Lnc-C/EBPβ Modulates Differentiation of MDSCs Through Downregulating IL4i1 With C/EBPβ LIP and WDR5

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Myeloid-derived suppressor cells (MDSCs), which play an important role in tumor and inflammatory diseases, are divided into two subsets CD11b+Ly6ChLy6G− monocytic MDSC (Mo-MDSC) and CD11b+Ly6Clo/Ly6G+ polymorphonuclear MDSC (PMN-MDSC) with different immunosuppressive function. However, it is poorly understood the mechanism(s) to control differentiation of Mo-MDSCs and PMN-MDSCs. Here, we found that lnc-C/EBPβ may promote PMN-MDSC but impede differentiation of Mo-MDSCs in vitro and in vivo. We demonstrated that lnc-C/EBPβ mediated differentiation of MDSCs was through downregulating multiple transcripts such as IL4i1. Lnc-C/EBPβ not only bound to C/EBPβ isoform LIP to inhibit the activation of C/EBPβ but also interacted with WDR5 to interrupt the enrichment of H3K4me3 mark on the promoter region of IL4i1. Data also imply that conserved homo lnc-C/EBPβ has a similar function with mouse lnc-C/EBPβ. Since MDSC subsets exert different suppressive function, lnc-C/EBPβ may be acted as a potential therapeutic target for inflammatory and tumor-associated diseases.

Keywords: MDSC, lncRNA, IL4i1, WDR5, H3K4me3

INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) have emerged as a major regulator of immune responses in cancer and other pathological conditions (1–4). In mice, MDSCs are broadly identified as CD11b+Gr1+ cells. The Gr1+ subsets may be more accurately identified based on Ly6C and Ly6G markers as CD11b+Ly6ChLy6G− monocytic MDSC (Mo-MDSC) and CD11b+Ly6Clo/Ly6G+ polymorphonuclear MDSC (PMN-MDSC) (5, 6). Human MDSCs including monocytic (CD14+) MDSCs and PMN (CD15+) MDSCs, are described as lineage negative cells that co-express CD11b and CD33 but lack HLA-DR (7). These MDSCs play a pivotal role in cancer progression and other relative diseases by suppressing both innate and adaptive immune responses. However, recent studies have shown that Mo-MDSCs are more prominent than PMN-MDSCs. Mo-MDSCs may rapidly differentiate to tumor-associated macrophages (TAM) in tumor environment (2). Furthermore, Mo-MDSCs can produce large amounts of NO, Arg-1, and immune suppressive cytokines (2). Thus, it is necessary to understand the mechanism(s) to control the differentiation of Mo-MDSCs and PMN-MDSCs.
MDSCs are generated from common myeloid progenitor cells in bone marrow (BM). However, their development is governed by a complex network of signals including promoting accumulation of immature myeloid cells and providing for the pathological activation of these cells (2, 8). Inflammation and tumor-dependent activation of transcription factors, transcriptional co-regulators, and chromatin modifying factors collaborates to determine the differentiation of MDSCs (4, 7). Cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin (IL–6) could in vitro induce development of MDSCs. Transcription factors such as CCAAT/enhancer binding protein β (C/EBPβ), phosphorylated signal transducer of activator of transcription 3 (phospho-STAT3), and C/EBP homologous protein (CHOP) centrally regulate MDSC expansion (1, 4, 7, 9, 10). Recent studies show that epigenetic modification such as lncRNAs (length >200 nucleotides) also play an important role in regulating differentiation and function of immune cells. These lncRNAs can be intergenic (between protein coding genes), intronic, and natural antisense transcripts, or transcribed from divergent enhancers and promoters (11). They may regulate gene expression in diverse biological processes through binding to chromatin-modifying factors, transcription factors (12). Multiple lncRNAs have been described in myeloid derive cells such as lnc-DC in dendritic cells (13), lincRNA-Cox2, lincRNA-EPS, and AS-IL-1a in macrophages (14–16), lncRNA-Morrbid in myeloid cells (17). However, effect(s) of lncRNA on differentiation and function of MDSCs is very little understood.

Our previous studies have shown that inflammatory or tumor associated factors may affect the development of MDSCs (18), and also found that differentiation and function of MDSCs may be regulated through microRNA(s), IncRNA and epigenetic modifying factors (19, 20). We here found that Inc-C/EBPβ, which was identified by us (21), had a novel role in the differentiation of MDSCs. We found that Inc-C/EBPβ may promote PMN-MDSCs but impede the differentiation of Mo-MDSCs. We demonstrate that Inc-C/EBPβ may downregulate IL4ii to affect the differentiation of MDSCs through binding with C/EBPβ LIP and WDR5. These may provide a novel target for controlling MDSC differentiation.

**MATERIALS AND METHODS**

**Mice, Human Samples, and Cell Lines**

CS7BL/6 mice were purchased from the Beijing Animal Center (Beijing, China) and maintained in a specific pathogen-free (SPF) facility. B6.SJL-CD45a (Ly5a) (CD45.1) mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). All animal experiments were carried out in accordance with Nankai University Guide for the Care and Use of Laboratory Animals. The peripheral blood and tissues samples from the patients and healthy human were obtained after signing informed consent at People Union Hospital (Tianjin, China). The collection and use of all human samples were approved by the Institute’s Human Ethics Committee of Nankai University and in accordance with the Declaration of Helsinki.

