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Lnc-chop Promotes Immunosuppressive Function of Myeloid-Derived Suppressor Cells in Tumor and Inflammatory Environments

Yunhuan Gao, Tiantian Wang, Yuanyuan Li, Yuan Zhang, and Rongcun Yang

Myeloid-derived suppressor cells (MDSCs) are major regulators of immune responses in cancer. Both C/EBP homologous protein (CHOP) and C/EBPβ play a critical role in regulating immunosuppressive function of MDSCs. In this study, we identified a novel long noncoding RNA termed as lnc-chop in MDSCs, which may interact with CHOP and the C/EBPβ isoform liver-enriched inhibitory protein. The binding of lnc-chop with both CHOP and the C/EBPβ isoform liver-enriched inhibitory protein promoted the activation of C/EBPβ and upregulated the expression of arginase-1, NO synthase 2, NADPH oxidase 2, and cyclooxygenase-2, which are related to the immunosuppressive function of MDSCs in inflammatory and tumor environments. Additionally, lnc-chop also promoted the enrichment of H3K4me3 on the promoter region of arginase-1, NO synthase 2, NADPH oxidase 2, and cyclooxygenase-2. These findings suggest an important role of lnc-chop in controlling immunosuppressive function of MDSCs in the tumor environment. The Journal of Immunology, 2018, 200: 000–000.

Myeloid-derived suppressor cells (MDSCs) generated in the bone marrow from common myeloid progenitor cells are one of the major components of the tumor microenvironment. These cells have emerged as critical regulators of immune responses in cancer and other pathological conditions (1–4). There are two different types of MDSC in both mice and humans. In mice, MDSCs are broadly identified as CD11b+Gr1+ cells. The Gr1+ subsets may be more accurately identified based on Ly6C and CD11b and CD33 but lack HLA-DR (7). These MDSCs play a pivotal role in cancer progression and other associated diseases by suppressing both innate and adaptive immune responses. They express high levels of arginase-1 (Arg-1), NO synthase 2 (NOS2), NADPH oxidase 2 (NOX2), and cyclooxygenase-2 (COX2), which may result in the production of NO and reactive oxygen species (ROS) (8, 9). NO reacts with multiple cellular compounds to produce many toxic and regulatory factors including H2O2 and the hydroxyl radical, damage nucleic acids, proteins, and lipids (10). MDSCs may also cause the elimination of key nutrition factors needed for T cell proliferation by depleting local environment L-arginine (11), sequestering L-cysteine (12), and/or reducing local tryptophan levels (2, 13).

Transcription factors C/EBPβ, C/EBP homologous protein (CHOP), and phospho-STAT3 centrally regulate MDSC function and expansion (14–16). C/EBPβ has three isoforms: liver-enriched activator proteins (LAP⁺ and LAP) and liver-enriched inhibitory protein (LIP). LAP⁺ and LIP function as transcriptional activators. LIP lacks DNA activation domains. However, it may form heterodimers with other family members to control gene expression (17). The expression of Arg-1, NOS2, NOX2, and COX2 may be regulated by C/EBPβ (18). CHOP encoded by Ddit3 is involved in the regulation of immunosuppressive function in MDSCs (14). Studies have found that low regulatory potential of CHOP⁻/⁻/(Ddit3⁻/⁻) MDSCs is correlated with an impaired synthesis of major molecules linked to MDSC activity, including Arg-1, PNT (production of peroxynitrites), and superoxide (14). MDSC activity also depends on phospho-STAT3, which directly induces the expression of Arg-1 and Gp91phox, and the production of ROS (19–21).

Epigenetic modification such as long noncoding RNA (lncRNA; length >200 nt) play an important role in immune cell differentiation and function. They may regulate gene expression in diverse biological processes through binding to chromatin-modifying factors, transcription factors, and other factors (22). Multiple lncRNAs have been described in myeloid-derive cells such as lnc-DC in dendritic cells (23), lncRNA-Cox2, lncRNA-EPS, and AS-IL-1a in macrophages (24–26), and lncRNA Morbid in myeloid cells (27). However, the effects of lncRNA on the differentiation and function of MDSCs are very little understood.
Our previous studies showed that the development of MDSCs is affected by inflammatory or tumor-associated factors (28). We also found that microRNAs and epigenetic modifying factors may modulate the differentiation and function of MDSCs (29, 30). In this study, we identify an uncharacterized lncRNA termed Lnc-chop. We found that Lnc-chop may interact with CHOP and the C/EBPβ isoform LIP to encourage the activation of C/EBPβ and regulate a large set of target transcripts in MDSCs to control immunosuppressive function and differentiation of MDSCs in inflammatory and tumor environments. These may provide a novel regulatory mechanism controlling differentiation and suppressive function of MDSCs.

