical surface microenvironment and to elucidate the possible high-risk HPV pathogenic mechanism from a new prospective by highlighting the functional cell discrepancies between these two types. We found that CD16$^+$ NK cells were abundant in the HPV16$^+$ group of women with more IFN-γ secretion

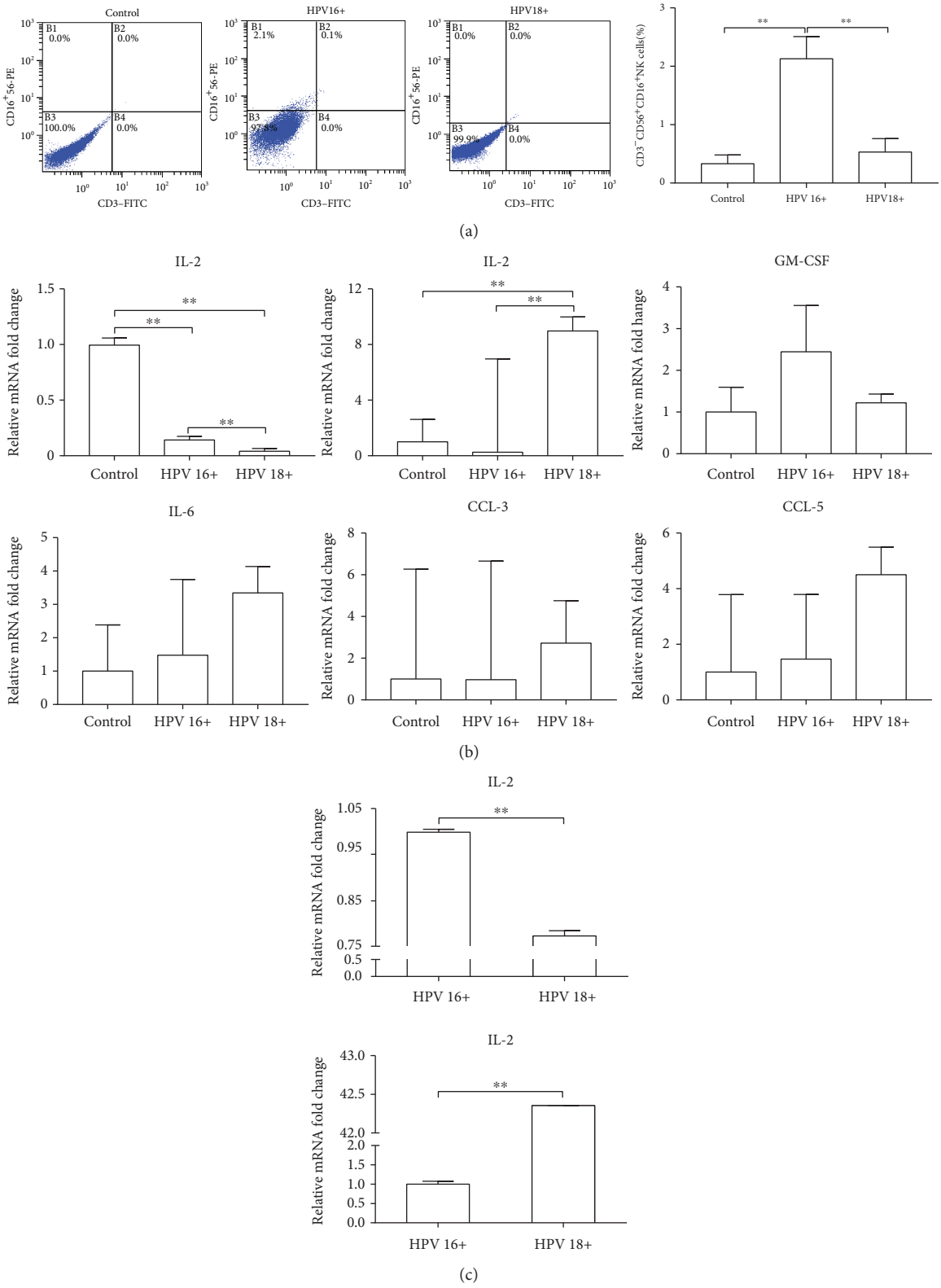

Figure 1: The differences of NK cells between HPV16- and HPV18-infected cervixes. (a) Distribution of $CD56^+CD16^+$ NK cells was analyzed in the cervical brush specimens by FACS. (b) Several soluble cytokines that represent NK cell involvement in the immune status were investigated in the cervical brush specimens by quantitative real-time PCR. (c) IFN-γ and IL-2 expressions were measured in the cervical conization tissue by quantitative real-time PCR. $^*P < 0.05$; $^{**}P < 0.01$.

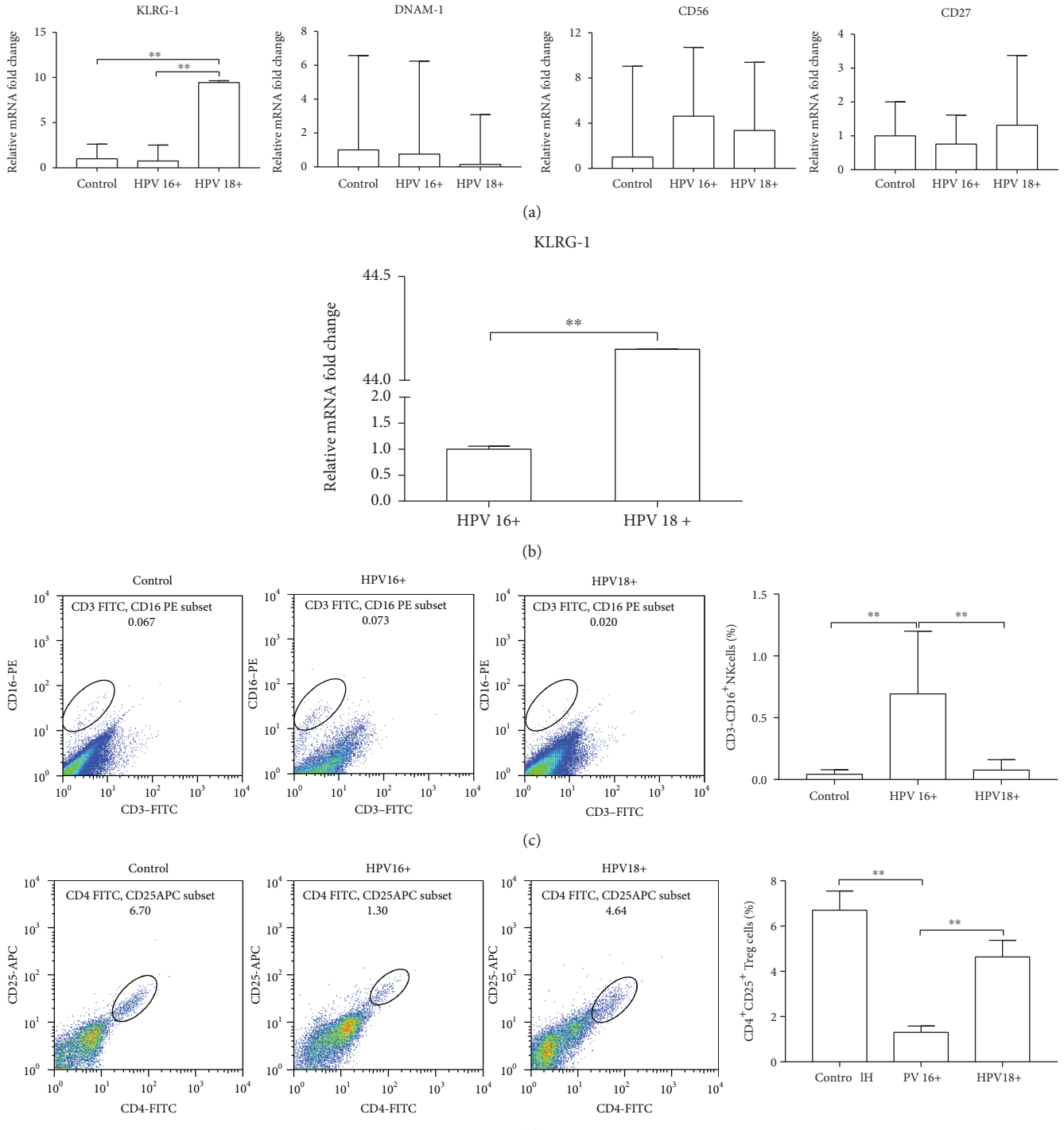

Figure 2: The different effects of NK cells between HPV16- and HPV18-infected cervixes. (a) Four typical cell membrane markers of NK cells were evaluated in the cervical brush specimens by quantitative real-time PCR. (b) KLRG-1 expression was measured in the cervical conization tissue by quantitative real-time PCR. (c) Distribution of $CD16^+$ NK cells was analyzed in the cervical brush specimens by FACS. (d) Distribution of $CD4^+CD25^+$ Treg cells was analyzed in the cervical brush specimens by FACS. $^*P < 0.05$; $^{**}P < 0.01$.

observed. CD16 is a vital membrane protein found on NK cells that can induce HPV-VLP endocytosis followed by degranulation and cytokine secretion (such as IFN-γ and tumor necrosis factor (TNF)) [6, 11]. Engagement of CD16 on NK cells results in its ADCC activation [12, 13]. There results imply that the chronic inflammation-induced antibody-dependent pathway is active in cervical NK cells.

However, these increased NK cells still failed to eliminate the mature HPV virus under circumstances that Treg cells were locally restricted. It is probably due to inadequate IL-2

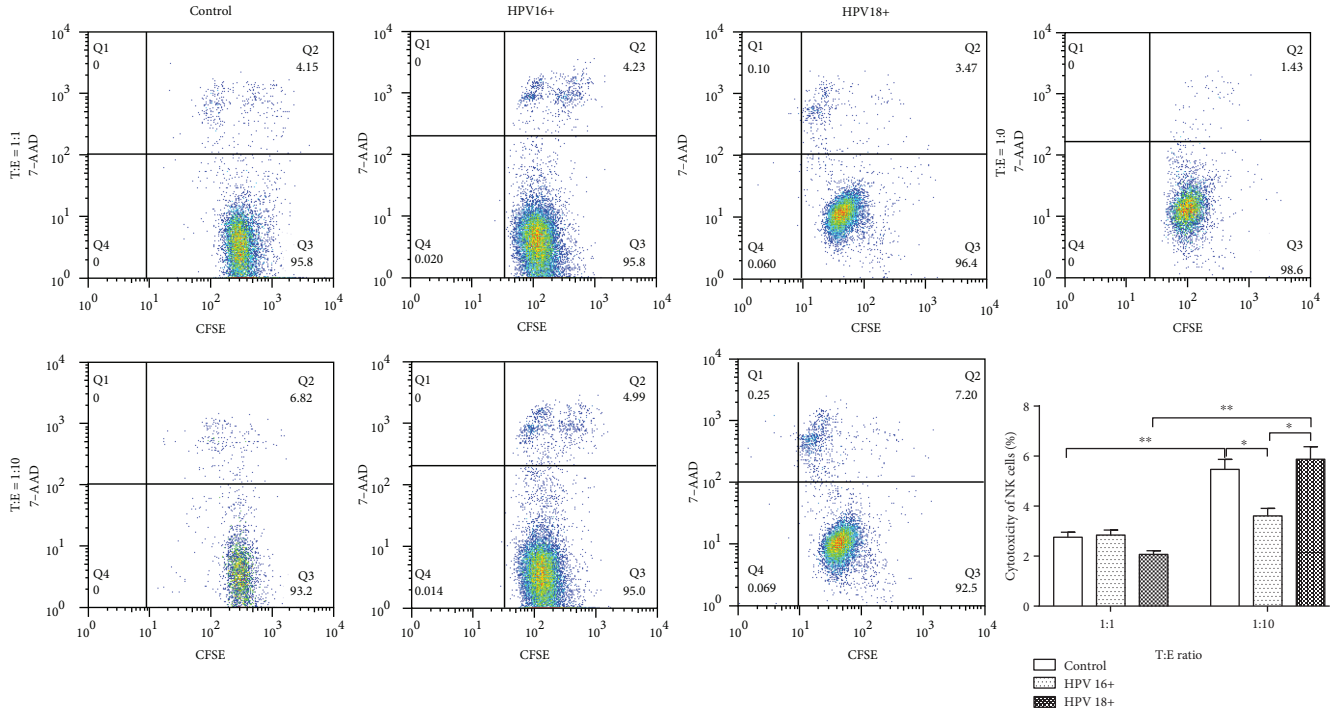

Figure 3: The cytotoxicity of NK cells derived from HPV16$^+$ or HPV18$^+$ women using HeLa cells labeled with CFSE. CD56$^+$CD16$^+$ NK cells were isolated from cervical brush specimens of women infected with either HPV16 or HPV18, respectively, via FACS. NK cells were cocultured with CFSE-HeLa in designated ratios in 24-well plates and incubated at a 37°C incubator for 4-6 hours. Cells were harvested and stained with 7-AAD. CFSE$^+$7-AAD$^+$ cells were examined by FACS. $^*P < 0.05$; $^{**}P < 0.01$.

production which is reported to positively regulate NK cell cytotoxic function and downstream differentiation [14], as well as virus-induced KLRG-1 expression. KLRG-1 belongs to the C-lectin superfamily and is known to be an inhibitory receptor of NK cells. KLRG-1$^-$ NK cells proliferate well, but they lack a mature phenotype [15]. Nevertheless, recent researches have emphasized that "NK cell education" which is part of its functional modification is mainly conducted via the ITIM/SHP1/SHP2/SHIP signaling pathway [16]. KLRG-1 is associated with the recruitment of SHIP and the KLRG/SHIP disturbance renders NK cells hyporesponsive and uneducated [17, 18]. Since effector CD8$^+$ T cells also express KLRG-1, we cannot rule out a role for exhausted CD8$^+$ T cells in an HPV-infected cervix [19]. We also found that HPV infection decreased IFN-γ transcription by immune cells, and this was consistent with other studies. It has been reported that the early gene products, E6 and E7, regulate local cytokine and chemokine expression, for instance the downregulation of IFN-γ and other components related to the signal transduction pathway, against virus infection [20].

Our study has some limitations: (1) cytokines were tested using quantitative real-time PCR, and this can be problematical. Some researchers collected fluid by cervicovaginal lavage in order to detect secretory proteins by ELISA. This is only feasible if vaginal diseases are first excluded; (2) the number of specimens collected was small. We enrolled women infected with either HPV16 or HPV18, but a large proportion of HPV women had a combined infection along with other low-risk types. In particular, the incidence of HPV18 itself was low in Eastern China. A future work will be focusing on evaluating other candidate cytokines (which in the present study showed a trend of change but with no statistical significance) such as IL-6, GM-CSF, CCL-5, and membrane protein DNAM-1 in HPV infection; (3) in the mixing of specimens for flow cytometry analysis, according to Hubert's study [21], the number of NK cells in cervical biopsies was too low to score, and they could only be counted in the stroma. Therefore, 4 specimens of different patients were mixed to produce a sample for flow cytometry analysis. Each group was repeated independently for four times.

One of the cervical cancer-preventive HPV vaccines—Cervarix™—is currently authorized for use in Europe and the United States. It is composed of viral components from the later period of gene expression, such as product L1 of HPV16 and HPV18 and an adjuvant, AS04. Although it has certain positive effects on the prevention of viral reinfection, its low concentration in the cervical environment is still an annoying problem and cannot be ignored. More importantly, it is far away from the curing the disease [22]. Another medicinal product used against high-risk HPV types in Canada is a quadrivalent HPV vaccine (Gardasil, Merck) which was recently proved to be 30-50% effective against lesions caused by all HPV types [23]. Over the past decade, many researchers have devoted time and effort into developing a therapeutic vaccine. Some have made gratifying results in animal models, but these vaccine applications are still confined to the laboratory. Clinical observations also suggest that antibodies in patients with persistent infection are mostly negative. Therefore, it is questionable whether humoral

immune status could be used as an indicator of regression for any infection. The efficacy of therapeutic vaccines is closely related to the T cell response, Th1 cytokine secretion, local infiltration of $CD4^+$ and $CD8^+$ T cells, and the function of NK cells in the cervix [24]. Based on this study, we raise another question that will such therapeutic vaccines possess equal capability for killing cervical cancer cells previously infected by either HPV16 or HPV18? We believe that drugs/-vaccines targeting specific HPV type will be a new promising direction for HPV treatment.

5. Conclusions

In summary, our data demonstrated that the number of NK cells was increased, but their cytotoxic function was abnormal in an HPV16-infected cervix. This is the first study emphasizing the unique immune profiles of the cervical microenvironment between two high-risk HPV types. The involved mechanisms may partially reveal the reason why HPV16 is the most likely to cause cervical cancer and may provide new potential strategies for its clinical management.

Abbreviations

HPV: Human papillomavirus
CIN: Cervical intraepithelial neoplasia
NK cell: Natural killer cell
IFN-γ: Interferon-γ
IL: Interleukin
GM-CSF: Granulocyte-macrophage colony-stimulating factor
CCL: CC-chemokine ligand
KLRG-1: Killer cell lectin-like receptor subfamily G member 1
DNAM-1: DNAX accessory molecule-1
ADCC: Antibody-dependent cellular cytotoxicity
ITIM: Immunoreceptor tyrosine-based inhibitory motif
SHP: SH2-domain-containing protein tyrosine phosphatase
SHIP: SH2-domain-containing inositol-5-phosphatase
Treg: Regulatory T cells.

Data Availability

All data supporting the results reported in the article are generated and archived in the facilities of the Department of Laboratory Medicine, the First Affiliated Hospital of Nanjing Medical University.

Conflicts of Interest

We declare that we do not have any commercial or associative interests that represent a conflict of interest in connection with the work submitted.

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Review Article

Cutting Edge: Probiotics and Fecal Microbiota Transplantation in Immunomodulation

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Probiotics are commensal or nonpathogenic microbes that confer beneficial effects on the host through several mechanisms such as competitive exclusion, antibacterial effects, and modulation of immune responses. Some probiotics have been found to regulate immune responses via immune regulatory mechanisms. T regulatory (Treg) cells, T helper cell balances, dendritic cells, macrophages, B cells, and natural killer (NK) cells can be considered as the most determinant dysregulated mediators in immunomodulatory status. Recently, fecal microbiota transplantation (FMT) has been defined as the transfer of distal gut microbial communities from a healthy individual to a patient's intestinal tract to cure some immune disorders (mainly inflammatory bowel diseases). The aim of this review was followed through the recent literature survey on immunomodulatory effects and mechanisms of probiotics and FMT and also efficacy and safety of probiotics and FMT in clinical trials and applications.

1. Introduction

Probiotics were defined in 2002 by experts from the Food and Agriculture Organization of the United Nations and the World Health Organization, and the definition was updated by the International Scientific Association in 2013 [1]. The definition states that probiotics are "live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host." Probiotic products are commonly known to be microecological preparations and are used to improve the structure of intestinal flora, inhibit the growth of harmful microorganisms, and enhance the immunity of the human body. To be considered microecologics, probiotics must satisfy the following conditions [2]: be live microorganisms; stay alive and stable before use after culture, production, and storage; be resistant to gastric acid, bile, and trypsin, and remain alive to colonize and proliferate in the intestinal tract; be scientifically proven to be beneficial to the host; and be proven to be safe and reliable or an member of the original intestinal microflora. Currently, the extensively studied and developed probiotics include the related bacteria of *Lactobacillus*, *Bifidobacteria, Escherichia coli (E. coli),* and *Enterococcus* and some yeasts [3].