Murine melanoma B16, breast cancer 4T1, colon cancer CT26, and human embryonic kidney cell line HEK 293T cells were obtained from the American Type Culture Collection (ATCC, USA). Murine ovarian tumor cell line 1D8 was from Dr. Richard Roden (The Johns Hopkins University School of medicine, USA).

Reagents

Recombinant murine GM-CSF and IL-6 were purchased from PeproTech (Rocky Hill, NJ). Mouse anti-C/EBPβ, anti-IL4ii, and anti-H3K4me3 were from Abcam (Cambridge, MA). Rabbit anti-WDR5 was from Cell Signaling Technology (Beverly, MA). Mouse anti-V5 was purchased from Thermo Fisher Scientific (Pittsburgh, PA). Anti-Gr1-PE, anti-CD11b-PerCP/Cy5.5, anti-Ly6G-PE, anti-Ly6C–FITC, anti-F4/80–FITC, anti-CD3–PerCP/Cy5.5, anti-CD45–APC, anti-CD45.1-Cy7, anti-CD33–PE, anti–HLA-DR–APC, anti-CD14–FITC, anti-CD15–PE were from BD Biosciences (San Diego, CA).

siRNAs, Lentiviruses, and Plasmid Construction

siRNAs were from Ribobio (Guangzhou, China). siRNA sequences for IL4ii and WDR5 were listed in Supplementary Table S1. ShRNA targets were chosen from the target sequences produced by BLOCK-iTM RNAi Designer (Invitrogen) and/or by i-Score Designer. ShRNA constructs were made using pGreenPuro™ shRNA cloning and expression lentivirus kit (System Biosciences Inc., USA) according to the manual. Control shNC was luciferase control shRNA from the kit. For packaging of lentivirus particles, shRNA lentivectors or Inc-C/EBPβ lentivectors together with pMD2G and psPAX2 packaging plasmids were co-transfected into 293T cells. The full-length sequences of C/EBPβ LAP/LIP, IL4ii, and WDR5 were amplified using PCR (Primer pairs used were described in Supplementary Table S1). The PCR products were cloned into the pcDNA3.1/V5-His TOPO® TA plasmid (Invitrogen, USA). The lentiviruses, siRNAs or plasmids were used to transduce or transfect MDSCs.

Transduction and Transfection

BMCs were collected from CS7BL/6 mice and cultured in six-well plate. Human monocytes were collected and isolated from peripheral blood. For transduction, the cells were infected with shRNA lentivectors [50 multiplicity of infection (MOI)] or Inc-C/EBPβ lentivectors (50 MOI) or control empty lentiviruses (50 MOI) in the presence of 8 μg/ml polybrene (Millipore, USA) by centrifugation and then cultured with complete medium for 24 h. For transfection, the cells were transfected with WDR5 siRNA (100 nM), IL4i siRNA (100 nM), negative control siRNA (100 nM) or pcDNA3.1/WDR5 (4 μg/ml), pcDNA3.1/IL4i (4 μg/ml), and empty pcDNA3.1 control (4 μg/ml) using Lipofectamine™ 3000 (Invitrogen, USA) or HiPerFect transfection reagent (siRNA transfection) (Qiagen, USA) according to the manufacturer’s instructions. The cells were then washed and cultured under GM-CSF or GM-CSF plus IL-6 or tumor supernatants for 4 days.
Generation of Inflammatory and Tumor Associated MDSCs

Inflammatory factors associated MDSCs were generated by culturing bone marrow cells of C57BL/6 mice in flask or 6-well plate for 4 days in the presence of 5% FBS medium containing GM-CSF (40 ng/ml) only or GM-CSF (40 ng/ml) plus IL-6 (40 ng/ml) (20). To prepare tumor cell supernatant-induced CD11b^+Gr1^+ MDSCs in vitro, 5 × 10^4 1D8, 4T1, CT26, or B16 tumor cells (upper chamber) were co-cultured with 2 × 10^6 BMCs (lower chamber) in a 24-transwell plate in the presence of GM-CSF (40 ng/ml) for 4 days. Human MDSC-like cells were generated according to previous reported methods (22).

Microarray

Microarray of coding mRNA in lnc-C/EBPβ knockdown MDSC was performed using Affymetrix GeneChip mouse Genome 430 2.0 array by Beijing Capitalbio Technology Co., Ltd. Briefly, total RNA was extracted using Trizol (Life Technologies). Contaminating DNAs were removed using RNase spin columns (Qiagen). The quality of isolated RNA samples was evaluated with an Agilent Bioanalyzer 2100 (Agilent technologies) and the purified RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Infinigen Biotechnology Inc.). Microarray of coding mRNA in lnc-C/EBPβ knockdown MDSC was performed using Affymetrix GeneChip mouse Genome 430 2.0 array. The R software (v.2.13.0) platform was applied to analyze the microarray data, and the limma (linear regression model) package was used to statistically analyze differentially expressed genes. The expression levels of mRNAs at each time point were compared with control. Genes having a fold change > 2 or < −2 and an adjusted p < 0.05 were considered as differentially expressed. Microarray GEO accession number GSE104571.