Materials and Methods

Mice and cell lines
C57Bl/6 mice were purchased from the Beijing Animal Center (Beijing, China). B6.129S6-Il6tm1(Wako) (IL-6−/−) and B6.SL-CD45a(Ly5a) (CD45.1) mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). OT-I and OT-II OVA-TCR transgenic mice were provided by Dr. L. Lu in Zhejiang University. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Murine melanoma B16, Lewis lung carcinoma, breast cancer 4T1 and human embryonic kidney cell line HEK293T cells were obtained from the American Type Culture Collection. Murine ovarian tumor cell line 1D8 was from Dr. R. Roden (Johns Hopkins University School of Medicine). B16-OVA was provided by Dr. L. Chen (Yale University).

Reagents
Recombinant murine GM-CSF, IL-6, and TNF-α were purchased from PeproTech (Rocky Hill, NJ). STAT3 inhibitor HO-3667 and Jak1 inhibitor filgotinib (GLPG0634) were purchased from Selleckchem (Houston, TX). OVA MHC class II peptide (323–339) and OVA MHC class I peptide (257–264) were purchased from Genscript (Piscataway, NJ). Mouse anti-C/EBPβ and anti-H3K4me3 were purchased from Abcam (Cambridge, MA). Mouse anti-CHOP, anti-NOS2, and rabbit anti-COX2 were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti–Arg-1 and anti–gp91-phox were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-CHOP, anti-NOS2, and rabbit anti-COX2 were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti–Arg-1 and anti–gp91-phox were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-CHOP was purchased from Thermo Fisher Scientific (Pittsburgh, PA). Anti–Gr1-PE, anti–CD11b-PerCP/Cy5.5, anti–Ly6G-PE, anti–Ly6C-PE, anti–CD3-PerCP/Cy5.5, anti–CD4-PE, anti–CD4-FITC, anti–CD8a-FITC, anti–CD45-allophycocyanin, anti–IFN-γ-PE, and anti–CD45.1-Cy7 were purchased from BD Biosciences (San Diego, CA).

Lentiviruses, plasmids, and microRNA
The short hairpin RNA (shRNA) target sequence 5′-gatcGCTGATGCTGTC-CATITCTtcaagagaTAGAATGGACAGCATCAGCTTTTTTa-3′ was chosen from the target sequences produced by BLOCK-it RNAi Designer (Invitrogen). The shRNA constructs were used in the pGreenPuro shRNA cloning and expression lentivector kit (System Biosciences) according to the manual. The control shRNA (shNC) is a luciferase control shRNA from the kit. For packaging of lentivirus particles, the shRNA lentivector or lentivector containing the pmD2G2 and pSPAX2 packaging plasmids were cotransfected into 293T cells. For plasmid construction, the target sequences of mouse C/EBPβ-LAP/LIP, CHOP, and lnc-chop were amplified using the primers depicted in Supplemental Table I. The PCR products were cloned into the pcDNA3.1/V5-His TOPO TA vector (Invitrogen). The C/EBPβ siRNA target sequence was 5′-GCCCTGGCAAGACTTGGTCAA-3′.

In vitro induction of inflammatory and tumor-associated MDSCs
Inflammatory factor-associated MDSCs were generated by culturing bone marrow cells of C57Bl/6 mice in a flask or six-well plate for 4 d in the presence of 5% FBS medium containing CM-CSF (40 ng/ml) or IL-6 (40 ng/ml) only, GM-CSF (40 ng/ml) plus IL-6 (40 ng/ml), or GM-CSF (40 ng/ml) plus TNF-α (20 ng/ml). To prepare the tumor cell supernatant-activated MDSCs in vitro, 5 × 104 ID8, 4T1, or 1D8 tumor cells (upper chamber) were cocultured with 2 × 106 bone marrow cells (BMCs; lower chamber) in a 24-transwell plate in the presence of GM-CSF (40 ng/ml) for 4 d. These in vitro-activated MDSCs were named iMDSCs.

Lentivirus transduction
BMCs were collected from C57BL/6 mice and cultured in six-well plate. The cells were infected with the lentiviruses in the presence of 8 μg/ml Polybrene by centrifugation and then cultured with complete medium for 24 h. The cells were then washed and cultured under GM-CSF or GM-CSF plus IL-6 or tumor supernatants for 4 d.