Currently, as a means of intestinal microecological regulation in addition to microecological preparations, fecal microbiota transplantation (FMT) has become popular in recent years. FMT refers to the transplantation of functional bacteria in the feces of healthy donors into the gastrointestinal tract of the patient to restore the balance of the intestinal microecology, which subsequently treats diseases associated with disorders of intestinal microorganisms. As far back as

traditional medical treatments in the fourth century of China, there have been relevant records of FMT treatment [4]. In the era of modern medicine, the earliest report of FMT was in 1958. Eiseman et al. successfully used FMT to treat a case with pseudomembranes [5]. The first report of FMT application in the treatment of *Clostridium difficile* (*C. difficile)* infection (CDI) was in 1983 [6]. In 2010, the United States recommended FMT as a treatment plan for CDI in their clinical guidelines [7]. FMT has now been deemed the primary therapy for refractory and relapsed CDI. In recent years, FMT has become a research focus on biomedicine and clinical medicine. FMT has also been clinically applied to inflammatory bowel disease (IBD), irritable bowel syndrome, chronic functional constipation, intestinal cancer, foodborne allergic gastroenteropathy, and so on [8], and researchers have achieved a certain clinical efficacy. Recently, some studies have shown that there is a very strong potential application for FMT in the field of nongastrointestinal diseases, such as treating arteriosclerosis, metabolic syndrome, diabetes, hepatic encephalopathy, neurodegenerative diseases, among others [9].

2. Probiotics and the Immune System

Relevant studies on the mechanism of probiotics mainly focus on the intestinal tract. However, the effect of probiotics is not confined to the initial infection site, and probiotics can work throughout the entire body via the immune system. In gut-associated lymphoid tissues (GALT), probiotic and antigen substances from its metabolites are phagocytized or internalized by M cells to form endosomes. Antigens in M cells are rapidly released and taken in by dendritic cells (DCs), which can transport the antigens to local lymph nodes and then activate naive T and B cells to differentiate into different effector subpopulations, initiating the release of the corresponding cytokines and displaying different immune functions.

A number of studies show that the mechanisms of probiotics include (1) enhancement of the chemical and biological barriers in the intestinal tract as well as regulation of the balance of intestinal flora. Through a space-occupying effect, competition, or antagonism [10–14], and by secreting antibacterial or bactericidal substances (e.g., bacteriocin), increasing digestive enzyme activity, producing organic acid, and so on [15], probiotics can exert an antibacterial effect, maintain the function of intestinal epithelial cells, prevent pathogenic bacteria adhesion, and inhibit the growth of pathogenic bacteria. (2) Through increasing the synthesis of tight junction proteins between epithelial cells [16, 17], probiotics stimulate and promote the expression and secretion of mucous glycoproteins [18], enhance the integrity of intestinal epithelial cells, strengthen the mechanical barrier function of the intestinal tract, and prevent the displacement of intestinal bacteria and endotoxins. (3) Probiotics regulate innate and adaptive immunity, including promoting the development and maturation of the immune system [19], enhancing the viability of macrophages and natural killer (NK) cells [20], stimulating the secretion of secretory immunoglobulin A (sIgA) [21], activating related immune responses mediated by Toll-like receptor (TLR) and nucleotide-binding oligomerization domain-containing protein- (NOD-) like receptors (NLR), regulating the T helper cell (Th)1/Th2 immune response, increasing the number of regulatory T cells (Treg) that secrete interleukin- (IL-) 10 and transforming growth factor (TGF)-β, and strengthening their function as well as reducing the level of allergen-specific IgE [22].

The role of probiotics in the immune system is complex. The immune stimulations induced by probiotics are manifested as an increase in the generation of immunoglobulins, enhanced activity of macrophages and lymphocytes, and stimulation of interferon- (IFN-) γ. Probiotics that inhibit the immune system are mainly embodied in their anti-inflammatory action. Figure 1 summarizes the dual function of probiotics in the immune system in in vitro and animal experiments.

Additionally, there is a mechanism behind positive and negative effects of probiotics on the immune system; yet, the exact molecular mechanisms for these commensal-host interactions are poorly described. Many immunomodulatory biologically active signaling molecules of probiotics are microbial-associated molecular patterns (MAMP) that interact with transmembrane host pattern recognition receptors (PRRs). TLR has been the most studied. In addition, extracellular C-type lectin receptors (CLRs) and intracellular NLR can also transmit signals by interaction with bacteria. Table 1 summarizes the immunomodulatory components of the most common probiotics, *Lactobacillus* and *Bifidobacterium*. However, the molecular basis of these effector-mediated strain-specific probiotics needs to be thoroughly investigated.

Importantly, studies have shown that there are some differences in the physiology and metabolism between probiotic strains from different species and that their effects on the human body are different. Even the functions of different strains from the same species can vary greatly. Similarly, different doses of the same strain can produce different effects. Additionally, there are some differences in function in different hosts. Therefore, the functions of probiotics need to be verified at the strain level to clarify the efficacy of the strain.

3. FMT and the Immune System

FMT can increase the microbial diversity of the intestines, maintain the intestinal microecological balance, and rebuild the function of the immune system. Related mechanisms may include (1) intestinal flora introduced from healthy donors that can maintain the intestinal epithelial integrity of patients, limit intestinal permeability, and inhibit intestinal epithelial cell apoptosis to reestablish the function of the intestinal barrier (this may be related to the mechanisms of the intestinal flora from donors that inhibit the adhesion between intestinal pathogens and intestinal epithelial cells (IECs), reduce the damage of IECs, and increase the production and expression of mucosal IgA and mucin by colonizing resistance and producing immunomodulatory molecules and bacteriocin, etc.); (2) the intestinal flora of the donors can also fight against proinflammatory cytokines by directly synthesizing anti-inflammatory factors, reducing local and

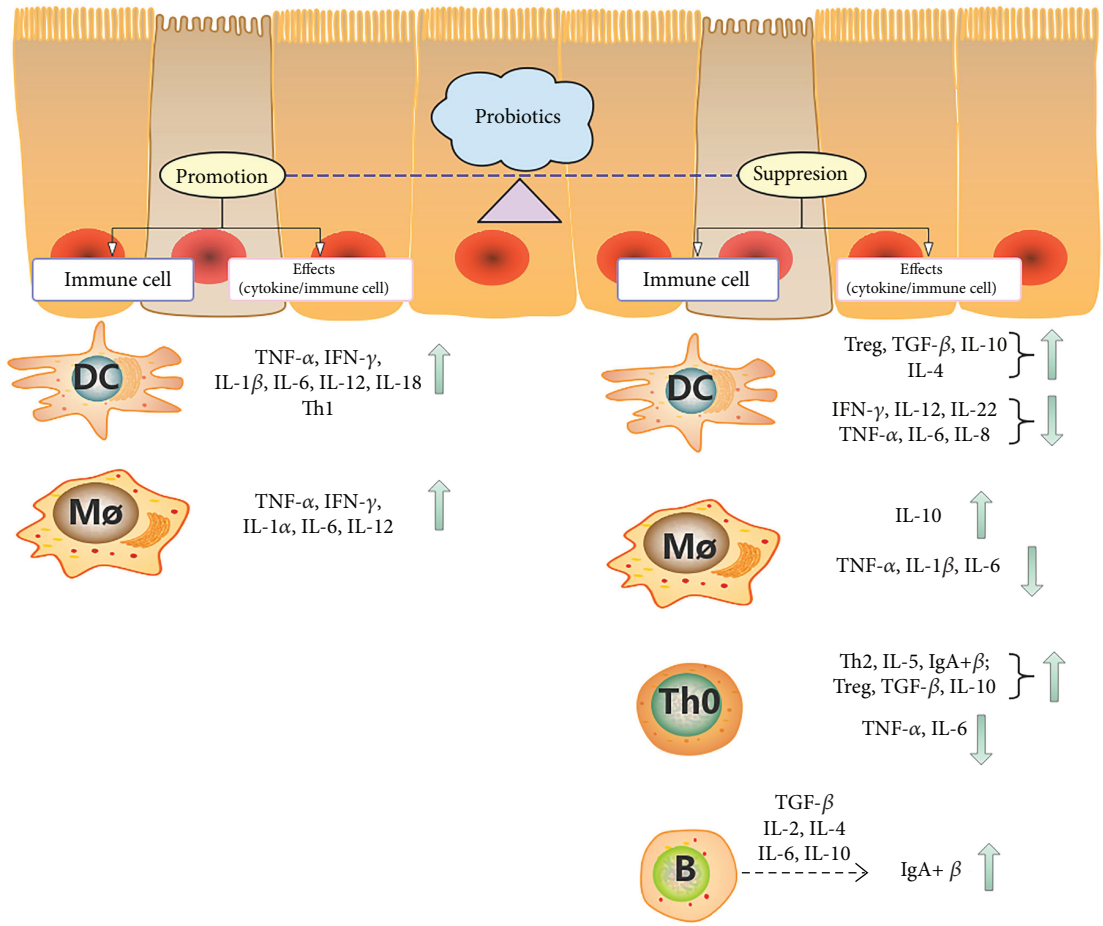

Figure 1: The dual functions of probiotics on the immune system in in vitro and animal experiments. ↑: activity enhanced or quantity increased; ↓: activity reduced or quantity decreased. Immune cell: immune cells on which probiotics directly stimulate. Effects: the immunological effect generated by immune cells stimulated by probiotics, mainly including the regulation on cytokines and the differentiation of related immune cell subpopulations.

systemic inflammatory responses; (3) FMT restores the metabolism of secondary bile acids in the intestines, which makes the metabolism of secondary bile acids in the gastrointestinal tract of patients similar to that of donors; (4) competition or antagonism with pathogenic bacteria; and (5) improving insulin resistance. As a result, the patient's immunity is improved [23–27]. Applications in patients confirmed that the effects of FMT on the intestinal microflora of patients are long lasting and mostly safe, with few adverse effects [28]. In addition, FMT can improve anxiety and depression through mechanisms associated with the brain-intestine axis and improve the quality of life of patients [29].

With FMT, the intestinal flora of healthy donors may maintain the microenvironment of recipients and eventually reconstruct the recipient's intestinal ecological balance. The mechanisms can affect the disease processes of gastrointestinal and extraintestinal diseases by altering the mucosal cell gene expression, the intestinal mucosal immune function, the intestinal ecological environment, and body metabolism, which regulate the immune response, the inflammatory response, and the number and activity of neurotransmitters.

4. Immunomodulatory Effects and Mechanisms of Probiotics and FMT

4.1. Th1/Th2 Balance. Th1 activates macrophages and neutrophils to promote an inflammatory response by secreting IL-2, IL-3, IFN-γ, and tumor necrosis factor- (TNF-) α. Th2 can secrete IL-4, IL-5, IL-6, IL-10, and IL-13 to activate mast cells and basophils to participate in allergic reactions. Many experiments have shown that probiotics can participate in the negative regulation of the immune system, such as anti-inflammation and antiallergy effects through affecting the Th1/Th2 balance.

4.1.1. Anti-inflammatory Effects. By oral administration of *Lactobacillus plantarum* (*L. plantarum*) A7 and *Bifidobacterium animalis* (*B. animalis*) PTCC 1631 to mice with autoimmune encephalomyelitis (EAE), Salehipour et al. found that naive T cells preferred to differentiate to Th2 cells because of increased production of transcription factor GATA3, which eventually led to the secretion of more IL-4 and IL-10 [30]. Mi et al. found that by orally administering *Bifidobacterium infantis* (*B. infantis*) to colorectal cancer mice

Table 1: The immunomodulatory components of Lactobacillus and Bifidobacterium.

Probiotic genera	Probiotic strains	Immunomodulatory components of probiotics	References
Lactobacillus	*L. acidophilus*, *L. amylovorus*, *L. bulgaricus*, *L. crispatus*, *L. casei*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L. pentosus*, *L. reuteri*, *L. paracasei*, *L. plantarum*, *L. rhamnosus*	(1) Lipoteichoic acid stimulates NO synthase (2) Lipoproteins and LTA can potentially signal through binding to TLR2 in combination with TLR6 (3) Unmethylated DNA fragments containing CpG motifs mediate anti-inflammatory effects via TLR9 signaling at the epithelial surface (4) Highly O-acetylated peptidoglycan might affect the release of NLR stimulating PG fragments and innate immune responses of antigen-presenting cells such as dendritic cells and macrophages (5) EPS and other cell wall polysaccharides could be recognized by CLRs that are involved in the recognition and capture of antigens by antigen-presenting cells such as dendritic cells and macrophages	[139–143]
Bifidobacterium	*B. animalis*, *B. breve*, *B. infantis*, *B. bifidum*, *B. lactis*, *B. catenulatum*, *B. longum*, *B. adolescentis*	(1) Lipoteichoic acid stimulates NO synthase (2) Bifidobacterial proteins are one of the targets of human immunoglobulins, notably IgA (3) Although no specific host receptors have been found, EPS has been recognized as an effector of the interaction between probiotics and the host immune system (4) *Bifidobacteria* possess genomes with high G+C proportions, and unmethylated CpG motifs derived from them can interact with the TLR 9 present on immune cells (5) The peptidoglycan hydrolase TgaA is shown to induce IL-2 production in the monocyte-derived dendritic cell, the key cytokine in Treg cell expansion (6) The specific interaction between pili and gastrointestinal mucosa	[41, 144–146]

NO: nitric oxide; PG: peptidoglycan; LTA: lipoteichoic acid; LPS: lipopolysaccharide; EPS: exopolysaccharides.

induced by dimethylhydrazine, CD4+IL-17+ cells were reduced, resulting in decreased secretion of IL-2, IL-12, and IFN-γ from Th1 and Th17. Therefore, *B. infantis* could inhibit intestinal mucositis caused by chemotherapy drugs in colorectal cancer mice [31]. In addition, Rebeca's research showed that after feeding *B. animalis* ssp lactis CNCM-I2494 to low-level inflammatory mice induced by dinitrobenzene sulfonic acid, the number of Th2 cells and the levels of IL-4, IL-5, and IL-10 increased, which significantly improved the barrier permeability diseases [32]. Interestingly, oral administration of *Clostridium butyricum* (*C. butyricum*) CGMCC0313.1 to nonobese diabetic mice resulted in a significant reduction of Th1 and IFN-γ secretion in the spleen and an increase of Th2 and IL-4 [33]. Additionally, the serum IgE and IL-4 levels in atopic dermatitis mice were reduced by oral administration of *Lactobacillus casei (L. casei)* variety rhamnosus (LCR35). Moreover, the recovery of the Th1/Th2 balance improves intestinal flora [34]. In the study by Zheng et al., after feeding *Bifidobacterium breve (B. breve*) to colitis mice, the expression levels of IL-4, IL-5, IL-13, and IL-23 message ribonucleic acid (mRNA) in colon tissue increased. In subsequent studies, they also cocultured peripheral blood mononuclear cells (PBMCs) with *B. breve* and found that Th1 and Th17 decreased and Th2 and Treg increased [35].

4.1.2. Antiallergic Effects. In the mouse model of ovalbumin (OVA) allergy, after oral administration of *Lactobacillus bulgaricus* (*L. bulgaricus*), *Streptococcus thermophilus* (*S. thermophilus*), and *Lactobacillus paracasei* (*L. paracasei*) ssp. paracasei CNCMI-1518, the number of Th2 cells and serum IgE decreased but serum IL-10 and IFN-γ increased in mice [36]. Similarly, after feeding *Lactobacillus rhamnosus* (*L. rhamnosus*) MTCC 5897 to OVA allergy mice, serum IL-4 decreased, whereas serum IFN-γ increased [37]. In a mouse model of whey protein hypersensitivity, oral administration of *Lactobacillus acidophilus* (*L. acidophilus*) and *Bifidobacterium bifidum* (*B. bifidum*) increased the levels of IFN-γ, IL-10, and IL-12 and decreased the level of IL-4 in the spleen [38]. Another experiment also showed that differentiation of Th1 increased in mesenteric lymph nodes (MLN) and the spleen and the serum histamine concentration decreased after oral administration of *Bifidobacterium lactis* (*B. lactis*), *L. casei*, *L. rhamnosus*, and *L. plantarum* to mice that were allergic to whey protein [39]. In addition, after feeding *L. plantarum* CJLP133 and CJLP243 to mice with allergic rhinitis caused by birch pollen (BP), the researchers found an increase in IFN-γ and decrease in IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid (BALF). At the same time, serum IL-4, IL-5, IL-13, IgE, and BP-specific IgG1 were also reduced [40].

4.2. Th17/Treg Balance. Probiotics can affect the Th17/Treg balance in the host immune system. When probiotics promote the differentiation of Th0 to Treg, the clinical effect is to negatively regulate the host immune system. Conversely, when probiotics promote the differentiation of Th0 to Th17, the clinical effect of probiotics is to positively regulate the host immune system. Treg can secrete TGF-β, IL-10, and IL-35 to participate in negative immune regulation. Th17 can secrete IL-17, IL-21, and IL-23 to participate in positive immune regulation. To provide a better understanding of

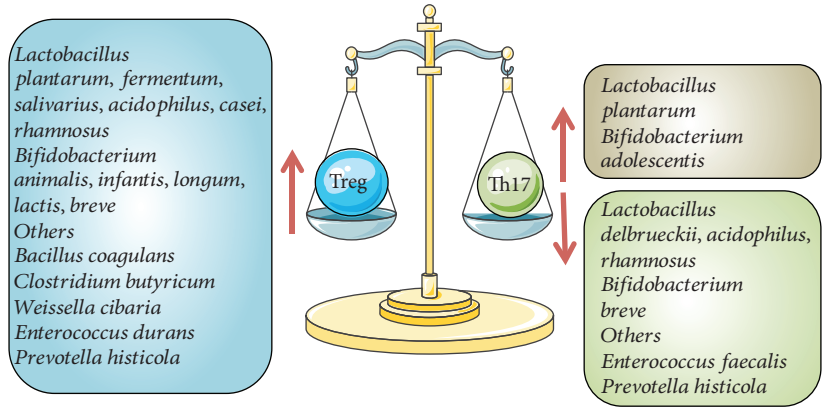

Figure 2: Effects of probiotics on the Th17/Treg balance. Treg can be increased by probiotics, such as *Lactobacillus* (*plantarum*, *fermentum*, *salivarius*, *acidophilus*, *casei*, and *rhamnosus*), *Bifidobacterium* (*animalis*, *infantis*, *longum*, *lactis*, and *breve*), and *Bacillus coagulans*, *Clostridium butyricum*, *Weissella cibaria*, *Enterococcus durans*, and *Prevotella histicola*. Th17 can be increased by probiotics, such as *Lactobacillus plantarum*, *Bifidobacterium adolescentis*. Conversely, Th17 can be decreased by probiotics, such as *Lactobacillus* (*delbrueckii*, *acidophilus*, and *rhamnosus*), *Bifidobacterium breve*, and *Enterococcus faecalis* and *Prevotella histicola*.

this section, we summarized the available literature in Figure 2.