Flow Cytometry

Cells were collected and rinsed twice with ice cold PBS, incubated with FITC-, PE-, percy5.5-, or APC-labeled antibodies for 30 min with ice cold PBS. Cell extracts were prepared with lysis buffer and centrifuged at 13,000 g for 10 min at 4°C. Protein samples were electrophoresed using 12% polyacrylamide gels and transferred to PVDF membranes. After the membranes were blocked with 5% skim milk powder for 1 h at room temperature, they were incubated with first antibody in TBST overnight at 4°C. Secondary antibodies with horseradish peroxidase (HRP) (1:5000) were labeled according to our previous method (23). The signals were checked by autoradiography film when HRP substrate was added to the membranes.

Immunostaining and RNA Fluorescence in situ Hybridization

Immunostaining and RNA Fluorescence in situ hybridization (RNA-FISH) were performed according to reported protocol (24). Cells were first slicked on sterile and 0.01% poly-lysine-treated slides in the bottom of 6-well tissue culture dish. After that, the slides were processed sequentially with ice-cold C TK buffer, CSK buffer, CSK+0.4% Triton X-100 buffer, and CSK buffer for 30 s for cell membrane perforation. The slides were then treated with 4% PFA for 10 min and cold 70% ethanol three times for cells fixation. After rinsed three times with ice cold PBS, the slides were blocked in pre-warmed 5% goat serum for 30 min at 37°C. Then, the slides were incubated with primary antibody at 37°C for 1 h, washed three times with 1× PBS/0.2% Tween-20 for 3 min on a rocker, and then incubated with secondary antibody at 37°C for 30 min. After washing three times with 1× PBS/0.2% Tween-20, the slides were fixed with 2% PFA at room temperature for 10 min. The slides were dehydrated by moving them through a room temperature ethanol series (85, 95, and 100% ethanol) for 2 min each, and air-dried at room temperature for 15 min and hybridized using the indicated probes overnight at 37°C in a humid chamber. After washing with 2× SSC/50% formamide, 2× SSC, and 1× SSC each for three times, DAPI dye was added. Finally, the slides were sealed, and then observed using confocal microscope.

Chromatin Immunoprecipitation-PCR

Chromatin immunoprecipitation (CHIP)-PCR was performed using EZ-CHIP™ chromatin immunoprecipitation kit (Millipore, USA) according to the reported method (25). MDSCs were crosslinked with 1% paraformaldehyde and incubated with rotation at room temperature. Crosslinking was stopped after 10 min with glycine to a final concentration of 0.125 M and incubated 5 min further with rotation. Cells were washed with ice cold PBS (containing 1% PMSF) 3 times and immediately resuspended in SDS lysis buffer (containing 1% PMSF). Cell lysates were sonicated for 40 cycles of 30 s ON and 30 s OFF in 10 cycle increments using a Biorupter (Diadenode) on ice. After pelleting debris, protein G agarose was added and incubated for 1 h at 4°C with rotation for preclearing. For immunoprecipitation, pre-cleared cell lysate was incubated with the indicated antibodies (1 μg per 2 million cells) overnight with the rotation at 4°C and protein G agarose was added for the final 2 h of incubation. Beads were washed with low salt, high salt, LiCl wash buffer, and chromatin immunocomplex was eluted using elution buffer through incubating at room temperature for 15 min. Reverse crosslinks of protein/DNA complexes to free
DNA were realized through adding 5 M NaCl and incubating at 65°C overnight. qPCR was performed on DNA purified after treatment with RNase (30 min, 37°C) and proteinase K (2 h, 55°C) after reversal of crosslinks.

**RNA Immunoprecipitation (RIP)**

RNA immunoprecipitation (RIP) was performed according to previously reported protocol (24). Briefly, the cells were harvested, washed, added ice cold IP lysis buffer (Thermo Scientific Pierce) containing 0.5% ribonuclease inhibitor (Invitrogen), and incubated on ice for 5 min with periodic mixing. Then the lysates were transferred into a microcentrifuge tube and centrifuged at 13,000 g for 10 min to pellet the cell debris at 4°C, and the supernatants were transferred into a new tube, and protein G agarose was added and incubated for 1 h at 4°C with rotation for pre-clearing. The immunoprecipitating antibody was added and incubated overnight at 4°C with rotation. Protein G agarose was pelleted by brief centrifugation (3,000 g for 1 min) and then washed sequentially with IP lysis buffer (containing 0.5% ribonuclease inhibitor). Finally, RNA was extracted from protein/RNA complexes on the beads using TRIzol reagent and dissolve in DEPC-water and quantified by qPCR.

**Immunoprecipitation-MASS**

Immunoprecipitation (IP)-MASS was performed according to our previous method (20). Briefly, the MDSCs were lysed in IP lysis buffer (Pierce) containing 10% PMSF. Protein A/G magnetic beads (Pierce) were first added into the cell lysates for pre-clearing. The supernatants were collected after centrifuging at 12,000 rpm and then immunoprecipitated overnight at 4°C with the indicated antibodies. Protein A/G Magnetic Beads were added into cell lysates and incubated for additional 3 hrs. After being washed with IP lysis buffer for five times, Protein A/G Magnetic Beads were denatured and resolved by SDS-PAGE gels, and followed by silver staining. The gel lanes containing the immunopurified samples were excised for liquid chromatography-tandem MS (LC-MS/MS) analysis by Tsinghua University.

**RNA-Protein Pull-Down Analyses**

RNA-protein pull-down analyses were performed using Pierce™ magnetic RNA-protein pull-down kit. MDSCs were harvested and cell lysates were prepared using IP lysis buffers (Thermo Scientific Pierce, USA) (26). Lnc-C/EBPβ was transcribed (NEB, manual HiScribe T7 in vitro transcription Kit) and labeled using RNA 3′ Deshiobiotinylation Kit (Thermo Scientific Pierce) in vitro. 50 ul beads and 50 pmol of labeled RNA were added into RNA capture buffer, and incubated for 30 min at room temperature with agitation to binding of labeled Lnc-C/EBPβ to streptavidin magnetic beads. After washing beads with an equal volume of 20 mM Tris (pH 7.5), 100 µL of 1× protein-RNA binding buffer was added into the beads and mixed well. 100 µL of master mix of RNA-protein binding reaction was added.
FIGURE 2 | Lnc-C/EBPβ promotes differentiation of PMN-MDSC but impedes Mo-MDSC in vitro. (A) Flow cytometry of inflammatory cytokine and tumor mediated CD11b^+Ly6G^-Ly6C^- and CD11b^+Ly6G^-Ly6C^+ MDSCs. 1D8, mouse ovarian carcinoma; 4T1, mouse breast cancer; B16, mouse melanoma. BMC, bone marrow cells; GM, GM-CSF. (B) Flow cytometry of Gr1^+CD11b^+, Gr1^CD11b^+, and Gr1^-CD11b^+ MDSCs in lnc-C/EBPβ knockdown or exogenous lnc-C/EBPβ treated MDSCs. The percentages and absolute number of cells were compared (n = 3). (C) Flow cytometry of CD11b^+Ly6G^-Ly6C^- and CD11b^+Ly6G^-Ly6C^+ MDSC in lnc-C/EBPβ knockdown or exogenous lnc-C/EBPβ treated MDSCs. The percentages and absolute number of cells were compared (n = 3). (D) Flow cytometry of (Continued)
to the RNA-bound beads, mixed by pipetting and then incubated 60 min at 4°C with rotation to binding of RNA-binding proteins to RNA. After washing beads with 100 ul wash buffer for twice, 50 µL of elution buffer was added, and incubated 30 min at 37°C with agitation. The samples were analyzed on a gel.

**Statistical Analyses**

Statistical analyses were performed using two-tailed Student’s t-test and GraphPad Prism 5 software. A 95% confidence interval was considered significant and defined as p < 0.05. * indicates p < 0.05, **p < 0.01, ***p < 0.001.

**RESULTS**

**Different Levels of Lnc-C/EBPβ Expression in PMN-MDSC, Mo-MDSC, and Macrophages**

Our previous studies have shown that lnc-C/EBPβ negatively regulates suppressive function of MDSCs (21). Since lncRNAs often exert multiple effects on the function and differentiation of the cells, we here investigated the role of lnc-C/EBPβ in the differentiation of MDSCs. We first examined the expression levels of lnc-C/EBPβ in different MDSC subsets. BMCs were cultured in vitro for 4 days in the presence of GM-CSF and IL-6. MDSC subsets were sorted by flow cytometry and analyzed using qRT-PCR, immunostaining, and RNA-FISH. Data showed that lnc-C/EBPβ could be detected in PMN-MDSC, Mo-MDSC, and F4/80+ macrophage. However, the level of lnc-C/EBPβ in PMN-MDSCs was significantly higher than in Mo-MDSCs and F4/80+ macrophages (Figures 1A, B). Importantly, isolated PMN-MDSCs from WT mice bearing melanoma B16, colon cancer CT26, ovarian cancer ID8, and breast cancer 4T1 had also higher levels of lnc-C/EBPβ than in Mo-MDSCs and F4/80+ macrophages (Figure 1C). These data indicate that there exist different levels of lnc-C/EBPβ in PMN-MDSCs, Mo-MDSCs, and macrophages.