4.2.1. Anti-inflammatory Effects. In experiments with mice that had autoimmune encephalitis (EAE), Ménard et al. found that after feeding *L. plantarum* A7 and *B. animalis* PTCC 1631, the transcription factor Foxp3 of naive T cells increased, resulting in increased Treg differentiation and IL-10 production [41]. In addition, a study by Kwon et al. showed that IRT5 (a mixture of five probiotics) not only increased the levels of Treg and IL-10 in superficial lymph nodes of EAE mice but also reduced the amount of Th17 and secretion of IFN-γ, TNF-α, and IL-17 [42]. In the study by Mangalam et al., after feeding *Prevotella histicola (P. histicola)* to EAE mice, they found that the amounts of Th1 and Th17 decreased in the MLN and spleen, while the numbers of Treg, regulatory dendritic cells (DCreg), and suppressive macrophages increased [43]. Therefore, what is the possible pathway through which probiotics affect T cells? The study by Haghikia and colleagues provides an answer. They fed propionic acid (a metabolite of probiotic) to EAE mice and found that the JNK1 and p38 pathways in naive T cells were inhibited, leading to increased expression of Foxp3 and IL-10 mRNA as well as the promotion of the differentiation of naive T cells to Treg [44]. In addition, in experiments with colitis mice, Qiu et al. [45], Rodríguez-Nogales et al. [46], and Kanda et al. [47] found that probiotics promoted naive T cell differentiation to Treg and increased IL-10 secretion. Moreover, after giving oral *L. acidophilus* to colitis mice induced by dextran sulfate sodium (DSS), they found that not only Treg and IL-10 were increased but also IL-17 was decreased in the spleen. Additionally, the levels of IL-6, TNF-β, IL-1β, and IL-17 also decreased in colon tissue [48]. In the same model, Kim et al. found that activation of nuclear factor kappa B (NF-κB) was inhibited and the endoplasmic reticulum (ER) pressure signal pathway was disturbed, leading to increased expression of IL-10 in the colon and increased levels of Th2 and Treg in the spleen [49]. In addition, studies have shown that after oral administration of a mixture of 12 probiotics, zinc, and CoQ10 to arthritic mice induced by collagen, Th17 decreased but Treg increased in the spleen. Moreover, the secretion of TNF-α, IL-1β, IL-6, and IL-17 decreased in the joint synovium. At the same time, the levels of IgG, IgG1, and IgG2a in the serum were reduced [50]. Cortes-Perez et al. found that after intragastric administration of *L. casei* BL23, the number of Foxp3+RORγt+T cells (type 3 Treg) increased [51]. By oral administration of *Weissella cibaria (W. cibaria)* WIKIM28 to mice with chronic inflammatory skin disease induced by 2,4-dinitrochlorobenzene, Lim et al. found that serum IgE decreased but Treg and IL-10 increased in MLN [52]. After feeding *L. acidophilus* to mice with ulcerative colitis, Chen et al. found that phosphorylation of STAT3 was inhibited, which subsequently caused increased secretion of IL-17 and TNF-α [53]. After feeding *C. butyricum* CGMCC0313.1 to autoimmune nonobese diabetic mice, $\alpha 4\beta 7$+ Tregs increased in the pancreatic LN. This change restored the intestinal microbial disorders caused by diabetes [33].

4.2.2. Antiallergic Effects. In the OVA-allergic mouse, Kim et al. showed that feeding *L. rhamnosus* (Lcr35) could result in increased Treg but decreased IL-4 and IL-17 in MLN, and the response of thymic stroma lymphocytes was weakened [54]. In addition, studies have shown that oral administration of *Enterococcus faecalis (E. faecalis)* FK-23 to OVA-allergic mice can reduce the number of IL-17-expressing CD4+ cells in the lungs, spleen, and intestine. Additionally, the total number of white blood cells and mast cells decreased in BALF [55]. Fu et al. discovered that after feeding *Bacillus coagulans (B. coagulans)* 09.712 to mice allergic to the prion troponin, the mTOR pathway was inhibited in naive T cells, which caused an increase in Foxp3 expression. Additionally, naive T cells differentiated into Treg, which increased the secretion of IL-10 by Treg and decreased the secretion of IL-17A and IL-6 by Th17 [56]. Furthermore, oral administration of *L. casei* DN-114 001 to allergic dermatitis mice increased the number of Treg in the skin and the levels of IL-10 in LN [57]. Salehipour et al. found that the number

of Treg was increased in MLN and the spleen, whereas serum histamine decreased but IL-10 increased after feeding *B. lactis*, *L. casei*, *L. rhamnosus*, *L. plantarum*, and sodium butyrate to mice allergic to whey protein [30]. In the study by Zhang et al., oral administration of *C. butyricum* CGMCC0313-1 increased the number of Treg and decreased the serum IL-4, IL-5, IL-13, and IL-17 levels in mice with an intestinal allergy induced by lactoglobulin [58].

4.2.3. Other Aspects of Negative Immune Regulation. In the study by Laskowska et al., feeding bokashi preparations (a mixture of 11 probiotics) to pregnant sows increased serum IL-10 as well as IL-10 and TGF-β in the colostrum [59]. Moreover, in some experiments, probiotics also regulate autoimmune diseases. For example, in systemic lupus erythematosus (SLE) mice induced by pristane, feeding *L. rhamnosus* and *Lactobacillus delbrueckii* (*L. delbrueckii*) reduced the expression of RORγ mRNA, downregulated Th1 and Th17 cells, and decreased the levels of IFN-γ and IL-17 [60].

4.2.4. Positive Immune Regulation. When probiotics promote the differentiation of Th0 cells into Th17 cells or inhibit the differentiation of Th0 cells into Treg, they can positively regulate the host immune system. Tan et al. found that feeding *Bifidobacterium adolescentis* (*B. adolescentis*) could increase the number of Th17 in the gut [61]. Xie et al. found that by oral administration of *L. plantarum* NCU116 to immune suppressive mice induced by high-dose cyclophosphamide, the expression of TLR-2 and TLR-6 mRNA increased in the small intestine, which resulted in an increase of Th17 cells and the IL-17, IL-21, IL-23, and TGF-β3 levels [62].

4.3. B Cells. B cells can differentiate into plasma cells or regulatory B cells (Breg). Plasma cells can synthesize and secrete antibodies and are mainly involved in humoral immunity. Breg can perform immunological negative regulation by producing IL-10 or TGF-β. When probiotics promote the differentiation of B cells into plasma cells, positive regulation of the immune system can be achieved. When probiotics promote the differentiation of B cells into Breg, they can negatively regulate the immune system. Shi et al. fed *C. butyricum* to OVA-allergic mice and found an increasing number of IL-10-producing OVA-specific B cells (OVAsBC). Furthermore, they cocultured OVAsBC, OVA, and *C. butyricum* and showed that OVAsBC differentiated towards Breg and the secretion of IL-10 increased [63]. In addition, studies have shown that *Lactobacillus helveticus* (*L. helveticus*) SBT2171 stimulated B cells isolated from mouse spleens, which could inhibit lymphocyte proliferation by inhibiting the JNK signaling pathway [64]. In addition, Sakai et al. showed that after oral application of *Lactobacillus gasseri (L. gasseri)* SBT2055, B cells could produce more IgA in Peyer's patch and small intestines of mice [65]. Through oral administration of VSL#3 (a mixture of multiple probiotics) to macaques, Manuzak et al. discovered that B cells could secrete more IgA in the colon and LN [66].

4.4. Dendritic Cells. DC is a type of professional antigen-presenting cell (APC) that can efficiently ingest, process, and present antigens. DC eventually presents antigens to T cells to affect the differentiation of T cells. Negative immune regulation can be performed when probiotics that affect DC present antigens or differentiate to DCreg.

4.4.1. Anti-inflammatory Effects. Mariman et al. showed that DC secreted high levels of IL-12p70, IL-23, and IL-10 after VSL#3 stimulated mouse bone marrow DC (BMDC) [67]. Moreover, the activation of TLR-2 receptors in DC caused the polarization of Th0 cells into Treg and high levels of IL-10 and TGF-β secretion in MLN after coculturing BMDC with probiotics [68]. Further evidence suggested that coculturing human peripheral blood mononuclear cells (PBMCs) with *Lactobacillus crispatus (L. crispatus)* SJ-3C-US increased the maturation of DC, the number of Treg, and the secretion of IL-10 [69].

4.4.2. Antiallergic Effects. In in vivo experiments, after feeding *B. infantis* to mice allergic to tropomyosin, Fu et al. also found that the maturation of DC and number of CD103+ DCreg cells increased, which promoted the expression of IL-10, TGF-β, and Foxp3 mRNA in Treg [70]. Some studies have shown that feeding *L. paracasei* L9 reduced the maturation of DC and increased the expression of CD103 and number of Treg in the MLN, Peyer's patch, and spleen of mice allergic to β-lactoglobulin [71]. In their in vitro experiments, Adam et al. extracted BMDC from mice allergic to house dust mites and then cocultured BMDC with the *Escherichia coli* Nissle 1917 strain. They found that activation of the TLR-4 pathway could promote DC differentiation. Additionally, activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways can promote DC to secret more IL-10 and IL-12 [72]. Some studies have also shown that after *E. coli* O83:K24:H31 stimulating DC from cord blood of pregnant women (CBDC), the differentiation and maturation of CBDC increased. In addition, CBDC expressed a higher level of CD83 and secreted more IL-10 [73].

4.4.3. Other Aspects of Negative Immune Regulation. By coculturing human PBMCs with *Lactobacillus reuteri (L. reuteri)* DSM 17938, Haileselassie et al. found that the expression of CCR7 increased in DC. Moreover, the expression of Foxp3 and IL-10 in Treg also increased [74]. In addition, research showed that after *Kluyveromyces marxianus* (a fungus that provides beneficial effects like probiotics) stimulated PBMCs, they found DC secreted more IL-12, IL-1, IL-6, and IL-10, which promoted the polarization of naive T cells to Treg [75].

4.5. Natural Killer Cells. NK cells are involved in antitumor, antiviral, hypersensitivity, and immunoregulation activities. When probiotics promote the production of NK cells, they can positively regulate immunity. Conversely, when probiotics inhibit the number of NK cells, they negatively regulate immunity. Johansson et al. stimulated PBMCs with *Staphylococcus aureus* (*S. aureus*) and then cocultured the cells with *L. rhamnosus* GG and *L. reuteri* DSM 17938. They found that *S. aureus*-induced T cells and NK cells to proliferate and produce IFN-γ, but probiotics *L. rhamnosus* GG and *L. reuteri* DSM 17938 inhibited this effect [76]. In the study by Gong et al., the cytotoxicity of NK cells was enhanced after feeding

mice *Bacillus subtilis (B. subtilis)* BS02 and BS04, and there were changes to CD4+ and CD8+ T cells as well as the level of IFN-γ [77]. Studies have shown that high expression of cytotoxic receptors and IL-22 in NK cells can be achieved by NK-92MIX cell coculturing with *L. plantarum* [78]. After oral administration of *Bifidobacterium longum (B. longum)* MM-2 to mice given an intranasal flu virus, Kawahara et al. found that the amounts of IL-6 and TNF-α in BALF were reduced. Additionally, the activity of NK cells in the lungs and spleen was elevated [79]. Some researchers used AJ2 (a mixture of 8 probiotics) to stimulate PBMCs and found that NK cells were activated, and the release of inflammatory cytokines was reduced [80].

4.6. Other Adaptive Immune Cells. In addition to acting on T cell and B cells, probiotics can also work on other adaptive immune cells, including follicular helper T cells (Tfh) and $\gamma\delta$ T cells. The main function of Tfh is to assist B cells in participating in humoral immunity. The main function of $\gamma\delta$ T cells is in innate immunity, as they can both recognize cancer antigens and kill cancer cells. Scharek-Tedin et al. fed *Bacillus cereus (B. cereus)* var. toyoi to weaned piglets. As a result, they found $\gamma\delta$ T cells were significantly reduced in the blood [81]. Arai et al. showed that feeding heat-killed *L. paracasei* MCC1849 could increase the number of Tfh in Peyer's patch of mice [82].

4.7. Other Innate Immune Cells. Other innate immune cells, including macrophages, neutrophils, and mast cells, could also be influenced by probiotics. Macrophages are a type of phagocytic cell whose main function is to phagocytose pathogens and activate immune cells to respond to pathogens. Neutrophils can perform chemotaxis, phagocytosis, and bactericidal actions and defense. Mast cells can secrete a variety of cytokines and participate in immune regulation. Mast cells can also release allergic mediators to mediate allergic reactions. Through in vivo experiments, Juan et al. showed that after feeding *C. butyricum* CGMCC0313-1 to OVA-allergic mice, the degranulation of mucosal mast cells was inhibited and the infiltration of lung inflammatory cells was also reduced. In BALF, MMP-9 was reduced and IL-10 was increased [83]. In addition, Kim et al. fed *L. acidophilus* to colitis mice and found that M2 macrophages increased in the peritoneal cavity [49]. Through in vitro experiments, some researchers found that coculturing mice bone marrow-derived neutrophils with *L. rhamnosus* GG could inhibit the phagocytic ability and the cytotoxicity of neutrophils [84]. Carasi et al. cocultured human PBMCs with *Enterococcus durans (E. durans)* (EP-1) and found that IL-6 secretion was significantly reduced, while IL-10 secretion increased. After feeding mice EP-1, they found that the expression levels of IL-17, IL-6, IL-1, IFN-γ, and CXCL1 were remarkably reduced in Peyer's patch [85]. Studies by Gong et al. showed that feeding mice with *B. subtilis* BS02 and BS04 could enhance the phagocytosis of monocytes in mice [77].

4.8. Immunomodulatory Effects of Probiotic Fungi. In addition to probiotic bacteria, some fungi also have immunomodulatory effects, which can improve the host microecological balance and regulate the host immune system. Smith et al. cultured *K. marxianus* and *S. boulardii* with DCs, respectively, they found that DCs secreted increased levels of IL-12, IL-1β, IL-6, and IL-10. Besides, they found that the use of these two fungi cell wall extracts, β-glucan, could stimulate DC receptor Dectin-1, allowing DCs to secrete IL-1β, IL-6, and IL-10, but not including IL-12. Finally, they cultured *K. marxianus* and *S. boulardii* with the DC-naive T cell cocultured system; they found that *K. marxianus* induced the differentiation of naive T cells to Foxp3+ Treg, increased secretion of IL-10, and controlled inflammation. Moreover, *S. boulardii* could induce differentiation of naive T cells to Th1, resulting in an increased secretion of IFN-γ [75]. Thomas et al. cocultured bone marrow-derived DCs from Crohn's disease (CD) and ulcerative colitis (UC) patients with *S. boulardii*; they found that DCs secreted less TNF-α but more IL-6 and IL-8 [86]. Interestingly, the same research team cocultured *S. boulardii* with DCs isolated from PBMCs; they found that DCs secreted less TNF-α and IL-6 but more IL-10, thereby inhibiting T cell proliferation [87]. By coculturing DCs with *S. boulardii* and *K. marxianus* CBS1553, respectively, Smith et al. found that both *S. boulardii* and *K. marxianus* CBS1553 can promote IL-12, IL-10, IL-6, TNF-α, and IL-1β secretion [88]. In addition, by, respectively, coculturing mouse bone marrow-derived DCs and spleen cells with β-glucan extracted from the cell wall of *Saccharomyces cerevisiae* (*S. cerevisiae*), Karumuthil-Melethil et al. found that DCs and spleen cells could secrete increased IL-10, TGF-β1, and IL-2 [89].

Xu et al. firstly stimulated mouse macrophages with LPS, and then added *S. cerevisiae*, and found that *S. cerevisiae* inhibited the production of IL-1α, IL-1ra, and IL-27 by macrophages, of which mechanism may be related to the inactivation of the mitogen-activated protein kinase and TLR2 pathway in macrophages [90]. In addition, by feeding *S. cerevisiae* IFST062013 to mice, Fakruddin et al. found that high doses of *S. cerevisiae* IFST062013 increased the expression of TLR-2 and IFN-γ genes in the intestinal mucosa of mice, while Foxp3, TGF-β, and IL-4 gene expression decreased. They also found an increase in IL-10 in mouse serum [91]. Maccaferri et al. cocultured *K. marxianus* B0399 with PBMCs and found more IL-1β, IL-6, MIP-1α, and TNF-α released. In another experiment, they used LPS to stimulate PBMCs with *K. marxianus* B0399 and found the ability of LPS to trigger an inflammatory response was attenuated by *K. marxianus* B0399. Besides, *K. marxianus* B0399 can significantly reduce the concentration of proinflammatory cytokines TNF-α, IL-6, and MIP-1α secreted by PBMCs; however, IL-1β was increased [92]. By giving oral administration of *Scytalidium acidophilum* (*S. acidophilum*) to broilers chickens, Huang et al. found an increase in serum IgA [93]. Interestingly, after mice were infected with *C. difficile*, the mice that continued to be infected with *Candida albicans* (*C. albicans*) expressed higher levels of IL-17A in infected tissues than the mice that were not continued to be infected with *C. albicans*. This improves the survival rate after *C. difficile* infection. *C. albicans* may be a potential probiotic [94].

By feeding mice with Tibetan mushroom (a drink which was produced by fermentation of more than a dozen bacteria and yeasts), Diniz et al. found that the granuloma induced by

Table 2: RCT clinical trials of probiotics and FMT treatment in IBD.

Researcher/country	Year	Single/multiple-center study	Strains of probiotics/delivery way of FMT	No. of enrolled patients	Diseases	Period of observation	Efficacy	Safety
Ahmed/UK [103]	2013	Single	*Lactobacillus acidophilus* LA-5, *Lactobacillus delbrueckii* subsp. *bulgaricus* LBY-27, *Bifidobacterium animalis subsp. lactis* BB-12, and *Streptococcus thermophilus* STY-31	20	CD and UC	2 months	No	Not mentioned
Krag/Denmark [99]	2013	Single	*Lactobacillus plantarum* 299v	74	UC	2 years	Yes	Yes
Bourreille/France [104]	2013	Single	*Saccharomyces boulardii*	165	CD	52 weeks	No	Yes
Petersen/Denmark [105]	2014	Single	*Escherichia coli Nissle*	100	UC	7 weeks	No	Not mentioned
Fedorak/Canada [100]	2015	Multiple	4 strains of *Lactobacillus*, 3 strains of *Bifidobacterium*, and 1 strain of *Streptococcus salivarius subspecies thermophilus*	119	CD	1 year	Yes	Yes
Yoshimatsu/Japan [101]	2015	Single	*Streptococcus faecalis* T-110, *Clostridium butyricum* TO-A, and *Bacillus mesentericus* TO-A	46	UC	1 year	Yes	Yes
Tamaki/Japan [102]	2016	Single	*Bifidobacterium longum* 536	56	UC	8 weeks	Yes	Yes
Jacob/USA [122]	2017	Single	Colonoscopy	20	UC	4 weeks	Yes	Yes
Meighani/USA [126]	2017	Single	Colonoscopy	201	CD and UC	2 years	Yes	Not mentioned
Karolewska-Bochenek/Poland [124]	2017	Single	Nasoduodenal tube or gastroscopy	10	CD and UC	2 weeks	Yes	Yes
Goyal/USA [123]	2018	Single	Upper and lower endoscopy	21	CD, UC, and IC	6 months	Yes	Yes
Mintz/USA [128]	2018	Single	Colonoscopy	26	UC	3 months	Yes	Not mentioned

cotton balls was significantly inhibited. Meanwhile, using carrageenan, dextran, and histamine to stimulate rats to get paw edema was also significantly reduced. But the experiment did not show which kind of bacteria or fungi was responsible for the anti-inflammatory function [95]. Zhang et al. fed mice which were allergic to peanut with ImmuBalance (a fermented soy product from *Aspergillus* and lactic acid); the results showed histamine and IgE levels were decreased in mice sera. Additionally, the amount of IL-4, IL-5, and IL-13 in mouse spleen cells was significantly reduced [96].

5. Efficacy and Safety of Probiotics and FMT in Clinical Trials and Application

5.1. Efficacy and Safety of Probiotics. To be effective at their likely sites of action, probiotics need to be able to survive stomach acid, bile, and digestive enzymes and to be viable for the duration of their shelf lives. Many products (e.g., yogurt) on supermarket shelves do not meet even these most basic standards [97]. To date, clinical trials have not been performed to test whether probiotics taken orally lose their efficacy over time. Additionally, probiotics are generally regarded as safe, but there may still be risks in certain disease populations [98]. To ensure patient safety, the participating patients were provided information both orally and in writing and were instructed to follow all instructions and attend clinical follow-ups with their usual gastroenterologist.

As described above, some probiotics have been shown to have anti-inflammatory effects and promote maintenance of the gut intestinal barrier in vitro and in murine models of IBD. This outcome may give credence to their use as a treatment option in human IBD. The results of clinical trials have been mixed, with some studies showing an improvement in the maintenance of remission or induction of remission with probiotics, while other trials have failed to show any benefit (summarized in Table 2). In a randomized controlled trial (RCT) designed by Krag et al., supplementation

with profermin (contained *L. plantarum* 299v) was found to be safe and well-tolerated and to definitely reduce the simple clinical colitis activity index (SCCAI) scores at a statistically and clinically significant level in patients with mild-to-moderate ulcerative colitis (UC) with a flare-up [99]. Fedorak et al. found early treatment (at day 90 after ileocolonic resection and reanastomosis) with VSL#3 had a larger impact on the prevention of Crohn's disease (CD) recurrence than late treatment (from days 90 to 365) [100]. Yoshimatsu et al. conducted a single-center RCT and found that probiotic (a bio-three tablet, containing *Streptococcus faecalis* (*S. faecalis*) T-110, *C. butyricum* TO-A, and *Bacillus mesentericus* (*B. mesentericus*) TO-A) therapy was useful for preventing relapses of inactive UC in patients who were already in remission [101]. In a multiple-center study, Tamaki et al. found that supplementation with *B. longum* 536 (BB536) was well-tolerated and reduced the UC disease activity index (UCDAI) scores, Rachmilewitz endoscopic index (EI), and Mayo subscores after 8 weeks in Japanese patients with mild to moderately active UC [102]. By contrast, Ahmed et al. designed a prospective randomized crossover study. They found that there was no difference in the colonic microflora between patients with CD or UC and that the spectrum of the gut microflora was not altered by oral synbiotic administration, which contained 4 strains of probiotics, *L. acidophilus* LA-5, *L. delbrueckii* subsp. bulgaricus LBY-27, *B. animalis* subsp. lactis BB-12, and *S. thermophilus* STY-31 [103]. In another prospective study, Bourreille et al. showed that although the probiotic yeast *S. boulardii* was safe and well tolerated, it did not appear to have any beneficial effects for patients with CD in remission after steroid or salicylate therapies [104]. Some clinical trials also proved that probiotics could not be used as the main treatment method for IBD. Petersen et al. used probiotics *E. coli* Nissle together to treat acute UC after the antibiotic ciprofloxacin. They found that there was no benefit in the use of *E. coli* Nissle as an add-on treatment to conventional therapies for active UC. Furthermore, treatment with *E. coli* Nissle without a previous antibiotic cure resulted in fewer patients reaching clinical remission [105]. Recently, a meta-analysis showed VSL#3 could be effective for inducing remission in active UC. Probiotics may be as effective as 5-aminosalicylates (5-ASAs) in preventing relapse of quiescent UC. The efficacy of probiotics in CD remains uncertain, and more evidence from RCTs is required before their utility is known [106].

In clinical trials of other inflammatory and immune diseases, probiotics also showed an immunomodulatory effect. Sindhu et al. provided 124 children with gastroenteritis *L. rhamnosus* GG (LGG) ATCC 53103 or placebo, and they found that LGG had a positive immunomodulatory effect for improving intestinal function in children with rotavirus and cryptosporidial gastroenteritis [107]. Maldonado-Lobón et al. carried out a 3-year study to show that early administration of the probiotic of *Lactobacillus fermentum* (*L. fermentum*) CECT5716 in an infant formula was safe, and differences were observed on the incidence of infectious and noninfectious diseases or disorders related to intestinal function [108]. In recurrent aphthous stomatitis (RAS), Mimura et al. found that a symbiotic treatment based on a fructooligosaccharide, *Lactobacillus*, and *Bifidobacterium* composition produced an alteration in the Th2 serological immune profile in the direction of Th1 and improved pain symptomatology [109]. Savino et al. used *L. reuteri* to treat patients suffering from infantile colic, and they found that infants with colic treated with *L. reuteri* for 30 days had significantly increased forkhead box P3 (FOXP3) expression, which could produce more Treg and, ultimately, reduced fecal calprotectin [110]. Dennis-Wall et al. determined whether consuming *L. gasseri* KS-13, *B. bifidum* G9-1, and *B. longum* MM-2 would improve quality of life during allergy season by increasing the percentage of Tregs and inducing tolerance [111]. Kim et al. identified a population of atopic dermatitis (AD) patients with a good clinical response to probiotic treatment. All patients were given *L. plantarum* CJLP133 once a day for 12 weeks. Their results suggested that a subgroup of patients with a specific AD phenotype showing an immunologically active state (high total IgE, increased expression of TGF-β, and high numbers of Treg) might benefit from probiotic treatment [112]. Sheikhi et al. also investigated immune state changes with probiotics in AD. They found that *L. delbrueckii* subsp. bulgaricus could modulate the secretion of Th1- and Th2-Treg-related cytokines in AD patients [113]. In addition to Th1- and Th2-Treg-related cytokines, Rø et al. found that perinatal maternal probiotic supplementation with a combination of LGG, *B. animalis* subsp. lactis Bb-12 (Bb-12), and *L. acidophilus* La-5 (La-5) reduced the proportion of Th22 cells in 3-month-old children with AD [114]. Another study showed that only probiotics had an effect on Th17, but no effect on the relative frequencies of Th1, Th2, and Treg cells among circulating PBMCs; on plasma cytokine levels; and on in vitro production of cytokines by T cells [115]. In addition to T cells, probiotics also could affect NK cells. Lee et al. found that daily consumption of dairy yogurt containing *L. paracasei* ssp. paracasei, B. lactis, and heat-treated L. plantarum could be an effective option to improve immune function by enhancing NK cell function and IFN-γ concentration [116]. In enthesitis-related-arthritis category of juvenile idiopathic arthritis (JIA-ERA), probiotic VSL3# capsules were well-tolerated but failed to show any significant immune (frequencies of Th1, Th2, Th17, and Treg cells in blood, serum cytokines IFN-γ, IL-4, IL-17, IL-10, TNF-α, and IL-6) or clinical effects [117]. Another study investigating immune responses among sedentary young males showed the total leukocytes, total lymphocytes, T lymphocytes, T-helper, T-cytotoxic, B lymphocytes, and NK cell counts in peripheral blood were not significantly affected by the probiotics [118]. Komano et al. found that heat-killed *Lactococcus lactis* (*L. lactis*) JCM 5805 (LC-Plasma) supplementation relieved morbidity and symptoms of URTI via activation of plasmacytoid DC (pDC) and decreased fatigue accumulation during consecutive high-intensity exercise in athletes [119].

As described above, the effects of probiotic treatment in human studies are often variable, and there are inconsistencies between different clinical trials, undoubtedly related to the fact that different multistrain probiotic combinations have been used in variable dose frequencies. It is therefore

difficult to draw clinically relevant conclusions about the effects of probiotics in human studies.

5.2. Efficacy and Safety of FMT. FMT is a complex intervention that involves multiple components, ranging from donor selection to the methods of transplantation (for example, colonoscopy) and several organizational levels, such as the use of stool banks or analysis of gut microbiota composition by a biologist [120]. The factors that could affect the efficacy and safety of FMT are unknown. In addition, multiple components of FMT (such as donor screening, methods for collecting stool, preparation, and transplantation) could differ among studies [121]. In IBD clinical trials (Table 2), Jacob et al. carried out a single FMT delivery by colonoscopy for active UC using a 2-donor fecal microbiota preparation. Mucosal CD4+ T-cell analysis revealed a reduction in both Th1 and Treg post-FMT [122]. Goyal et al. found that a single FMT was relatively safe and could result in a short-term response in young patients with active IBD. Responders possessed increased fusobacterium prior to FMT and demonstrated more significant microbiome changes compared to nonresponders after FMT [123]. Karolewska-Bochenek and colleagues also proved that FMT had beneficial effects on pediatric UC and CD colitis, and FMT was well-tolerated and safe. However, they emphasized that a proper protocol of FMT administration was crucial for treatment efficacy [124]. In the same year, Pai and Popov summarized an optimal and detailed multiple-center RCT protocol of FMT for pediatric IBD [125]. For CDI patients with IBD, Meighani et al. revealed that FMT could provide a good alternative treatment option with high success rates for recurrent or refractory CDI in patients with well-controlled IBD who fail standard antimicrobial therapy [126]. Khanna et al. showed that CDI patients with IBD had a higher proportion of the original community after FMT and lacked improvement of their IBD symptoms and increased episodes of CDI in a long-term follow-up [127]. Another pilot study suggested that the microbial imbalances in CDI + UC recipients more closely resemble those of the CDI-only recipients compared to the UC-only recipients after a single FMT [128].

In an age of reductionist science and targeted therapeutic interventions, FMT seems oddly unsophisticated. However, FMT has been shown to be a highly efficacious, safe, and cost-effective therapy for immune diseases, especially IBD.

6. Conclusions and Future Perspectives

Probiotics have a large spectrum and have been used in main diseases, such as IBD, necrotizing enterocolitis (NEC), irritable bowel syndrome IBS, diarrhea, and other gastrointestinal diseases, in vivo and in vitro. Due to their ability to regulate systemic immune function, probiotics have recently attracted attention in the development of new treatments for obesity, insulin resistance syndrome, type 2 diabetes mellitus and nonalcoholic liver steatosis, hepatic encephalopathy, autism and chronic kidney disease, allergic asthma, atopic dermatitis (AD), acne, rheumatoid arthritis, prevention of dental caries, preventive treatment of an infection, and other fields. In addition, the use of probiotic strains as carriers of antigen delivery is a viable alternative strategy to overcome the shortcomings of vaccines. However, despite their active role in various tumor diseases, probiotics also have side effects associated with anticancer therapies.

The immunomodulation induced by probiotics is a complex interaction between different hosts and microorganisms, so the immunomodulatory characteristics of specific probiotics cannot be generalized. Presently, the composition, dosage, course of treatment, specific mechanism of action, and efficacy of probiotics used in clinical treatment have not been standardized. Overall, probiotics are generally considered safe, but there is growing evidence of widespread concern about the safety of probiotics. In 2002, a joint report by the World Health Organization and the United Nations Food and Agriculture Organization showed that probiotics can cause four side effects, namely, systemic infection, harmful metabolic activity, excessive immune stimulation, and gene transfer in susceptible individuals. Recently, two reports in September 2018 also noted the unknown aspects of the safety of probiotics at this stage and raised concerns in the scientific community about studying adverse reactions to probiotics. Zmora et al. [129] emphasize that the colonization of probiotics is highly personalized and that different individuals have different sensitivity to different probiotic colonization. The host microbiome influences probiotic colonization through competitive rejection of the same species and specific immune mechanisms. The intake of probiotics did not significantly affect the composition of the symbiotic community but instead stimulated the response of the host immune system. Therefore, we suggest that it is necessary to develop personalized probiotics from the perspective of the specificity of the intestinal flora and host physiology. When a clinical application of probiotics is selected, it should gradually transform from empirical treatment to evidence-based treatment, and suitable individualized treatment plans should be developed for patients using evidence-based treatments.

Suez et al. [130] reported that in mice and mixed probiotic intervention in healthy subjects and fecal bacteria autograft (aFMT) of antibiotics might improve the recovery of the intestinal flora after disturbance; the study illustrated that compared with spontaneous recovery, probiotic preparations significantly delayed the host's feces and the reconstruction of the intestinal mucosa flora and host the transcriptome of recovery. Moreover, this study showed that it is difficult to be fully recovered; this is mainly because of the soluble factors that secreted probiotic bacteria inhibition, and probiotics in the potential beneficial effects of antibiotic therapy possibly will be offset by intestinal mucosa recovery effect. It is important to note that microbiome transplantation enables rapid and nearly complete recovery of host-microbiome and transcriptome within a few days. This suggests that, compared with probiotics or prebiotics, fecal bacteria transplantation as the most direct method of intestinal flora intervention may be more effective and feasible.

Since 2013, when it was included in the FDA's official treatment guidelines for relapsing *C. difficile*, fecal transplants have seen more comprehensive development worldwide. Compared to the standard use of probiotics, FMT can be explored faster and further in this area. At present, the

standardization of donor screening, microflora separation and preparation, transplantation, and other aspects involved in the FMT process has begun to take shape. Recently, a large number of studies [131–135] have proposed the step-up treatment strategy of FMT: when the single FMT and multiple FMTs (greater than or equal to 2) are not effective, FMT can be combined with conventional drug therapy (such as glucocorticoid, cyclosporine, anti-TNF-beta antibody, and whole intestinal nutrition). The efficacy of each step can be improved in the next step. This FMT stepwise treatment strategy is suitable for refractory IBD, immune-related diseases [135], and severe or complex CDI [131], especially for patients who are not responsive to conventional therapeutic drugs. At the same time, severe adverse events caused by FMT can be caused by infectious microorganisms in donor feces, which is because many infectious diseases in the donor are still difficult to be excluded. Therefore, FMT-related adverse events in specific populations should be prevented, especially those with low immunity. During FMT treatment through the digestive tract, improper fecal bacteria infusion technology and process may also lead to nausea, vomiting, aspiration, and other adverse events. In order to prevent FMT transmission diseases, strict donor screening should be carried out, and FMT treatment decisions, methods, short-term and long-term follow-up safety evaluation, and supervision will be the focus of future research.

To sum up, personalized probiotics intervention and standardized fecal bacteria transplantation should be challenges and prospects for future research on the intervention model of intestinal flora. Furthermore, increasing evidence shows that the microbiome has potential effects outside the intestinal tract, such as vagina and sinus tract [136], urethra, [137] and skin [138]. Therefore, future research should focus on a specific use of microbiome in different organs.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Wenjie Zeng and Jie Shen collected the literatures and drafted the initial manuscript. Liangxin Peng and Hongbo Xu assisted to make the figures and tables. Tao Bo and Moussa Ide Nasser revised the manuscript and edited the language. Quan Zhuang and Mingyi Zhao conceptualized and guaranteed the review. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work. Wenjie Zeng and Jie Shen, these authors contributed equally to this study.

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Review Article

Functions of Macrophages in the Maintenance of Intestinal Homeostasis

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Intestinal macrophages constitute the largest pool of macrophages in the body and have emerged as crucial sentinels for pathogen recognition and elimination. The source and development of intestinal macrophages, as well as their distinct properties have been well documented. Intestinal macrophages exert their functions in the maintenance of intestinal homeostasis by shaping host-microbiota symbiosis, managing gut inflammation, crosstalking with T cells, and facilitating wound repair. Recently, nutritional regulation of intestinal macrophages has attracted substantial attention and is becoming a promising approach to disease prevention and control. Understanding the mechanisms employed by intestinal macrophages in mediating intestinal immune homeostasis and inflammation, as well as the mode of action of dietary nutrients in the modulating functions of intestinal macrophages, represents an opportunity to prevent and control inflammatory bowel diseases.

1. Introduction

The gastrointestinal tract mucosa is continually exposed to a high load of antigens, ranging from dietary proteins and commensal microbiota to clinically important pathogens, viruses, and toxins. A single layer of intestinal epithelial cells form a barrier between the lamina propria and the luminal contents of the intestine. Intestinal macrophages that reside in the subepithelial lamina propria (LP) represent the most abundant mononuclear phagocytes in the body and have emerged as crucial sentinels for the maintenance of intestinal homeostasis [1]. As the first phagocytic cells of the innate immune system, intestinal macrophages engulf and clear pathogens, cellular debris, and bacterial products, constantly maintaining a balance between immunity against foreign pathogens and tolerance to commensals [2]. Nonetheless, the cellular and molecular mechanisms by which this critical balance is achieved remain relatively unknown. Due to the crucial role of macrophages in the initiation and development of intestinal immunity, therapeutically manipulating macrophages are becoming an attracting way for disease prevention and treatment. In this review, we focus our attention on intestinal macrophages, describing the recent insights into the role of intestinal macrophages in maintaining gut homeostasis and managing gut inflammation. Finally, we will discuss the nutritional modulation of intestinal macrophage function and the potential of nutritional strategies aimed at manipulating intestinal macrophages to ameliorate inflammatory bowel disorders.

2. Intestinal Macrophages

Intestinal macrophages, which constitute the largest pool of macrophages in the body, are the most abundant mononuclear phagocytes in the LP. Macrophages in the intestine are identified by the expression of F4/80 and CD64 markers, as well as the integrin CD11b [3, 4]. Mature intestinal macrophages also express high levels of the chemokine receptor CX3CR1 [5]. However, with the deepening research on the intestinal mucosal immune system, these characteristic markers have not been able to distinguish intestinal macrophages from other cells. For instance, dendritic cells share

many phenotypic characteristics with macrophages, such as MHCII and CD11b [6, 7]. Thus, additional markers need to be discovered to distinguish intestinal macrophages from other cells.

3. Source and Development of Intestinal Macrophages

Intestinal macrophages, which are thought to play a pivotal role in orchestrating intestinal mucosal immune responses, have received relatively little research attention compared with other tissue macrophages. Macrophages are present in virtually the entire body. In contrast to macrophages from many other tissues, those in the LP of the intestine are continuously replenished from recruited Ly6C$^+$ blood monocytes under steady state or in response to inflammation [8]. These peripheral-blood monocytes develop from hematopoietic stem cells in the bone marrow. During monocyte development, hematopoietic stem cells divide and differentiate in to monoblasts, then promonocytes, and finally monocytes in the presence of macrophage colony-stimulating factor [9]. The CCL2-CCR2 axis plays a critical role in the migration of Ly6C^{hi} monocytes from the bone marrow to the peripheral blood [10, 11]. Bone-marrow monocytes have been classified into two principal subsets with distinct migratory properties in mice [12]. In steady state condition, Ly6C^{hi} monocytes enter the gut mucosa and differentiate into mature CX3CR1^{hi}F4/80$^+$ macrophages via a CX3CR1^{int} transitional stage. These CX3CR1^{hi} macrophages produce PGE2 and help maintain integrity of the gut epithelial layer [13]. Additionally, CX3CR1^{hi} macrophages also secrete interleukin-10 (IL-10), an anti-inflammatory cytokine that maintains mucosal homeostasis [14, 15]. Likewise, lamina propria macrophages drive differentiation of regulatory T (T_{reg}) cells in the intestinal mucosa through production of IL-10 [16]. Signaling mediated by the IL-10 receptor plays a pivotal role in the hyporesponsiveness of murine or human intestinal macrophages. Macrophage-derived IL-10 also maintains survival and expansion of inducible FoxP3$^+$ T_{reg} cells in the LP, which are crucial for tolerance of orally ingested antigens in mice [17]. Impaired production of IL-10 would result in macrophage hyperactivity and inflammatory bowel disease in mice and humans [18, 19]. The IL-10–IL-10R axis, especially IL-10 receptor, is indispensable for gut homeostasis. Macrophages unable to sense IL-10, due to loss of IL-10 receptor, play a central role in the development of severe spontaneous colitis [20]. When intestinal homeostasis is disturbed by infection or inflammation, the normal pattern of monocyte differentiation is disrupted. Ly6C^{hi} monocytes and their CX3CR1^{int} derivatives are recruited to the intestinal mucosa in large numbers during incidents of acute colitis [6]. The CX3CR1^{int} macrophages produce large amounts of TNF-α, IL-6, IL-12, and IL-23, as well as iNOS, rendering them responsive to TLR stimulation to become proinflammatory effector cells [5, 21, 22]. In addition, Ly6C^{hi} monocytes may recruit other innate effector cells via production of chemokines. For example, Waddell et al. (2011) found that Ly6C^{hi} monocytes orchestrated the recruitment of eosinophils through secretion of CCL11 (eotaxin) in a mouse model of dextran sodium sulfate- (DSS-) induced colitis. Importantly, these elicited Ly6C^{hi} monocytes are able to directly control the pathogenic effects of neutrophils and, in particular, the production of TNF-α and ROS by neutrophils in a PGE2-dependent manner [13].