**Lnc-C/EBPβ Promotes PMN-MDSC but Impedes Differentiation of Mo-MDSC**

MDSCs could be induced by inflammatory cytokines and tumor cells (Figure 2A). Compared with GM-CSF alone, GM-CSF plus IL6 mediated MDSCs contained lower percentage of Ly6C+ cells (Figure 2A). Our previous studies found that the expression of lnc-C/EBPβ may be regulated by IL6. And lnc-C/EBPβ may potentially promote the differentiation of PMN-MDSC (Ly6G+CD11b+Ly6Cneg) (21). Consistent with this finding, lnc-C/EBPβ knockdown inhibited BMCs to differentiate into Gr1+CD11b+ MDSCs; whereas exogenous lnc-C/EBPβ promoted the differentiation of Gr1+CD11b+MDSCs (Figure 2B), indicating that lnc-C/EBPβ may promote the differentiation of PMN-MDSCs. Lnc-C/EBPβ transduction decreased the fraction of CD11b+Ly6ChighLy6C− Mo-MDSC subpopulation; whereas the percentages and absolute cell number of Mo-MDSCs increased in lnc-C/EBPβ silencing bone marrow cells (BMCs) (Figure 2C), suggesting that lnc-C/EBPβ not only promotes the differentiation of PMN-MDSCs but also impedes differentiation of MDSCs into Mo-MDSCs. Notably, F4/80+ macrophages significantly increased after lnc-C/EBPβ knockdown. The exogenous lnc-C/EBPβ also inhibited the differentiation of MDSCs into F4/80+ macrophages (Figure 2D). We further proved the validity of lnc-C/EBPβ lentivirus by qRT-PCR and fluorescence microscopic imaging of green fluorescent protein in lentivirus infected cells (Figure 2E), and also excluded the effect of off-target by a dose-dependent experiment (Figure 2F), different shRNA to transfect BMCs and inflammatory responses (Supplementary Figures 1A–C). All of these indicate that lnc-C/EBPβ may inhibit BMCs into Mo-MDSC/macrophages. To further confirm the effects of lnc-C/EBPβ on the differentiation of MDSCs, we next employed a mouse CD45.1+ bone marrow cell (BMC) chimera model to investigate the effects of lnc-C/EBPβ on MDSC differentiation in vivo. CD45.1+ BMCs from homozygous mice were transduced with lnc-C/EBPβ shRNA/lentiviruses and then injected into B16 tumor-bearing WT mice via tail vein at the indicated time (Figure 3A). Once mice were injected by exogenous lnc-C/EBPβ transduced CD45.1+ BMCs, the proportion, and absolute cell number of CD11b+Ly6ChighLy6C− Mo-MDSC populations significantly decreased in the tumor-bearing mice; whereas increased Mo-MDSCs could be observed in those tumor bearing mice injected with lnc-C/EBPβ knockdown CD45.1+ BMCs (Figure 3B). PMN-MDSCs increased in these mice injected with lnc-C/EBPβ transduced CD45.1+ BMCs but decreased in the mice with lnc-C/EBPβ knockdown CD45.1+ BMCs (Figure 3B). CD45.1+ cells could be detected in the tumor of mice injected by lnc-C/EBPβ–transduced BMCs (Figure 3C), indicating successful establishment of the mouse model. To further confirm the effects of lnc-C/EBPβ on the differentiation of BMCs, mice were also injected by exogenous lnc-C/EBPβ transduced CD45.1+ BMC (Figure 3D). The proportion and absolute cell number of CD11b+Ly6ChighLy6C− Mo-MDSC populations significantly decreased in the spleen of mice; whereas increased Mo-MDSCs could be seen in the mice injected with lnc-C/EBPβ knockdown CD45.1+ BMCs (Figure 3E). PMN-MDSCs increased in mice injected with lnc-C/EBPβ transduced CD45.1+ BMC but decreased in mice with lnc-C/EBPβ knockdown CD45.1+ BMC (Figure 3E). CD45.1+ cells
Lnc-C/EBPβ promotes differentiation of PMN-MDSC but impedes Mo-MDSC in vivo. (A) Schematic of the experiment. Lnc-C/EBPβ lentivirus treated CD45.1+ BMCs were injected into B16 tumor bearing WT mice in tail vein, and after 21 days the tumor CD45.1 cells were analyzed. (B) Flow cytometry of MDSCs in mouse tumor after injecting Lnc-C/EBPβ lentivirus treated CD45.1+BMCs. The percentage changes and absolute cell number of CD45.1+ cell of MDSC subsets were compared (n = 6). (C) Confocal microscopy of CD45.1+ cells in tumor. Green, CD45.1; blue, DAPI; NC, isotypic Ab. Scale bars = 100 µm. (D) Schematic of the
could be detected in the spleen of mice injected by lnc-C/EBPβ transduced BMCs (Figure 3F). Taken together, lnc-C/EBPβ not only promotes the differentiation of PMN-MDSCs but also impedes MDSCs to Mo-MDSCs.

Lnc-C/EBPβ Mediated Differentiation of MDSCs Is Through Downregulating Expression of Interleukin 4 Induced Gene-1