4. The Distinct Properties of Intestinal Macrophages

The epithelial surface of the gastrointestinal tract is exposed to a great mass of bacteria as well as a large number and diversity of dietary antigens. The primary role of intestinal macrophages is to act as innate effector cells in the intestinal LP. To cope with this large antigenic load that may potentially cross the intestinal LP, macrophages in the intestine form some functional adaptations to preserve local tissue homeostasis [23]. Unlike their progenitor cells and blood monocytes, human intestinal macrophages show greatly diminished expression of costimulatory molecules, such as CD40, CD80, and CD 86 [24]. In addition, human resident intestinal macrophages exhibit greater phagocytic activity without initiating an inflammatory response due to their low, or even absent, expression of innate response receptors, including receptors for LPS (CD14), Fcα (CD89), Fcγ (CD64, CD32, and CD16), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) [25]. This hyporesponsiveness enables intestinal macrophages to act as efficient scavengers without inducing inflammation that would normally occur and impair intestinal homeostasis when macrophages encounter pathogens. Finally, human intestinal macrophages also lack the triggering receptor expressed on myeloid cells-1 (TREM-1) [26]. TREM-1 is a cell surface molecule expressed on peripheral blood neutrophils and monocytes/macrophages. This cell surface molecule is an efficient amplifier of inflammation because TREM-1-mediated activation causes enhanced expression of proinflammatory mediators (e.g., TNF, IL-1β, and IL-6) or an upregulation of several cell surface molecules indicating oxidative burst (e.g., CD40, CD86, and CD 32) [27]. The absence of TREM-1 expression on human intestinal macrophages probably contributes to the low level of inflammation observed under physiological conditions, which can be regarded as a further adaptation of intestinal macrophages to the specific environment of the intestinal LP.

5. Functions of Intestinal Macrophages

5.1. Shaping Host-Microbiota Symbiosis. Given the trillions of microorganisms that live in the intestine, the intestinal immune system must continually sustain a balance between immunity to pathogens and tolerance of commensals to prevent needless immune responses against inoffensive bacteria. A question arises about how the immune system discriminates between pathogenic and commensal bacteria. One explanation is that the immune system can discriminate between commensals and pathogens through recognition of symbiotic microbial molecules. *Bacteroides fragilis* is a prominent gut commensal. The symbiosis factor, polysaccharide A (PSA) of *B. fragilis*, is essential for *B. fragilis* to suppress T-helper 17 (Th17) responses during homeostatic colonization

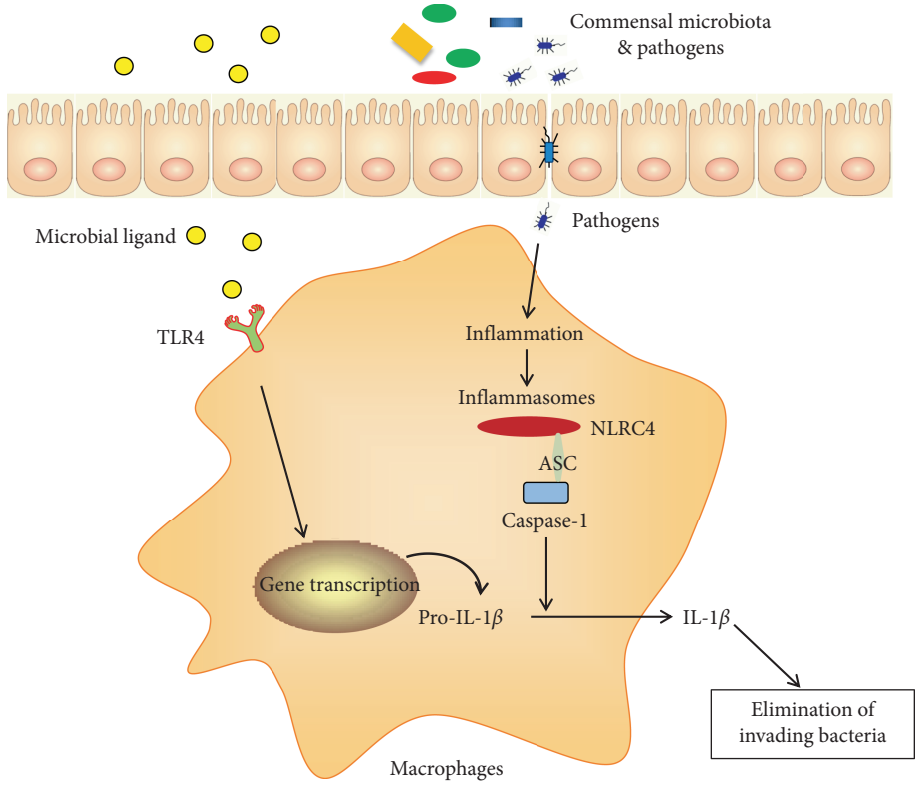

Figure 1: The role of intestinal macrophages in shaping host-microbiota symbiosis. The gastrointestinal tract is colonized by a dynamic community of microorganisms. Intestinal macrophages are anergic to microbial ligand from commensals and consistently produce a precursor to interleukin 1β (pro-IL-1β). Nod-like receptor NLRC4 and the adaptor ASC are crucial components of inflammasome by transmitting pathogenic danger signals to caspase-1 activation. Active caspase-1 is essential for the cleavage of pro-IL-1β into its mature and biologically active form. Mature IL-1β is critical in the elimination of invading bacteria in the intestine.

[28]. In addition, resident macrophages are hyporesponsive to Toll-like receptor (TLR) stimulation but constantly produce pro-IL-1β, whereas pathogens but not commensals could elicit mature IL-1β through the NLRC4 inflammasome. Inflammasomes are molecular platforms inducing the activation of caspase-1, which lead to the secretion of mature and biologically active IL-1β [29] (Figure 1). Additionally, intestinal macrophages can also help maintain intestinal homeostasis by inducing production of anti-inflammatory cytokines, as well as engulfing and degrading commensals [25].

5.2. Managing Gut Inflammation. An increasing body of evidence suggests that macrophages located in the intestinal mucosa have an important role in maintaining the tolerance of commensals while staying responsive to pathogens [2]. However, disorders in enteric bacterial recognition by intestinal macrophages can result in chronic intestinal inflammation, such as inflammatory bowel diseases (IBDs) [30]. Proinflammatory macrophages ($CX3CR1^{int}$ cells) isolated from an inflamed intestine produce large amounts of IL-1β, IL-6, TNF-α, IL-23, and NO [13, 31–33]. Besides contributing to tissue damage, these factors mediate the bactericidal function of macrophages. TNF-α has many functions such as activation and chemotaxis of neutrophils to kill microbes [34]. NO, synthesized by iNOS, is a short-lived gas that possesses beneficial roles in antibacterial activity of macrophages against pathogens [35]. Heme-oxygenase-1 (HMOX-1) is an antioxidant and anti-inflammatory enzyme produced by $CX3CR1^{+}$ macrophages. Previous studies reported that HMOX-1 also helps to control inflammation in the intestine via enhancing phagocytic activity of macrophages [36]. It is well recognized that IL-23 is essential for host defense during the early phase of infection. For example, during the early phase of *Citrobacter rodentium* infection, invasion of the pathogen leads to secretion of IL-23 [37]. IL-23 can stimulate IL-22 production under several infectious conditions [38], and IL-22 seems to be indispensable in protecting the integrity of the intestinal epithelial layer. IL-22 also plays a key role in preventing the spread of pathogens by inducing antimicrobial peptides and chemokines that recruit immune cells to the site of infection [39]. Therefore, proinflammatory intestinal macrophages are essential for protection against pathogenic bacterial infections such as salmonellosis and colibacillosis [25, 40].

5.3. Crosstalk with T Cells. Macrophages can also maintain immunological homeostasis via induction or expansion of regulatory T cells in the intestine [41, 42]. $FoxP3^{+}$ T_{reg} cells play a critical role in intestinal homeostasis. Mice deprived of T_{reg} cells are more susceptible to colitis [43]. In the LP, $CD11^{b+}F4/80^{+}CD11^{c-}$ macrophages induce differentiation of $FoxP3^{+}$ T_{reg} cells via a mechanism dependent on retinoic acid, IL-10, and transforming growth factor-β

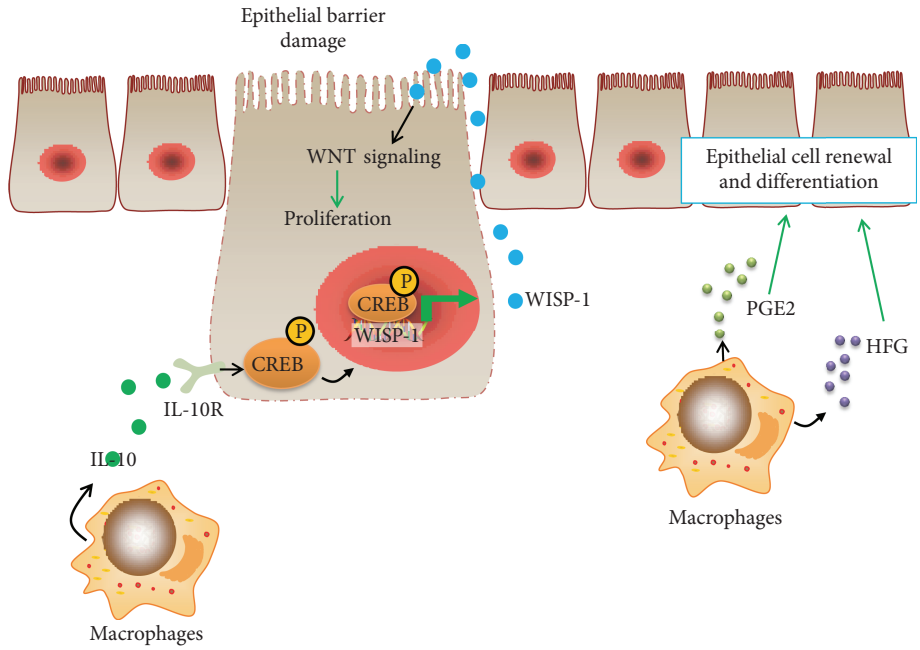

Figure 2: Macrophages contribute to the coordination of wound healing. Macrophages recruited to the sites of intestinal injury produce IL-10, resulting in the activation of cAMP response element-binding protein (CREB) signaling. This signaling enhances secretion of WNT-1-inducible signaling protein 1 (WISP-1) that in turn promotes WNT signaling, epithelial cell proliferation, and wound healing in the intestine. Additionally, intestinal macrophages also secrete prostaglandin E2 (PGE2) and hepatocyte growth factor (HGF), which stimulate renewal and differentiation of the intestinal epithelium.

(TGF-β) [16]. In parallel, the number of FoxP3$^+$ T_{reg} cells in the intestine is correlated with macrophage numbers [44]. Moreover, these FoxP3$^+$ T_{reg} cells have also been reported to have the ability to inhibit inflammatory activity of Th1 and Th17 cells in inflamed intestines [45]. Collectively, these studies emphasize the function of macrophages as a bridge between innate and adaptive immunity against infections in the intestine.

5.4. Wound Repair. Epithelial damage concerned with the impairment of the intestinal mucosal layer occurs following mechanical injury and is a characteristic of inflammatory bowel disease. Repair of the mucosal layer is crucial for alleviating gut inflammation and regaining intestinal homeostasis. Macrophages contribute to the coordination of tissue repair [46] (Figure 2). Macrophages are a major source of IL-10 for healing intestinal mucosa. IL-10 activates the cAMP response element-binding protein (CREB) signaling. This signaling promotes secretion of WNT1-inducible signaling protein 1 (WISP-1) that in turn enhances β-catenin/TCF signaling, epithelial cell proliferation, and repair in the intestine [46]. In addition, Cosín-Roger et al. found that the STAT6-dependent macrophage phenotype accelerated wound healing in the intestinal mucosa of mice treated with 2,4,6-trinitrobenzenesulfonic acid (TNBS) through eliciting the Wnt signaling pathway [47]. Moreover, macrophages have been reported to secrete several mediators including PGE2 and hepatocyte growth factor that can promote renewal and differentiations of intestinal epithelial cells [48, 49]. However, the role of macrophages is a double-edged sword for health. Intestinal macrophages have been reported to be crucial determinants of gut carcinogenesis [50]. Tumor-associated macrophages efficiently trigger angiogenesis that provides nutrition and oxygenation to tumor cells [50]. A better understanding of the modulation of macrophage phenotypes and their tumor-promoting functions would contribute to a promising design of tumor-associated macrophage-centered therapeutic interventions.

6. Influence of Nutrition on Intestinal Macrophage Function

An important role for enteral nutrients in modulation of intestinal macrophages is emerging. Many diet-derived luminal metabolites that are processed by gut microbiota, such as short-chain fatty acids (SCFAs), vitamins, and bile acids, have been demonstrated to regulate immune cell functions in the intestine. In addition, certain nutrients derived from the diet, without processing by microbiota, also possess immunomodulatory functions [51, 52]. Not surprisingly, the effects of dietary nutrients on the regulation of intestinal macrophages have attracted substantial attention in recent years.

6.1. Fatty Acids. Short-chain fatty acids (SCFAs) including acetate, propionate, and butyrate are metabolites of gut bacterial fermentation of dietary fiber that are not digested by host in the small intestine [53]. Increasing evidence suggests that SCFAs have a potential to modulate the immune response in the intestine. Administration of SCFA can alleviate intestinal inflammation and lesions in patients with colitis or in murine colitis models [54, 55]. These immunomodulatory effects of SCFA are probably due to

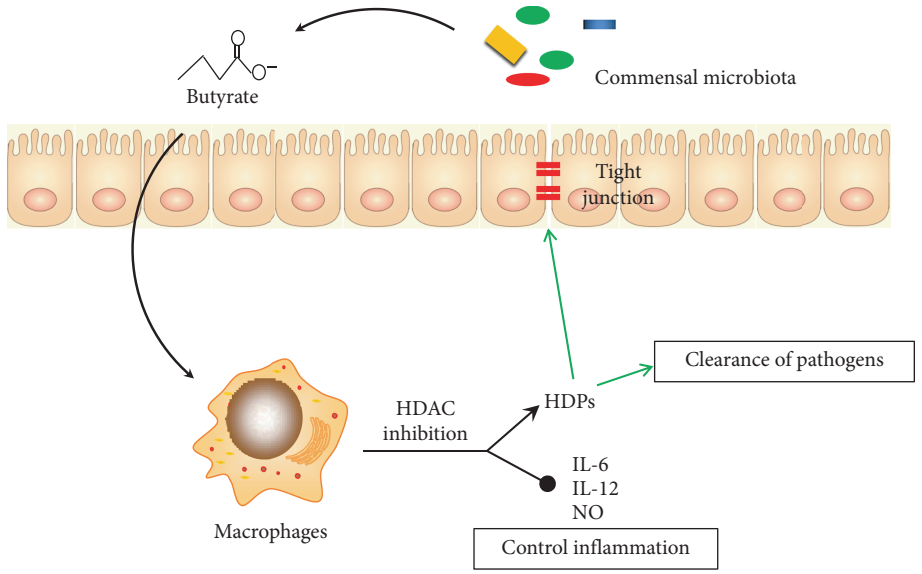

Figure 3: Mechanism of butyrate in modulating intestinal macrophage function. The microbial metabolite butyrate causes intestinal macrophages to reduce secretion of proinflammatory mediators such as IL-6, IL-12, and nitric oxide (NO) via inhibition of histone deacetylase (HDAC). This effect drives the intestinal immune system to be tolerant of commensals. Butyrate is also a strong inducer of host defense peptides (HDPs) that have pleiotropic functions in the maintenance of intestinal homeostasis, such as upregulation of tight junction protein expression in the intestinal epithelium, and clearance of pathogenic bacteria.

their anti-inflammatory properties [56–58]. Recent work has demonstrated that butyrate can modulate intestinal macrophage function, thereby contributing to homeostasis in the intestines [2] (Figure 3). Treatment of macrophages with butyrate results in downregulation of LPS-induced proinflammatory mediators, such as IL-6, IL-12, and nitric oxide. These effects are attributed to inhibition of histone deacetylases by butyrate [2].

6.2. Functional Amino Acids. A deficiency of dietary amino acids is known to cause malnutrition and then impair the intestinal immune system, increasing susceptibility of the host to infectious disease. Accumulating evidence indicates that dietary amino acids have the capability of regulating intestinal macrophage functions [52, 59]. For instance, deprivation of enteral nutrients related to total parenteral nutrition results in a decrease in the number of IL-10-producing macrophages in the small intestine of mice. Whereas dietary amino acids are able to directly regulate replenishment of intestinal macrophages and their IL-10 secretion [52]. However, further studies are needed to elucidate the mechanism by which dietary amino acids modulate macrophage function. It was found that dietary histidine prevented the development of colitis in an IL-10-deficient murine model. The protective effects of histidine were due to its suppression of NF-κB activation in macrophages, thereby inhibiting the production of proinflammatory cytokines such as TNF-α and IL-6 [59]. Furthermore, previous studies have demonstrated that specific amino acids, such as arginine and glutamine, are required for the phagocytic activity of macrophages [60, 61]. Oral administration of tryptophan has been shown to promote phagocytosis by macrophages, which might contribute to increased resistance to pathogenic infections in rats [62]. New knowledge about the role of amino acids in regulation of intestinal macrophage function is important for the development of effective strategies to prevent immunodeficient diseases.

6.3. Vitamins. Vitamin A and its derivative, retinoic acid (RA), modulate a broad spectrum of immune functions. Retinoic acid, the active metabolite of vitamin A, is produced by many subsets of intestinal antigen-presenting cells (APCs) including macrophages and dendritic cells. It has been recognized for decades that vitamin A insufficiency is related to increased susceptibility to various types of infections and impairment of both the innate and adaptive immune systems [63, 64]. Emerging evidence demonstrates that RA has an indispensable role in modulating the functions of APCs in the intestine [65, 66]. Wang et al. reported that RA suppressed IL-12 production while increasing IL-10 production in macrophages [67]. However, vitamin A deficiency was found to exacerbate inflammation in a rat model of colitis [68]. In addition, vitamin A deficiency decreased phagocytic activity and bactericidal capacity of macrophages [65]. Nevertheless, oral administration of RA can inhibit in vivo growth of *Mycobacterium tuberculosis* via downregulating tryptophan-aspartate-containing coat protein (TACO) gene transcription [69]. A previous study demonstrated that downregulation of TACO gene transcription can restrict entry/survival of *M. tuberculosis* in macrophages [70].