LncRNAs may exert its role through regulating gene expression (15, 27, 28). To find lnc-C/EBPβ associated gene(s), which potentially affect the differentiation of MDSCs, we analyzed gene expression pattern of lnc-C/EBPβ knockdown MDSCs using a microarray (Figure 4A). Data showed that lnc-C/EBPβ knockdown affected multiple gene expression such as interleukin 4-induced gene-1 (IL4i1). As shown (Figure 4A, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104571), IL4i1 was remarkably upregulated in lnc-C/EBPβ knockdown MDSCs. The higher levels of expression could be further confirmed by qRT-PCR and immunoblot in these lnc-C/EBPβ knockdown cells (Figure 4B). IL4i1 plays a critical role in the differentiation of monocyte/macrophages (29–31). Isolated PMN-MDSCs from WT mice bearing B16 tumor had much lower level of IL4i1 than Mo-MDSC and F4/80+ macrophages (Figure 4C), implying a potential role of IL4i1 in regulating the differentiation of MDSC subpopulations. We next used loss- and gain-of function studies to investigate the effects of IL4i1 on the differentiation of MDSC. IL4i1 knockdown decreased the proportion of CD11b+Ly6ChiLy6G− Mo-MDSC subpopulation; whereas the percentages of Mo-MDSC increased in exogenous IL4i1 transfected BMCs (Figure 4D). The proportion of CD11b+Ly6CchiLy6G− Mo-MDSC populations significantly increased in the spleen of mice injected by exogenous IL4i1 transfected CD45.1 BMC. The increased CD11b+Ly6CchiLy6G− Mo-MDSC could not be seen in the mice injected with IL4i1 knockdown CD45.1+ BMCs (Figure 4E). F4/80+ macrophages significantly decreased after IL4i1 knockdown; whereas exogenous IL4i1 promoted differentiation into F4/80+ macrophages (Figure 4F). The exogenous IL4i1 also inhibited the differentiation Ly6GhighCD11b+Ly6CLow/− PMN-MDSCs; but IL4i1 knockdown promoted the differentiation of PMN-MDSCs (Figures 4D–F). We further confirmed the validity of siRNA and excluded the effect of off-target by using different kinds and doses of siRNAs to transfect BMCs (Figure 4G, Supplementary Figure 1D). Thus, IL4i1 promotes the differentiation of Mo-MDSCs but impedes MDSCs into PMN-MDSCs. Since lnc-C/EBPβ may downregulate the expression of IL-4i1, these data indicate that IL4i1 is involved in lnc-C/EBPβ mediated differentiation of MDSCs.

Binding of Lnc-C/EBPβ With C/EBPβ Isoform LIP and WDR5 Is Required for Lnc-C/EBPβ Mediated Downregulation of IL4i1

We next sought to determine underlying molecular mechanism(s) by which lnc-C/EBPβ regulates expression of IL4i1, which impedes the differentiation of Mo-MDSC. Consistent with previous findings (21), lnc-C/EBPβ could bind with C/EBPβ isoform LIP to impede the activation of C/EBPβ (Figures 5A,B). Genome browser images showed that there had the binding site of C/EBPβ in the 5′ region of IL4i1 (Figure 5C). In the lnc-C/EBPβ knockdown cells, CHIP-qPCR showed the increased C/EBPβ; whereas extrinsic lnc-C/EBPβ reduced the enrichment of C/EBPβ in the 5′ region of IL4i1 (Figure 5D). All of these support that binding of lnc-C/EBPβ with C/EBPβ isoform LIP may be involved in the regulation of IL4i1 gene.

Epigenetic modification plays a critical role in the gene expression such as that lysine methylation in the histones regulates the activation and repression of transcription (32). We next used C/EBPβ as a bait to investigate whether lnc-C/EBPβ also bind with other molecules, which are related to epigenetic modification. IP-MASS analyses showed that there were remarkable differences not only in the nuclear proteins but also in their signal molecule networks between GM-CSF and GM-CSF plus IL-6 mediated MDSCs (Figure 5E, Supplementary Figure 2). Data showed that WDR5 was a C/EBPβ targeting molecule (Figures 5E–K) and confirmed by co-immunoprecipitation (Figure 5F). Importantly, RIP and pull-down also confirmed the binding of lnc-C/EBPβ with WDR5 (Figures 5G,H). The interaction of WDR5 with lnc-C/EBPβ was also detected by FISH and immunostaining (Figure 5I). Thus, lnc-C/EBPβ may bind with epigenetic factor WDR5. Since WDR5 is responsible for depositing the H3K4me3 mark on the promoter of active genes (33) and positively correlated with transcriptional activity, it also is possible for the binding of lnc-C/EBPβ with epigenetic component WDR5 to promote gene expression. Genome browser images showed there also has the binding site of H3K4me3 mark in the 5′ region of IL4i1. Using CHIP-qPCR, we investigated the effects of lnc-C/EBPβ on the enrichment of H3K4me3 mark. In the lnc-C/EBPβ knockdown cells, CHIP-qPCR showed the increased enrichment of H3K4me3 mark; whereas exogenous lnc-C/EBPβ reduced this enrichment in the 5′ region of IL4i1 (Figure 5J). Therefore, the binding of lnc-C/EBPβ with WDR5 affects the enrichment of H3K4me3 mark on the IL4i1 promoter and ultimately affect the expression of IL4i1. Further studies exhibited that WDR5 was indeed involved in the differentiation of MDSCs. WDR5 knockdown promoted the fraction of PMN-MDSC.
FIGURE 4 | Lnc-C/EBPβ mediated differentiation of MDSCs depends on IL4i1. (A) Gene expression in Lnc-C/EBPβ knockdown treated MDSC. (B) QRT-PCR (upper) and immunoblotting (lower) of IL4i1 in Lnc-C/EBPβ knockdown MDSCs. (C) QRT-PCR of IL4i1 in the MDSC subsets isolated from WT mice bearing B16 tumor. (D) Flow cytometry of CD11b+Ly6G−Ly6C−, CD11b+Ly6G−Ly6C+, and CD11b+Ly6G+Ly6C+ MDSCs in IL4i1 knockdown cells or exogenous IL4i1 treated MDSCs. The percentages were compared (right, n = 3). (E) Flow cytometry of MDSCs in mouse spleen after injecting IL4i1 knockdown or exogenous IL4i1 treated CD45.1+ BMCs. Percentage (Continued)
subpopulation; whereas the percentages of Mo-MDSCs increased in exogenous WDR5 transfected CD45.1+ BMCs in vitro and in vivo (Figures 5L–N). We also further confirmed the validity of WDR5 siRNA, and excluded the effect of off-target by using different kinds and doses of siRNAs to transfect MDSCs (Figure 5O, Supplementary Figure 1E). Notably, other transcription factors and regulators such as STAT3, STAT5, S100A8/A9, E2F3, IRF8 etc. which play an important role in the expansion and differentiation of MDSCs (1) were also potentially modulated by lnc-C/EBPβ (Supplementary Figure 3A, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104558).