Vitamin D is also a strong modulator for macrophage functions. Zhang et al. found that vitamin D suppressed the production of proinflammatory cytokines in macrophages via targeting MAPK phosphatase-1 [71]. In addition, vitamin D(3)-1,25-dihydroxyvitamin D(3) directly stimulates the host defense peptide cathelicidin expression through the vitamin D receptor, which is required for the antimicrobial activity against *M. tuberculosis* in macrophages [72, 73]. Host

defense peptides (HDPs) constitute an important component of the innate immune system and provide immediately effective and nonspecific defenses against infections [74]. Oral supplementation of compounds that induce HDP synthesis has recently become a novel and promising strategy to prevent and control infections in both humans and animals [75, 76]. Myeloid cells, especially macrophages and neutrophils, are major sources of most HDPs. Therefore, the induction of HDPs represents another important mechanism in enhancing macrophage function by vitamin D.

7. Conclusion

Macrophages are indispensable modulators of the innate immune system because they maintain a delicate balance between immunity against pathogenic bacteria and tolerance of commensals in the intestine. Nutritional modulation of intestinal macrophages is becoming a promising approach to disease prevention and has attracted considerable attention. A better understanding of mechanisms employed by intestinal macrophages in maintaining intestinal homeostasis and the action of enteral nutrients in the regulation of intestinal macrophages will facilitate the development of nutritional strategies in gut health improvement as well as prevention and control of inflammatory bowel disorders.

Conflicts of Interest

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

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Research Article

Comparative Serological Study for the Prevalence of Anti-MERS Coronavirus Antibodies in High- and Low-Risk Groups in Qatar

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Infection with Middle East respiratory syndrome coronavirus (MERS-CoV) could be asymptomatic or cause mild influenza-like illness. Therefore, the prevalence of MERS-CoV infections in the general population could be underestimated, which necessitates active surveillance to determine the epidemiological importance of asymptomatic cases. The aim of this study is to evaluate the performance of various serological assays and to estimate the seroprevalence of anti-MERS-CoV antibodies in high- and low-risk groups in Qatar. A total of 4858 samples were screened, including 4719 samples collected from healthy blood donors (BD) over a period of five years (2012-2016), 135 samples from baseline case contacts (CC) collected from individuals in close contact with three positive PCR-confirmed patients (CP), and four samples from MERS-CoV CP. Initial screening using anti-MERS-CoV IgG (IgG rS1-ELISA kit) revealed ten reactive samples from BD (10/4719, 0.21%), one from CC (1/135, 0.74%), and three from CP (3/4, 75%). Samples from CP but not from BD were also reactive by whole-virus anti-MERS-CoV IgG ($n = 3/4$) and IgM ($n = 1/4$) indirect immunefluorescent tests (IIFT) and pseudoparticle neutralization test (ppNT). The reactive sample from CC was also confirmed by ppNT. Surprisingly, one out of thirteen (7.7%) randomly selected IgG rS1-ELISA-negative BD samples from the initial screening was reactive by the IgM-IIFT (but not by the IgG-IIFT) and was subsequently confirmed by ppNT. All IgG rS1-ELISA-reactive samples from BD exhibited considerable reactivity to the four circulating human coronaviruses (HKU1, OC43, 229E, and NL63). Cross-reactivity with SARS was only reported for samples from CP using IgG and IgM-IIFT. In conclusion, we report a low prevalence of anti-MERS antibodies in the general population, which coincides with the low number of all reported cases by the time of our study (2017) in Qatar ($n = 21$). The false-positive results and the observed cross-reactivity between MERS-CoV and other circulating human coronavirus necessitate more detailed evaluation of available serological assays.

1. Background

Middle East respiratory syndrome coronavirus (MERS-CoV) is a human beta-coronavirus (HCoV) that is originally identified in the Kingdom of Saudi Arabia (KSA) in 2012. So far, the WHO has reported 2229 cases of MERS-CoV infections in 27 countries, with a fatality rate of about 36% ($n = 791$) [1].

MERS-CoV-specific antibodies are widely found in dromedary camels (*Camelus dromedarius*) along with viral shedding of similar viruses detected in human. Accordingly, dromedaries are considered the primary source of MERS-CoV transmission to humans, although the original source for the virus is still unknown [2–4].

Phylogenetic analysis groups coronaviruses into four genera: alpha-, beta-, gamma-, and delta-coronaviruses. Bats are considered the natural reservoirs of these viruses. Although SARS-CoV is closely related to bat CoV (BtCoV) HKU3, and MERS-CoV is closely related to Pipistrellus BtCoV HKU5 and Tylonycteris BtCoV HKU4, the serologic and antigenic relationship between these viruses is unclear. Generally, coronaviruses across subgroups demonstrate a low level of cross-reactivity for the S protein and limited preservation of cross-neutralizing epitopes [5, 6]. However, few studies have demonstrated cross-reactivity among these Betacoronavirus. It has been shown that mouse hyperimmune sera to SARS-CoV harbor low levels of neutralizing activity against MERS-CoV [5]. Further, sera samples from SARS patients demonstrated 60.7% (17/28) binding and 25% (7/28) neutralizing activities to MERS-CoV, suggesting cross-reactivity within subgroup viruses [7].

Following its first isolation, several laboratory diagnostic tests for MERS-CoV have been developed [8–11]. Molecular tests such as RT-PCR and sequencing are majorly used in diagnosing MERS-CoV infections [12]. The United States Centers for Disease Control and Prevention (CDC) limits the use of serological tests for investigational or surveillance settings and not for diagnosis [13]. They established a two-phase serological test approach to detect anti-MERS antibodies based on ELISA (targeting S1 antigen) followed by whole-virus IgG and IgM IIFT and microneutralization test for confirmation. The microneutralization assay is highly specific and it is the gold standard for measuring specific neutralizing antibodies against MERS-CoV in sera samples. Nonetheless, compared with the ELISA and IIFT, the microneutralization assay requires a BSL3 facility, which is not available at many places, and it is labor-intensive and time-consuming, requiring at least 5 days before results are available [13, 14].

In the State of Qatar, twenty-one cases have been reported until 2017, including seven deaths (33.3%). Interestingly, 95% ($n = 20$) of the cases in Qatar were reported in males compared to only one female case. Thirteen of the MERS cases were reported in camel farm owners and workers, and five were suspected human-to-human transmissions, of which three were nosocomial infections (Ministry of Public Health-Qatar, personal communication).

Qatar was the first nation to report on the isolation and full genome sequencing of MERS-CoV from camels [3]. In a separate study from Qatar, Reusken et al. reported that ~ 7% (20/294) of persons with camel contact have antibodies reactive with MERS-CoV S1 antigen, compared to zero reactive in control or noncase contact samples. Using 90% plaque-reduction neutralization test (PRNT90), only 10 of the 20 (5%) MERS-CoV S1 antibody-reactive samples were confirmed positive [15].

Due to the uncertain epidemiological picture of MERS-CoV among Qatar population, we designed a staged serologic surveillance study for MERS-CoV consisting of initial screening by anti-MERS-CoV IgG rS1-ELISA kit followed by evaluation of reactive samples using whole-virus indirect immunofluorescence assays (IgM- and IgG-IIFT) and ppNT. We also tested the cross-reactivity of IgG rS1-ELISA-reactive samples with the four circulating human coronaviruses using ELISA and IIFT. This study targeted three groups: (i) low-risk group constituted of 4719 samples obtained from blood donors (BD) collected over a period of five years (2012-2016), (ii) high-risk group represented by 135 samples obtained from baseline case contacts (CC) collected from individuals who were in close contact with confirmed cases during the acute phase (first week), and (iii) four samples from PCR-confirmed MERS-CoV patients (CP). The high-risk group is defined by the individuals that were in direct contact with the confirmed cases either at work, house, or hospital (medical staff), prior or after symptom development. Our findings suggest that MERS-CoV is not heavily circulated among the population of Qatar. Additionally, the presence of antibody responses to other human coronaviruses resulted in false-positive results in binding assays, which mandate the need for more evaluation studies of the currently available diagnostic serological assays.

2. Methodology

2.1. Patient Samples. In total, 4858 plasma samples were analyzed in this study. Samples were distributed as follows: 4719 plasma samples were collected from BD during previous studies [16–20] over a period of five years (2012-2016; age: 19-88 years; mean age 37 years), 135 plasma samples were collected from individuals that were in CC to four CP (age: 14-49 years; mean age 31 years), and four plasma samples were collected from CP (age: 30-70 years; mean age 52). The CC individuals represented the patient's family members, healthcare workers, and camel farm coworkers. Samples from CC were collected within the first week of the patient's admission to hospital. This study was approved by Qatar University-IRB Review Exemption No. QU-QU-IRB 622-E/16.

2.2. Serological Testing. Initially, all plasma samples were screened for the presence of anti-MERS-CoV (S1 subunit) IgG using a commercial IgG rS1-ELISA kit (rS1-ELISA, Euroimmun, cat no. EI 2604-9601G). Since samples from CC were collected within the first week of primary case identification, these samples ($n = 135$) were also tested for the presence of IgM antibodies using whole-virus anti-MERS-CoV IgM IIFT kit (IgM-IIFT) (Euroimmun, cat no. FI 2604-1010 M). The anti-MERS-CoV (IgM/IgG) IIFT is based on MERS-CoV-infected eukaryotic cells and the anti-MERS-CoV ELISA (IgG) on purified S1 antigens of MERS-CoV. As recommended by the WHO, all borderline and reactive samples were then tested for the presence of anti-MERS-CoV antibodies using whole-virus indirect immunofluorescence assay (IgM- and IgG-IIFT) (Euroimmun, cat no. FI 2604-1010). Further, the borderline and reactive samples in addition to 13 randomly selected IgG rS1-ELISA-negative samples (served as negative controls) were screened with an

Table 1: Characteristic profile of the study population.

	BD (2012-2016)			CC (2015-2016)			CP (2015-2016)		
	Number	Age range (mean)	Exposure	Number	Age range (mean)	Exposure (n)	Number	Age range (mean)	Exposure
Qatari males	906	19-88 (37)	Unknown	11	14-49 (31)	Family contact (37), healthcare worker (73), camel farm worker (25)	2	29-69 (51)	Camel farm
Qatari females	22			3			0		
Non-Qatari males	3736			93			2		
Non-Qatari females	55			28			0		
Total	**4719**			**135**			**4**		**4858**

in-house recombinant-S1 protein IIFA IgG (rS1-IIFA; Institute of Virology, Charité - Universitätsmedizin Berlin, Germany; as described by Corman et al. 2012 [8]) in order to reduce the possibility of cross-reactivity of human sera with the full MERS virus antigens presented by Vero cells in whole-virus IIFT. Final confirmation was performed using pseudoparticle neutralization test (ppNT) against two MERS-CoV strains, the EMC strain (GenBank JX869059) and the Jordan N3 strain (GenBank KC776174), as previously described [21]. The determination of cross-reactivity of borderline and reactive samples against other human coronaviruses was performed using: (i) commercially available whole-virus IgM/IgG IIFT for SARS-CoV (Euroimmun, cat no. FI 2601-1010 G/M), (ii) prototype whole-virus IgG IIFT kit for HCoV-229E (Euroimmun, prototype kit), (iii) in-house ELISA for HKU1-CoV using recombinant S1 protein (Sino Biological Inc., catalog # 40021-V08H), and (iv) IgG rS1-IIFT for all other human-CoV (Institute of Virology, Charité - Universitätsmedizin Berlin, Germany) [22].

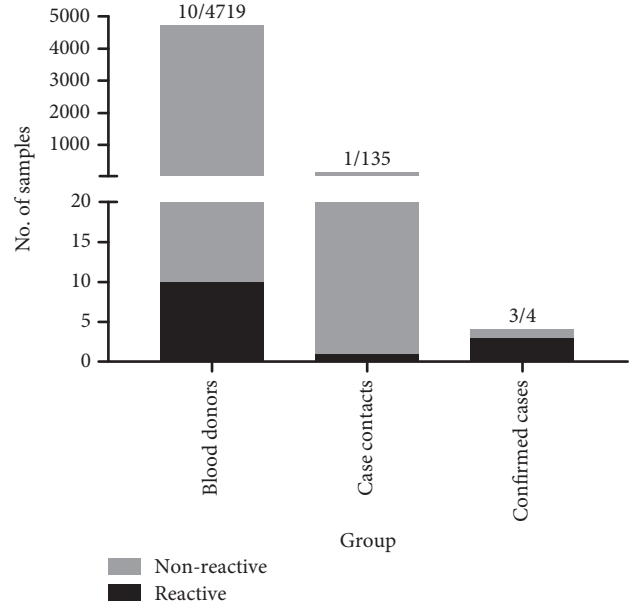

Figure 1: Number of reactive samples using rS1-ELISA (IgG) screening. A total of 4858 plasma samples were initially screened for anti-MERS S1 IgG using rS1-ELISA. The graph shows the number of reactive samples in three groups: blood donor ($n = 4719$), case contacts ($n = 135$), and confirmed cases ($n = 4$).

3. Results

The demography and characteristic profiles of the study population are summarized in Table 1. Initial screening for anti-MERS-CoV antibodies using IgG rS1-ELISA revealed 10/4719 (0.21%) and 1/135 (0.74%) reactive samples from BD (three borderline and seven positive samples) and CC, respectively. On the other hand, 3/4 CP (75.0%) were reactive with IgG rS1-ELISA assay (Figure 1; Table 2). Since CC samples were collected within the first week of primary case identification, samples were also tested by IgM-IIFT and all were negative.

As recommended by the WHO, borderline and reactive samples were then tested for the presence of anti-MERS-CoV IgG using whole-virus and recombinant (r) S1-IIFT. Analysis with whole-virus IgG-IIFT confirmed only two (2/10) samples from BD as well as three (3/4) samples from CP. Interestingly, none (0/10) of the above BD reactive samples tested positive with neither rS1-IIFT nor ppNT assay. The positive IgG rS1-ELISA CC sample was only tested by ppNT and it was positive. All of the randomly selected IgG rS1-ELISA negative BD samples were also negative by whole-virus and rS1 IgG-IIFT (Tables 3 and 4).

To determine the status of infection (recent versus older), all IgG rS1-ELISA reactive samples were further evaluated for the presence of IgM antibodies as an indication for recent infections using whole-virus IIFT (IgM-IIFT), and only one was marginally reactive and that was from a CP (Table 4). Strikingly, one of the 13 randomly selected IgG rS1-ELISA IgG-negative BD samples from the initial screening was found reactive for IgM antibodies (using IgM-IIFT) with a titer of 320. Positivity of this sample was further confirmed with ppNT, with EC50 titer of 500 (Table 3). The sample was obtained from a 35-year-old Syrian citizen residing in Qatar.

Discrepancies in the results obtained from different binding assays could be due to cross-reactivity with other viruses. Hence, we evaluated the cross-reactivity of rS1-ELISA-reactive samples for IgG antibodies against all currently known human coronaviruses. All tested BD samples including the negative controls from the initial screening exhibited

Table 2: Number of reactive samples for anti-MERS S1 IgG using rS1-ELISA.

Sample source	Year of collection (no. screened)	No. borderline/no. screened (%)	No. reactive/no. screened (%)
BD	2012 (120)	*1/120 (0.83)*	0/120 (0)
	2013 (28)	0/28 (0)	0/28 (0)
	2014 (611)	0/611 (0)	*1/611 (0.16)*
	2015 (3383)	*1/3383 (0.03)*	*5/3383 (0.15)*
	2016 (577)	*1/577 (0.17)*	*1/577 (0.17)*
Subtotal	4719	*3/4719 (0.08)*	*7/4719 (0.13)*
CC	May-2015 (100)	0/100 (0)	*1/100 (1)*
	Feb-2016 (10)	0/10 (0)	0/10 (0)
	June-2016 (25)	0/25 (0)	0/25 (0)
Subtotal	135	0/135 (0)	1/135 (0.74)
CP	Mar-2015 (1)	0/1 (0)	*1/1 (100)*
	May-2015 (1)	0/1 (0)	*1/1 (100)*
	Feb-2016 (1)	0/1 (0)	*1/1 (100)*
	May-2016 (1)	0/1 (0)	0/1 (0)
Subtotal	4	0/4 (0)	*3/4* (75)
Total	**Total (4858)**	**3/4858 (0.06)**	**12/4858 (0.25)**

Positive samples are shown in *italic*.

Table 3: Comparative serological analysis of reactive and borderline samples from blood donors (BD).

		rS1-ELISA*	Full virus IIFT		rS1-IIFT	ppNT
	Sample identifier	IgG (OD, ratio, endpoint titer)	IgG titer	IgM titer	IgG titer	IC50 titer (EMC/JordanN3)
Reactive ($n = 7$)	BD 2014/597	*(0.905, 2.114, 201)*	0	0	0	<50
	BD 2015/1303	*(0.397, 1.3, 101)*	*10000*	0	0	<50
	BD 2015/3004	*(0.439, 1.26, 201)*	0	0	0	<50
	BD 2015/3119	*(0.402, 1.06, 401)*	0	0	0	<50
	BD 2015/3380	*(0.477, 1.1, 101)*	*10000*	0	0	<50
	BD 2015/3513	*(0.497, 1.39, 401)*	0	0	0	<50
	BD 2015/4435	*(0.661, 1.74, 201)*	0	0	0	<50
Borderline ($n = 3$)	BD 2012/2644	*(0.333, 0.83, 101)*	0	0	0	<50
	BD 2015/1816	*(0.456, 0.823,201)*	0	0	0	<50
	BD 2015/4708	*(0.408, 1.07, 101)*	0	0	0	<50
Selected negative (showing 3/13) **	BD 2015/2859	(0.034, 0.076, <101)	0	0	0	<50
	BD 2015/2988	(0.039, 0.112, <101)	0	0	0	<50
	BD 2015/3379	(0.065, 0. 16, <101)	0	*320*	0	*531/502*

*Initial screening was done with rS1-ELISA, and reactive samples were further tested with various serological assays as indicated above. **13 negative samples from the initial screening with rS1-ELISA were selected for comparison, and one was found positive with full virus IgM and ppNT. Positive samples are shown in *italic*.

reactivity to at least 3 of 4 human coronaviruses. All rS1-ELISA-reactive samples were reactive to the four seasonal coronaviruses: 229E, HKU1, OC43-CoV, and NL63 (Table 5). The reactivity was also high in the negative controls from the initial screening reaching 100% (13/13) for 229E, 92% (12/13) for HKU1 and OC43, and 84% (11/13) for NL63 (partial data is shown in Table 5). None of the tested BD samples were reactive to SARS-CoV using whole-virus or rS1-IIFT IgG. Similarly, all samples from CP were also highly reactive with other human coronaviruses. Interestingly, two of the CP samples had considerable reactivity to SARS-CoV with titers of 320 and 3200 using IgG rS1-IIFT (Table 5).

Discrepancies in cross-reactivity were also observed among different serological tests for human coronaviruses. For example, one sample from CP tested negative with whole-virus IIFT IgG for HCoV-229E, but it was reactive with recombinant S1 protein of the same virus using similar assay. Similarly, two samples from CP showed reactivity to SARS-CoV in the IgG rS1-IIFT, whereas only the sample with higher antibody titer reacted with the whole-virus IgG-IIFT assay. Further, all samples from CP reacted with HKU1 spike protein in rS1-ELISA, but only two samples yielded positive reaction with the IgG rS1-IIFT (Table 5).

Table 4: Comparative serological analysis of reactive samples from CC and CP.

Sample identifier	rS1-ELISA IgG (OD, ratio, endpoint titer)	Full virus IIFT IgG titer	Full virus IIFT IgM titer	rS1-IIFT IgG titer	ppNT IC50 titer (EMC/JordanN3)
CC May.2015	*(0.61, 1.5, 101)*	Quantity not sufficient	Quantity not sufficient	Quantity not sufficient	*76/149*
CP Mar.2015	*(1.084, 2.86, 201)*	*10000*	0	*>10000*	*630/1707*
CP May.2015	*(0.412, 1.37, ND*)*	*3200*	0	*320*	*199/688*
CP Feb.2016	*(2.229, 6.517, ≥ 3201)*	*>32000*	*100*	*>10000*	ND

Positive samples are shown in *italic*. ND: not determined. *This samples showed controversial results in rS1-ELISA IgG and was considered positive based on IIFT.

Table 5: Cross-reactivity of reactive samples with rS1-ELISA and other human coronaviruses.

	Sample identifier	Whole-virus IIFT IgG titer			rS1-ELISA titer IgG		rS1-IIFT titer IgG		
		229E	SARS	HKU1	229E	OC43	SARS	NL63	HKU1
Reactive BD	BD 2014/597	*≥320*	0	*≥100*	*320*	*320*	0	*3200*	*3200*
	BD 2015/1303	*320*	0	*≥101*	*3200*	*3200*	0	*3200*	*320*
	BD 2015/3004	*≥1000*	0	*≥101*	*320*	*3200*	0	*3200*	*3200*
	BD 2015/3119	*≥320*	0	*≥101*	*320*	*320*	0	*320*	*320*
	BD 2015/3380	*320*	0	*≥101*	*3200*	*3200*	0	*320*	*320*
	BD 2015/3513	*≥1000*	0	*≥101*	*3200*	*3200*	0	*3200*	*3200*
	BD 2015/4435	*≥320*	0	*≥101*	*320*	*3200*	0	*3200*	*3200*
	BD 2012/2644	*≥1000*	0	*≥101*	*320*	*320*	0	*320*	*320*
Borderline BD	BD 2015/1816	*≥320*	0	*≥101*	*3200*	*3200*	0	*3200*	*3200*
	BD 2015/4708	ND	ND	*≥101*	*3200*	*3200*	0	*320*	*3200*
Selected negative BD (showing 3/13)	BD 2015/2859	ND	ND	ND	*3200*	*3200*	0	*3200*	*3200*
	BD 2015/2988	ND	ND	ND	*320*	*320*	0	0	*320*
	BD 2015/3379	*320*	*(IgM = 0)*	0		(IgM = 0)	*≥101*	*3200*	*3200*
0	*320*	*320*							
Reactive CP	CP Mar.2015	*100*	0	*≥101*	*3200*	*320*	*320*	*320*	*320*
	CP May.2015	*1000*	0	*≥101*	*3200*	*3200*	0	*320*	*3200*
	CP Feb.2016	0	*1000*	*≥101*	*3200*	*>10000*	*3200*	0	0

Positive samples are shown in *italic*. ND: not determined.

4. Discussion

Qatar reported a relatively low number of MERS cases in comparison to neighboring countries despite the fact that MERS-CoV continues to circulate in camels [23, 24]. In the absence of a clear epidemiological view of MERS-CoV, we present here a comparative serological study for the prevalence of anti-MERS coronavirus antibodies in high- and low-risk groups in Qatar.

Following the WHO recommendation, we run initial screening for IgG antibodies using rS1-ELISA, and reactive samples were then confirmed with full virus and rS1-IIFT IgG, followed by ppNT. Combined results from different serological tests indicate the low presence of neutralizing anti-MERS-CoV antibodies in the general population in Qatar (1/4719), while the rate increases to 1 : 135 in the high-risk group (CC). Our results revealed a few interesting observations. First, the only confirmed positive sample from the BD group was accidentally detected when we tested 13 randomly selected negative samples (originally selected to serve as a negative control) from the initial screening (using rS1-ELISA-IgG) for IgM response, where screening for IgM is not typically done in similar studies [14]. This mandates the development and utilization of assays that measure both classes of the antibodies for screening processes. Nonetheless, our results coincide with the low number of reported cases in Qatar ($n = 21$) compared to the neighboring countries such Saudi Arabia that has the highest number of reported MERS cases worldwide ($n > 1700$) [25, 26]. In a similar study using a similar approach in Saudi Arabia, 15/10009 (0.15%) were confirmed positive for anti-MERS antibodies in the general population. These numbers are slightly higher than what we observed in our study (1/4719; 0.02%); however, the low number of positive samples in both studies prevents a significant statistical analysis [14].

Another interesting observation was the seropositivity in CC samples. Although those samples were collected within the first week of primary case identification, only one sample was positive for IgG but not for IgM antibodies, indicating previous exposure to the virus. It also

confirms that infection with MERS-CoV can go unnoticed and that surveillance studies shall be done systematically to include screening for IgM and IgG responses. Similar to the above observation in the general population, the rate of seropositivity in the high-risk group (CC) in our analysis was lower (1/135; 0.74%) than that observed in Saudi Arabia (7/227; 3.08%). Several factors could explain the difference between both studies including the difference in sample size as well as the demographics and livestock population in both countries.

One out of four MERS CP samples did not show antibody response using rS1-ELISA, whole virus, and rS1-IIFT assays. The sample was collected during the acute infection phase, which explains the absence of anti-MERS-CoV IgG response. A recent study from South Korea indicated that humoral response to MERS-CoV, as measured by binding and neutralizing assays, wean rapidly after one year of infection and becomes undetectable in about 67% of those who show mild illness upon infection [27]. In another study from Korea, it was shown that none of the asymptomatically infected individuals showed seroconversion; however, the seroconversion rates gradually increased with increasing disease severity reaching 60.0%, 93.8%, and 100% in symptomatic infection without pneumonia, pneumonia without respiratory failure, and pneumonia progressing to respiratory failure, respectively [28]. Such studies indicate that human humoral immune response to MERS-CoV is a complicated phenomenon that requires further investigation. It further implies that the infection rate with MERS-CoV in the Middle East could be underestimated and that the fatality rate associated with MERS-CoV infection is most likely overestimated.

As observed in other studies, the rS1-ELISA resulted in significant false-positive results. While 10/4719 (0.2%) samples were positive in this assay, only 2/10 (20%) were positive with whole-virus IgG-IIFT and null were confirmed with rS1-IIFT and ppNT (0%). Similarly, out of 10009 tested samples in the Saudi Arabia study, 152 were positive with rS1-ELISA (1.5%), 17 of which (11%) were positive with whole-virus IIFT and 15 were confirmed with neutralization assay [14]. These results indicate cross-reactivity between MERS-CoV and other human coronaviruses. To test this further, we employed several binding assays to test the reactivity of our samples with five known human coronaviruses, namely, SARS, HKU1, 229E, OC43, and NL63 CoV. Interestingly, all MERS-reactive samples from first screening, as well as most of the randomly selected negative control samples, had cross-reactivity to at least 3/4 seasonal human viruses, but none of them were cross-reactive with SARS-CoV. On the other hand, two of the CP samples were cross-reactive with SARS-CoV with intermediate titers. Accordingly, it is not clear which of the human coronavirus is inducing this cross-reactivity with MERS-CoV, which mandates further investigation. We also observed discrepancies in cross-reactivity when using different assays, where rS1-based IIFT were more sensitive than full virus counterparts. That could be due to the higher concentration of the specific S1 protein in the first assay, which also ensures the use of defined regents (purified proteins) for developing screening assays.

On the other hand, cross-reactivity between SARS and MERS-CoV has been reported earlier. A 2013 study by Chan et al. indicated that 17/28 (60.7%) of SARS patients had significant binding antibody titers (using IIFT), of which seven (25%) had anti-MERS (EMC) neutralizing antibodies at low titers, which significantly correlated with that of HCoV-OC43 [7]. In the same study, bioinformatics analysis demonstrated a significant B-cell epitope overlapping the heptad repeat-2 region of spike protein between the two viruses [7].

We acknowledge few limitations to our study including the use of ELISA procedure to screen for anti-MERS-CoV IgG response, dominance of males over females (4642 versus 77), and non-Qataris over Qataris (3791 versus 928), as well as the screening at one-time point. Our findings affirm on the use of neutralization assay, and to a lower extent spike protein-specific IIFT, as confirmatory tests considering the high cross-reactivity among different human coronaviruses. Such findings were similar to what has been recently reported by Drosten et al. who showed that excess in IgG detection by anti-MERS rS1-ELISA IgG was not confirmed with IgG-IIFT assay [29].

5. Conclusion

Our results indicate a high discrepancy between different assays available in the market to screen for anti-MERS-CoV antibodies. ELISA-based assay seems to be more prone to produce false-positive results, while results of IIFT should always be confirmed by neutralization test. Most of the available ELISA assays utilize S1 protein, which is part of the whole protein and in many cases is not well characterized before use. Using affinity purified and structurally defined protein might be more reliable to produce accurate results. The recent determination of several ectodomain structures from many coronaviruses should be instrumental to design better immunogens [30–36], which can also be utilized to develop more specific screening assays. Lastly, further investigation of the possibility of MERS-CoV transmission through a blood transfusion from asymptomatic donors should also be assessed, though no cases have been reported so far with evidence of blood transfusion as a source of MERS-CoV infection.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The findings achieved herein are solely the responsibility of the authors.

Conflicts of Interest

All authors declare that they have no conflict of interest to disclose.

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Research Article

Lymphocytic Choriomeningitis Virus Infection Demonstrates Higher Replicative Capacity and Decreased Antiviral Response in the First-Trimester Placenta

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Lymphocytic choriomeningitis virus (LCMV) is a rodent disease that can be transmitted to humans. A majority of persons infected with LCMV have only minor symptoms; however, it can cross the placental barrier during pregnancy and cause congenital defects in the fetus. Some viral infections early in gestation are hypothesized to lead to worse outcomes compared to those acquired during late gestation; however, LCMV has not been studied in this context. In the present study, differences in immunomodulation between the first- and third-trimester placental explants infected with LCMV were measured. LCMV replication was observed in the first-trimester chorionic villi, but not in term. The term placenta exhibited a robust innate immune response to infection by LCMV, marked by induction of *ifn-α*, *il-6*, and *tnf-α* gene expression which was not seen in the first-trimester explants. Cytokine secretion was also only seen in term explants. The results indicate that the first-trimester and term placentas differ in their permissiveness for LCMV infection, inversely correlating with the innate antiviral responses. This has implications for developing effective mechanisms that protect the fetus from infection based on stage of development.

1. Introduction

Lymphocytic choriomeningitis virus (LCMV) is an arenavirus native to rodents which is shed at high levels through excrement [1]. Although mice are the most common reservoir for LCMV, humans can acquire it by direct contact with fomites, through breathing in aerosolized virus or through organ transplantation [2]. Infection with LCMV as an adult or child is similar to symptoms of meningitis and will lead to a full recovery. However, if contacted during pregnancy, this single-stranded RNA virus can cause transplacental human fetal infections with serious clinical consequences [3]. Like many congenital pathogens, LCMV has a tropism for fetal neural and retinal tissue, causing issues with brain development including microencephaly, periventrictular calcification, cerebellar hypoplasia, and hydrocephalus [4, 5]. Meta-analysis demonstrated that children with congenital LCMV infection have a 35% mortality rate by approximately 2 years of age; those who survive have long-term neurological impairment and/or vision impairment [5]. The incidence of congenital LCMV is unknown, and infants with suspected congenital infection are not commonly tested for this viral pathogen. However, 9% of mice carry LCMV and 5% of humans are seropositive for the virus [6, 7], indicating that it may be an underdiagnosed etiology.

Congenital viral infections generally manifest with more severe fetal disease following the first-trimester maternal infection, when compared to infection later in gestation. For example, fetuses infected with Zika virus during the first trimester are known to be at increased risk for structural abnormalities [8, 9]. In a rat model of LCMV, pups introduced to the virus early in gestation (days 1-10) had more frequent and severe neuropathologies compared to pups exposed later in gestation [10]. In part, this effect has been attributed to the teratogenic impact of infection during early fetal development—most evident after early

transplacental rubella and varicella infections [11, 12]. However, maternal-fetal immune interactions evolve throughout pregnancy [13], possibly changing the placental response to viral pathogens as pregnancy progresses.

Currently, no standard *in vitro* models exist for the study of the pathophysiology of human congenital viral infections. LCMV serves as a model virus for induction of robust innate and $CD8^+$ T cell immune responses in the murine model and has been shown to activate innate immunity through Toll-like receptor- (TLR-) 2 [14, 15]. Human placental tissue explants, especially those from the first and third trimesters, are useful models for studying viral infection as well as development, toxicology, and cellular interactions [16]. Other investigators have studied infection of the first-trimester placental explants with human immunodeficiency virus (HIV) and human cytomegalovirus (CMV) which are known to transit the placenta [17, 18].

The present study is aimed at using human placental explants to model LCMV infection and study differences in the innate immune response during the first and third trimesters. The working hypothesis is that antiviral responses will be activated in placental tissues from term pregnancies, but not in those from early pregnancy. In addition, a robust immune response from placental tissue may suppress LCMV replication.

2. Materials and Methods

2.1. Viruses. This study utilized LCMV strain Armstrong RHK 11.7.1989 (Sealy Center for Vaccine Development, University of Texas Medical Branch), which was propagated on Vero E6 cells (ATCC). Titer was determined by plaque assay in Vero E6 cells with 0.5% agarose overlay, followed by neutral red staining on day 4 postinfection.

2.2. Human Placental Explants. This study was approved by the University of Texas Medical Branch (UTMB) Hospital Institutional Review Board. Tissue was collected through a deidentified biobank, providing only the gestational age at collection and confirmed live singleton pregnancy. Eligible patients were enrolled in the study at the time of term cesarean delivery (≥37 weeks) or elective termination of pregnancy (5-14 weeks). Patients were at least 18 years of age and were excluded from the study if they had fever, preterm labor, HIV, syphilis, hepatitis B or C, or other clinically evident infections. Tissues were handed off aseptically, and chorionic villi were dissected from beneath the chorionic plate and washed in phosphate-buffered saline (PBS). They were cut into explants approximately 1-3 mm in diameter and suspended in 75 μm Netwells (Corning) containing RPMI 1640 with 1 mM HEPES, penicillin, streptomycin, amphotericin B, and 10% FBS. Explants were maintained at 37°C with 8% O_2 as previously described [16]. Explants were inoculated with 2×10^4 pfu/mL LCMV (1 : 1000 stock dilution) for 2 hours before the media was changed. The media was sampled daily for viral titer. Explants for tissue harvest were washed in cold PBS and lysed on ice by sonication, and supernatant was collected by centrifugation to remove cellular debris. Supernatant was passed through a 200 μm filter prior to dilution in RPMI 1640 for plaque assay.

2.3. RT-PCR. 200 mg explant tissue was incubated with 12 μg/mL polyI:C with FuGENE transfection agent (Promega, Madison, Wisconsin) or 2×10^4 pfu/mL LCMV for 24 hours and transferred into RNAlater (Thermo Fisher Scientific, Waltham, Massachusetts). Total tissue RNA was extracted using RNAqueous kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time amplification was carried out using the Applied Biosystems 7500 Fast System with TaqMan® reagents according to the manufacturer's instructions (Thermo Fisher Scientific). TaqMan® gene expression assays were used with probes for *ifn-γ* (Hs0017443_m1), *ifn-α* (Hs00356648_s1), *il-6* (Hs00174131_m1), *tnf-α* (Hs00174128_m1), *il-29* (Hs00601677_g1), and *il-28* (Hs00820125_g1). Expression was compared to that of *β*-actin, and four seperate subjects were used for each experiment run in triplicate.

2.4. Protein Determination. Placental explants with 12 μg/mL polyI:C with FuGENE or 2×10^4 pfu/mL LCMV were incubated for 24 h, and media was collected to determine baseline secretion of interferon- (IFN-) α, interleukin- (IL-) 2, IL-6, IFN-γ, and tumor necrosis factor- (TNF-) α. Concentrations of proteins were measured by Bio-Plex assay according to the manufacturer's instructions (Bio-Rad, Hercules, California). Lactate dehydrogenase (LDH) cytotoxicity assays were used to quantify cell death (Thermo Fisher Scientific). Four separate placentas from the first trimester and term were analyzed in duplicate.

2.5. Statistical Analysis. ELISA and RT-PCR data are expressed as mean ± standard deviation. Comparisons between the first and third trimesters were completed by a nonparametric *t*-test. For multiple comparison analysis, one-way ANOVA with the Bonferroni-Sidak correction was utilized. Significance was defined as a p value ≤ 0.05.

3. Results

3.1. LCMV Does Not Replicate in the Term Placenta. First, placental explants were infected with 2×10^4 pfu/mL LCMV, and then media was collected for up to 5 days to measure viral secretion. Figure 1(a) demonstrates that explants from 5-12 weeks of gestation actively secreted the virus; however, term (third trimester) placental explants did not support replication of LCMV (Figure 1(b)). Additionally, tissue lysates demonstrated viral replication in the first trimester tissue but not in the third trimester in 4-8 days of postinfection (Figure 1(c)). The lack of replication in the term placenta versus the first trimester was not due to differences in cell viability as measured by LDH secretion (Figure 1(d)). Although a trend was visible, no difference in LDH secretion was observed between the first and third trimesters and infected versus uninfected explants ($p = 0.1515$).