H3K4me3 mark in the 5’ promoter region of these genes was remarkable enrichment (Supplementary Figure 3B), implying that the modulation of lnc-C/EBPβ on these genes also is through WDR5. Taken together, lnc-C/EBPβ may promote PMN-MDSCs but impede differentiation of Mo-MDSCs through interrupting the expression of differentiation associated genes such as IL4i1.

Human Lnc-C/EBPβ Has a Similar Function With Murine Lnc-C/EBPβ

Since human lnc-C/EBPβ is high homology with mouse lnc-C/EBPβ (21), we examined whether human lnc-C/EBPβ had also similar function with mouse lnc-C/EBPβ. Human MDSC-like cells were generated from human peripheral blood monocytes (PBMCs) after co-culturing with GM-CSF plus tumor supernatants or IL-6 (22). Interestingly, IL4i1 was also detected in these human MDSC-like cells. Furthermore, lnc-C/EBPβ could modulate the expression of IL4i1 (Figures 6A,B). To examine the modulation of lnc-C/EBPβ on IL4i1 in vivo MDSCs, we isolated the MDSCs from patients with colorectal cancer, and examined the relationship between the expression of lnc-C/EBPβ and IL4i1. Lin− HLA-DR− CD33+ CD11b+ MDSCs were isolated from the blood of patients with glioblastoma, breast cancer, colon cancer, lung cancer and kidney cancer (34). Consistent with others (35–37), CD11b+CD14+ Mo-MDSC and Lin− HLA-DR− CD11b+CD15+ PMN-MDSC populations were much higher in patients with tumor as compared to tumor free-individuals (Figure 6C). Higher levels of lnc-C/EBPβ could be detected in Lin− HLA-DR− CD11b+ cells isolated from peripheral blood of patients with colorectal cancer as compared to Lin− HLA-DR− CD11b+ cells from tumor free healthy individuals (Figure 6D). QRT-PCR showed that there indeed existed negative relationship between lnc-C/EBPβ and IL4i1 in different MDSC subpopulations (Figures 6E,F). Finally, we also detected the expression of lnc-C/EBPβ in the samples of patients with colorectal cancer. FISH and immuno-staining of colitis/carcinoma tissues demonstrated the expression of lnc-C/EBPβ in CD11b+ monocytes/macrophages (Figure 6G) and nuclear localization of lnc-C/EBPβ in the mononuclear cells of colitis and carcinoma (Figure 6H). Since lnc-C/EBPβ may regulate the expression of MDSCs-associated genes in vitro, a high level of lnc-C/EBPβ may play a role in the differentiation and function of MDSCs in patients with colorectal cancer. Thus, human lnc-C/EBPβ is also involved in the differentiation of human MDSCs.

DISCUSSION

Previous data have already shown that transcription factor C/EBPβ plays a central role in regulating the differentiation and immune suppressive function of MDSCs (7, 38), and that WDR5 is responsible for depositing H3K4 methylation mark on the promoter region of active genes (33). We here demonstrate that the binding of lnc-C/EBPβ with C/EBPβ and WDR5 affects the activity of C/EBPβ and enrichment of H3K4me3 to promote PMN-MDSCs but inhibit the differentiation of Mo-MDSCs. As lnc-C/EBPβ is present in human monocyte-derived cells, it may be acted as a potential therapeutic target for inflammatory and tumor-associated diseases.

Our data show that lnc-C/EBPβ may bind with C/EBPβ and WDR5 to affect differentiation of MDSCs through regulating expression of IL4i1. IL4i1, a regulator of M2 macrophage polarization, may contribute to the regulation of monocyte/macrophage programming (29, 31, 39). C/EBPβ has been implicated in the differentiation of Mo-MDSCs (38, 40). Its deficiency affects mostly the differentiation of Mo-MDSCs (40). Mice lacking C/EBPβ in the hematopoietic system have lower frequencies of splenic Mo-MDSCs in MCA203 fibrosarcoma-bearing mice. Indeed, while the interaction of lnc-C/EBPβ with C/EBPβ and LIP affects LAP activity, MDSCs may not differentiate into Mo-MDSCs. WDR5 is involved in multiple biological processes and cell differentiation. It may mediate self-renewal and reprogramming of embryonic stem cell (41), and regulate immune cells through histone H3K4me3 (42, 43). Others also found that WDR5 binds with lncRNA HOTTIP to drive histone H3K4me3 and gene transcription (44).