3.2. LCMV Induces Antiviral Responses in the Term, but Not in the First-Trimester, Placenta. Using real-time, quantitative

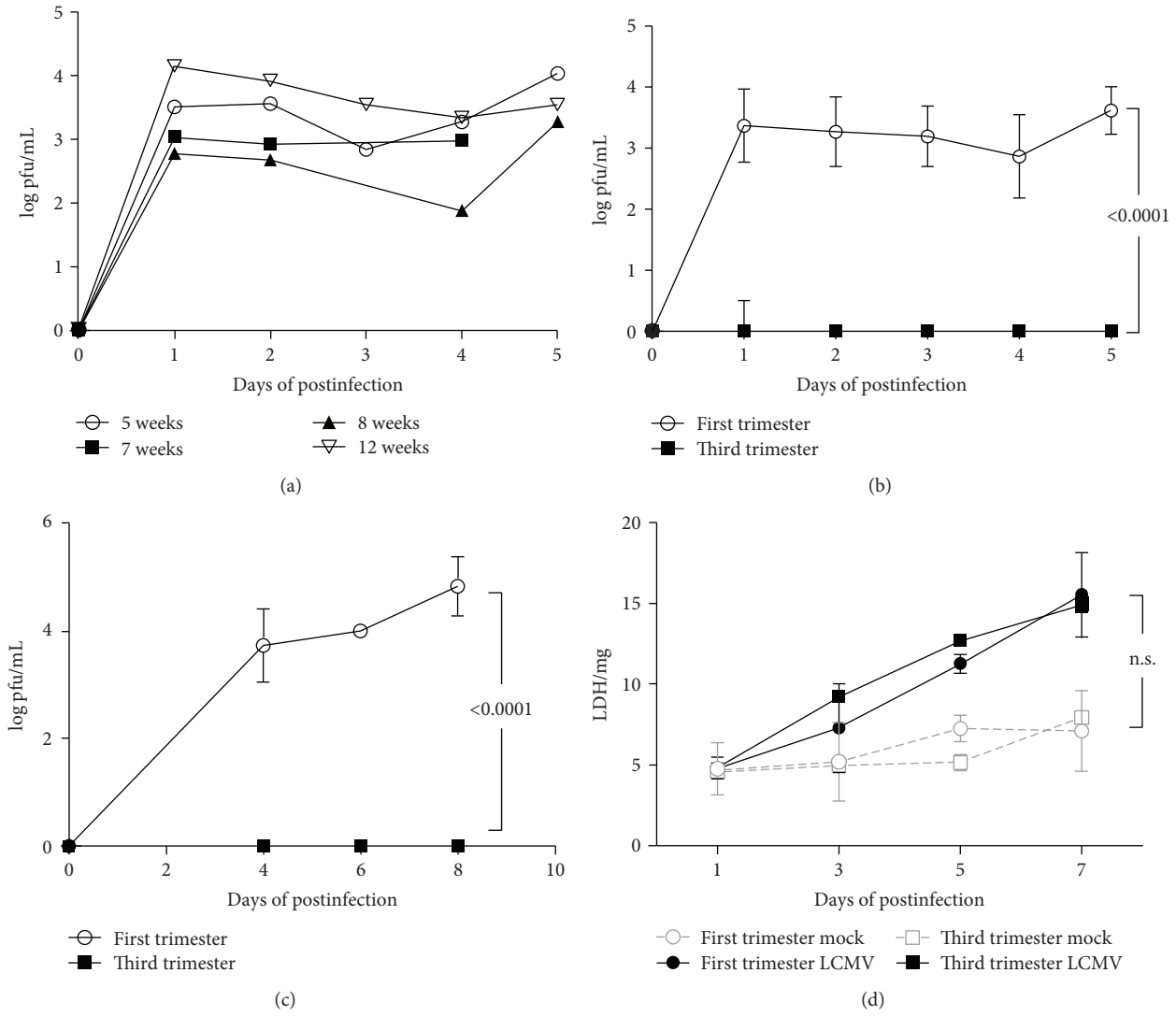

Figure 1: LCMV replication is more robust in the first-trimester placental explants. (a) The first-trimester human placental explants are permissive to viral replication at multiple gestational ages ($n = 1$/group). (b) LCMV titer placental villus explant tissue lysates from the first trimester and term. (c) LCMV viral secretion into the media starting at day 4 after infection. (d) LDH release by the first-trimester and term placentas. Data is presented as the mean and standard deviation of four different subjects in each group. Significance ($p \leq 0.05$) was determined by ANOVA.

RT-PCR, we analyzed induction of antiviral responses in the term and first-trimester placenta after treatment with polyI:C (synthetic RNA analog that signals through TLR-3) or LCMV for 24 hours. Each experiment was performed using tissue from four different subjects. Graphs represent the evaluation of differences between trimesters, analyzed by ANOVA (Table 1). Basal cytokine mRNA levels were not altered between the first-trimester and third-trimester tissues. LCMV induced mRNA expression of *ifn-α* (Figure 2(a)), *il*-6 (Figure 2(b)), and *tnf-α* (Figure 2(c)) in the term, but not the first-trimester, placenta. In response to TLR agonist polyI:C, *il*-6 and *tnf-α* had a more robust mRNA production following stimulation in the term placenta than in the first-trimester explants. Interestingly, *ifn-γ* followed an opposite pattern, trending toward lower transcript production in the third-trimester compared to the first-trimester tissue (Figure 2(d)). Expression of type III interferons *il-28* and *il-29* was also examined in LCMV infection. No increase in mRNA expression was demonstrated after LCMV infection in the first- or third-trimester tissue (Figures 2(e) and 2(f)). However, polyI:C did activate *il-28* transcription more robustly in the third-trimester explants compared to the first.

3.3. The First-Trimester Explants Do Not Secrete Cytokines following LCMV Infection. Bio-Plex assays were utilized to measure cytokine secretion of IFN-*α*, interleukin- (IL-) 6, IFN-*γ*, and TNF-*α* following infection with LCMV or stimulation with polyI:C. Each sample was run in duplicate, using the placentas from four individual subjects. Again, the graphs represent the differences in comparisons between trimesters. IFN-*α* concentration was under the limit of detection for this assay in both term and the first-trimester explant cultures. Increased TNF-*α* and IL-6 secretion was observed with the third-trimester placenta after LCMV or polyI:C incubation

Table 1: ANOVA comparisons between the first-trimester and third-trimester explants.

Comparison	Mean (relative expression or pg/mL)	p value
ifn-α		
Neg.	1 vs. 1	0.999
PolyI:C	1.7 vs. 1.2	0.9777
LCMV	0.3 vs. 8.1	*0.0002*
ifn-γ		
Neg.	1 vs. 1	0.999
PolyI:C	4.4 vs. 3.3	*0.0178*
LCMV	1.5 vs. 1	0.4358
il-6		
Neg.	1 vs. 1	0.999
PolyI:C	3.6 vs. 42.4	*<0.0001*
LCMV	0.8 vs. 16	*<0.0001*
tnf-α		
Neg.	1 vs. 1	0.999
PolyI:C	2.9 vs. 5.8	*<0.0001*
LCMV	1.5 vs. 9.8	*<0.0001*
il-28		
Neg.	1 vs. 1	0.999
PolyI:C	5.8 vs. 10.6	*<0.0001*
LCMV	0.8 vs. 0.7	0.999
il-29		
Neg.	1 vs. 1	0.999
PolyI:C	12.6 vs. 15.8	*<0.0001*
LCMV	1.9 vs. 2.9	0.2201
TNF-α protein		
Neg.	1000 vs. 3536	0.11
PolyI:C	1000 vs. 12,604	*<0.0001*
LCMV	1000 vs. 6983	0.0002
IL-6 protein		
Neg.	1449 vs. 23,737	*<0.0001*
PolyI:C	2386 vs. 50,633	*<0.0001*
LCMV	2043 vs. 42,250	*<0.0001*
IFN-γ protein		
Neg.	0 vs. 0	0.9999
PolyI:C	0 vs. 414	*<0.0001*
LCMV	0 vs. 15.3	*<0.0001*

(Figures 3(a) and 3(b)). Conversely, this cytokine response was not seen in the placentas from the first trimester. PolyI:C induced a strong IFN-γ response in the term placenta but not in the first-trimester placenta (Figure 3(c)). This indicates that the antiviral immune response to LCMV and polyI:C was more robust in the term placenta than in the first-trimester tissue.

4. Discussion

Congenital viral infections can lead to fetal complications that can negatively affect the health of the baby, including birth defects or death. In addition, pregnant women infected by viruses, including varicella, measles, or flu, are at greater risk of complications and even death compared to non-pregnant women [19, 20]. Clearly, pregnancy impacts the maternal immune response, but mechanisms for increased maternal and fetal susceptibility to certain pathogens are unclear. Congenital viral pathogens can cross the fetal-maternal interface and infect trophoblast cells, Hofbauer cells, chorionic villi, and amniotic fluid, which have been extensively reviewed [21]. Lymphocytic choriomeningitis virus (LCMV), although less well characterized, is no exception. Though the incidence of LCMV infection during pregnancy is unknown, it has been shown to cause microcephaly, periventricular calcification, cerebellar hypoplasia, and hydrocephalus due to its tropism for neural tissue [3–5]. Data presented here characterize differences in LCMV permissiveness and antiviral responses in human explants from the first-trimester and term placenta.

First, LCMV was determined to replicate effectively and be secreted into the media of the first-trimester explants, regardless of gestational week. However, viral replication was not detected in tissue or media from term explants, and this effect was not due to increased cell death. It is possible that the term placenta may have developed protective mechanisms to deal with LCMV infection compared to the first trimester. This result corresponds with data from other viral infections that indicate early infection leads to worse fetal outcomes [8–10], adding new data that demonstrates LCMV infection is no exception to previous findings.

During LCMV infection, TLR2 and MyD88 knockout mice have shown decreased cytokine production and cytotoxic T cell responses resulting in persistent infection [15]. TLR2 expression in the human placenta is localized mainly to endothelial cells and macrophages; however, expression of TLR2 on syncytiotrophoblasts and fibroblasts is also seen [22]. Infection in the murine placenta also increases the expression of TLR2 as a protective mechanism [23]. PolyI:C is a well-defined double-stranded RNA analog that signals through intracellular TLR3, leading to expression of type I interferons (IFN-α/β), TNF-α and IL-6. The data demonstrate an increase in mRNA expression of innate cytokines in the third-trimester explants compared to the first-trimester explants exposed to both LCMV and polyI:C. Relative gene expression of *ifn-α*, *il-6*, and *tnf-α* in the term placentas infected with LCMV was higher compared to the first-trimester gene expression and followed a similar pattern to polyI:C stimulation. mRNA expression of type II interferon *ifn-γ* and type III interferons, *il-28* and *il-29*, was increased with polyI:C stimulation but not with LCMV infection, suggesting that these may not be critical regulators of innate responses to this virus. In the human placenta, IFN-γ expression decreases as pregnancy progresses, whereas the IFN-γ receptors are expressed throughout [24, 25], indicating that antiviral actions of this cytokine by trophoblasts are more likely to be crucial early in gestation but can respond to the release of IFN-γ by other immune cells, such as natural killer cells.

Interestingly, no protein production of IFN-α was observed, although TNF-α and IL-6 cytokine secretion was

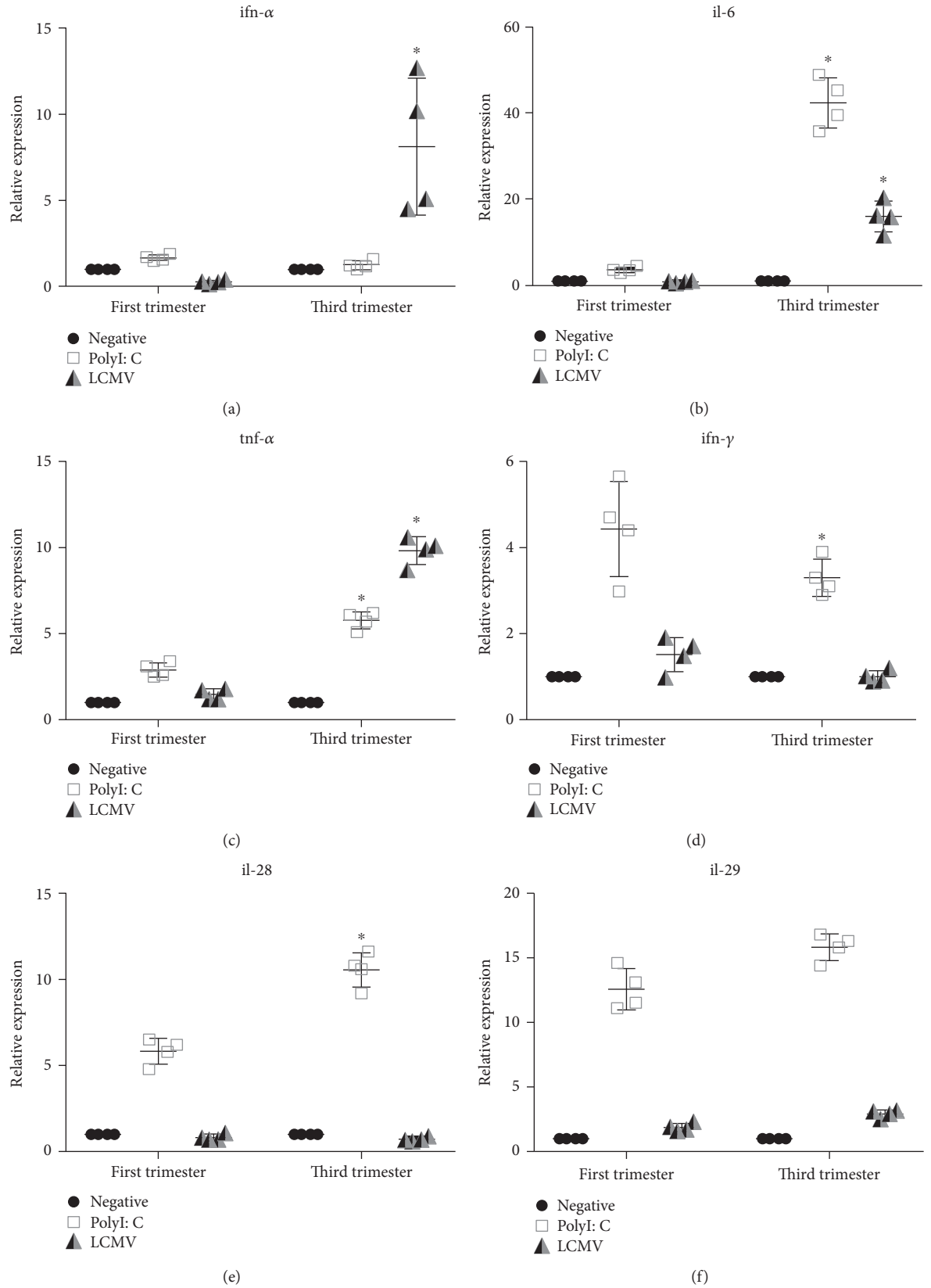

Figure 2: Innate immune responses are activated in term, but not in the first-trimester, human placental explants infected with LCMV. Term and the first-trimester placental explants were analyzed for mRNA expression by RT-PCR. (a) *ifn-α*, (b) *il-6*, (c) *tnf-α*, (d) *ifn-γ*, (e) *il-28*, and (f) *il-29*. Graph represents relative quantities and standard deviation of mRNA normalized to *β*-actin. Significance is defined as a p value ≤ 0.05 comparing the first vs. third trimester, denoted by * ($n = 4$ per group).

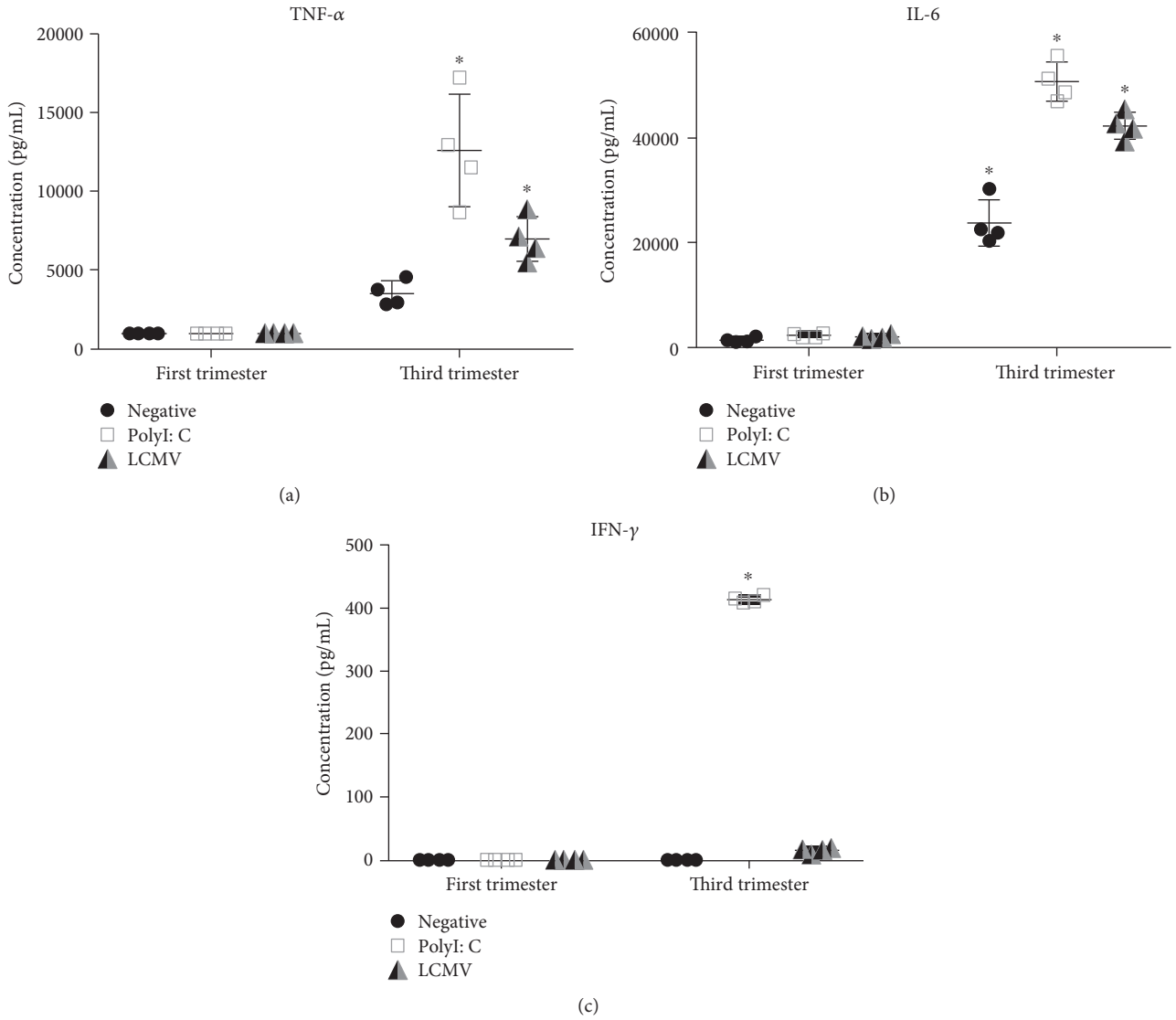

Figure 3: Cytokine secretion is seen in response to LCMV in term placental explants but not in the first-trimester tissue. Term and the first-trimester placental explants were analyzed for cytokine secretion after infection. (a) TNF-α, (b) IL-6, and (c) IFN-γ. Mean and standard deviation are shown. Significance is defined as a p value ≤ 0.05 comparing the first vs. third trimester, denoted by * ($n = 4$ per group).

noted in term explant cultures, but not in the first-trimester cultures. Type I interferon responses are important modulators of viral infection in the placenta, and viruses have found ways to evade this response. In IFN-α/β receptor (IFNAR) knockout animals, viremia and death occurred in the dams and fetuses during pregnancy; however, transfer of embryos with functional IFNAR rescued these animals from demise [26]. Type I responses have also been shown to cause harm. IFNAR$^{+/-}$ fetuses, but not IFNAR$^{-/-}$, infected with Zika virus were reabsorbed due to abnormal development of placental vasculature, cellular apoptosis, and hypoxia initiated by IFNAR signaling [27, 28]. Therefore, there is a critical balance between protective and harmful inflammations through type I interferons in placental viral infection.

Limitations of the current study include the use of tissue explants, which do not discern which cell types are infected by LCMV and the small sample size used for each assay. Additionally, the experiments do not detail whether infection occurs at the apical or basal side of infected cells since the LCMV receptor is known to change its location throughout placental development [29]. At this point, we can only hypothesize that interactions between LCMV and TLRs lead to NF-κB or MyD88 activation and the release of TNF-α or IFN-α. Future experiments are needed to clarify the mechanism of cytokine induction by LCMV as well as the mechanism(s) of suppression of this response in the first-trimester placenta.

5. Conclusions

Together, the data do suggest that innate immune response to LCMV infection of the human placenta is more vigorous in the third trimester than in the first trimester. The absence of viral replication in term placental explants may be

attributable to the robust innate antiviral response in this tissue. Such findings parallel the clinical observation of decreased transplacental transmission and less severe fetal phenotypes of viral pathogens acquired in later gestation. Continued research into LCMV as a model of human congenital infection and immunity is warranted.

Data Availability

The raw data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

The study protocol was approved by the UTMB Hospital Institutional Review Board.

Consent

All subjects included in this study provided their written informed consent.

Disclosure

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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