That lnc-C/EBPβ impedes MDSCs to differentiate into Mo-MDSC may have an important significance in the inflammation and tumor. Multiple reports have described the presence of MDSCs in patients with tumors such as colon cancer, lung cancer, breast cancer, pancreatic adenocarcinomas, urothelial carcinoma, kidney Cancer, glioblastoma (2, 34). Studies have shown that Mo-MDSCs have higher suppressive activity than PMN-MDSCs.
FIGURE 5 | Binding of lnc-C/EBPβ with C/EBPβ isoform LIP and WDR5 is required for Lnc-C/EBPβ mediated Differentiation of MDSC. (A) RIP in V5-tagged LAP or LIP and lnc-C/EBPβ cotransfected HEK293T cells. RIP was performed using anti-V5 antibody and then PCR for lnc-C/EBPβ. pcDNA3.1, control plasmid; PC, positive control; NC, water. (B) Immunoblot of LAP and LIP in HEK293T cells. LIP/pcDNA3.1 (without V5-tagged), V5-tagged LAP/pcDNA3.1, and different concentrations of lnc-C/EBPβ pcDNA3.1 plasmids were cotransfected into 293T cells. IP was performed using V5 antibodies, and then immunoblot using anti-C/EBPβ (specific for both LAP and LIP). (C,D) CHIP-PCR on the promoter of IL4i1 of MDSCs. (E) IP-MASS of C/EBPβ interacting partners associated (Continued)
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FIGURE 5 | with WDR5 were listed (lower). (F) Immunoblot of C/EBPβ and WDR5 interaction in GM-CSF alone and GM-CSFplusIL-6 mediated MDSCs. (G) RIP-PCR for Inc-C/EBPβ used WDR5 antibody in differently generated MDSCs. (H) RNA-protein pull-down in Inc-C/EBPβ and WDR5-V5 co-transfected HEK293T cells. No RNA and antisense RNA as control. (I) Immunostaining and RNA-FISH in mouse MDSC (M-MDSC, left) and human monocytes (Hu-MDSC, right). Red, WDR5; Green, Inc-C/EBPβ; Blue, nuclei. (J) CHIP-PCR in the H3K4me3 enrichment region on the promoter of IL4i1. (K) Immunoblotting of LAP and LIP in transfected HEK293T cells using anti-C/EBPβ antibody. 293T cells did not express LAP and LIP. (L) Flow cytometry of Gr1+CD11b+, Gr1hiCD11b+, and Gr1lowCD11b+ MDSCs in WDR5 knockdown or exogenous WDR5 treated MDSCs. The percentages were compared (n = 3). Error bars in (C,D,J,L–O) represent standard deviations from 3 independent measurements. Two-tailed, paired T-test was used in (C,D,J,L–O). *p < 0.05; **p < 0.005; ***p < 0.001. NS, no significant.

FIGURE 6 | Human Inc-C/EBPβ has a similar function with mouse. (A) QRT-PCR of IL4i1 in Inc-C/EBPβ knockdown human MDSC-like cells. (B) Immunoblotting of IL4i1 in Inc-C/EBPβ knockdown human MDSC-like cells. (C) Flow cytometry of Lin−HLA-DR−CD14+CD11b+ MDSCs subsets in healthy individuals and patients with colon and rectal cancer. (D) QRT-PCR of human Inc-C/EBPβ in MDSC subsets isolated from the human colon, rectal cancer and healthy individuals. (E) QRT-PCR of human Inc-C/EBPβ in MDSC subsets isolated from the human colon and rectal cancer. (F) QRT-PCR of human IL4i1 in MDSC subsets isolated from the human colon and rectal cancer. (G) Immunostaining and RNA-FISH in the human colon cancer and para cancer tissues. Red, CD11b; Green, Inc-C/EBPβ; Blue, nuclei. One representative of 12 patients with colitic cancer. Scale bar = 200 µM. Error bars in (A,D–F) represent standard deviations from 3 independent measurements. Two-tailed, paired T-test was used in (A,D–F). *p < 0.05; **p < 0.005; ***p < 0.001. NS, no significant.
In the tumor, Mo-MDSCs are more prominent than PMN-MDSC, and furthermore Mo-MDSCs may rapidly differentiate to tumor-associated macrophages (TAM) (2). These cells can produce large amounts of NO, Arg-1, and immune suppressive cytokines (2), which have much longer half-life than ROS produced by PMN-MDSCs (2, 38). Less immunosuppressive function of PMN-MDSCs than Mo-MDSCs is also confirmed in a single cell (34). In addition, PMN-MDSCs also have a short half-life (45). Thus, our data provide a new insight that will help direct development of novel immunotherapeutic methods for tumor through modulating the expression of lnc-C/EBPβ to control differentiation of MDSCs into Mo-MDSCs.

DATA AVAILABILITY

The datasets generated for this study can be found in Gene Expression Omnibus, GSE104571 GSE104558.

ETHICS STATEMENT

All animal studies were conducted according to the Institutional Animal Care and Use Committee of the Model Animal Research Center. Animal experiments were approved by the Institute’s Animal Ethics Committee of Nankai University.

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AUTHOR CONTRIBUTIONS

RY designed the research and wrote the paper. YG conducted in vivo and in vitro experiments and immunosayass, participated in the study design, and performed the statistical analysis. WS was involved in vitro and in vivo assay. DZ, XZ, and SZ treated patient’s samples. YZ offered assistances for the animal experiments. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01661/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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