Flow cytometry
Cells were collected and rinsed twice with ice-cold PBS, incubated with FITC-, PE-, PerCP/Cy5.5-, or allophycocyanin-labeled Abs for 30 min in PBS with 1% FBS according to our previous method (31). After washing twice, cells were resuspended in PBS and analyzed using a FACSscan flow cytometer (BD Biosciences). Dead cells were eliminated through 7-ami-noacridine D staining.

Real-time PCR
Total RNA was extracted from the cells using TRIzol reagent (Invitrogen). First-strand cDNA was generated from total RNA using oligo-dt/random primer mix and reverse transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was conducted using QuantiTect SYBR Green PCR master mix (Qiagen) and specific primers in an ABI Prism 7000 analyzer (Applied Biosystems). Specific primers for mRNA/lncRNA are listed in Supplemental Table I. GAPDH was detected as an endogenous control. The fold changes were calculated using the comparative threshold cycle method according to the manufacturer’s instructions (Applied Biosystems). All reactions were run in triplicate.

Western blot
Western blots were performed as described previously (31). Primary Abs were used at 1:2000 dilutions and secondary Abs conjugated with HRP were used at 1:5000 dilutions.

Arginase activity, NO, H2O2, and ROS detection
The immunosuppressive function of MDSCs was analyzed according to our previously reported methods (29). Briefly, for arginase activity, cells were lysed for 30 min with 100 μl of 0.1% Triton X-100 at 4˚C. Following lysis, 100 μl of 25 mM Tris-HCl and 10 μl of 10 mM MnCl2 were added and the mixture was heated for 10 min at 56˚C. Subsequently, the lysates were incubated with 100 μl of 0.5 M L-arginine (pH 9.7) at 37˚C for 120 min. The reaction was stopped with 900 μl of H2SO4 (96%)/H3PO4 (85%)/H2O (1:3:7). Urea concentration was measured by absorbance at 540 nm after addition of 40 μl of 9% α-isotyrotopropiosphenolone, followed by heating at 95˚C for 30 min. A standard curve was generated by serial dilution of 120 mg/ml urea. Arginase activity (U) was defined by the amount of enzyme that catalyzes the formation of 1 μg of urea per minute. For NO production, the total NO in the cell lysate was measured using a nitrate/nitrite assay kit. Equal volumes of cell lysate (60 μl), 2 mM NADPH (5 μl), flavin adenine dinucleotide (10 μl), and nitrate reductase (5 μl) were incubated at 37˚C for 30 min, followed by addition of 30 μl of lactate dehydrogenase buffer and lactate dehydrogenase solution. After incubation for 30 min at 37˚C, 50 μl of Griess reagent I and II was added, the solution was incubated at room temperature for 10 min, and absorbance at 540 nm was measured. Nitrite concentrations were quantified by comparing the absorbance values to a standard curve generated by serial dilution of 100 μM sodium nitrite. H2O2 production was evaluated using an Amplex red hydrogen peroxide/peroxidase assay kit (Invitrogen). Briefly, 1 × 105 cells were resuspended in Krebs–Ringer phosphate (containing 50 μM Amplex red reagent and 0.1 U/ml HRP). After addition of PMA (30 ng/ml), the absorbance at 560 nm was measured using a microplate reader at 37˚C. Absorbance values for the test samples were normalized to a standard curve generated by serial dilution of 10 μM H2O2. For ROS detection, the oxidation-sensitive dye DCFDA was used to measure ROS production by MDSCs. Cells were incubated at 37˚C in RPMI 1640 medium in the presence of 2.5 μM DCFDA for 30 min. For PMA-induced activation, cells were simultaneously cultured with DCFDA and 30 ng/ml PMA and then flow cytometric analysis was performed.
production of IFN-γ was measured by an ELISA kit (Biotech) according to the manufacturer’s instructions. As a negative control, 2 × 10^5 OT-I or OT-II cells were cultured with RPMI 1640 only. As a positive control, 2 × 10^5 OT-I or OT-II cells were cultured with 1 μg/ml peptide.

5′- and 3′-RACE for lnc-chop

A FirstChoice RNA ligase-mediated RACE kit (Ambion) was used to obtain the full sequence of lnc-chop. For the 5′ end, total RNA from mouse MDSCs was treated with calf intestinal phosphatase to remove 5′ phosphate from non-capped transcripts, resulting in 5′ capped transcripts and RNA with 5′ hydroxyl ends. Then, the 5′-7-methylguanosine cap structure was removed by tobacco acid pyrophosphatase, resulting in a 5′ mono-phosphate transcript exclusively from intact 5′ transcripts. An RNA adaptor with 5′ and 3′ hydroxyl groups was then ligated to the 5′ mono-phosphate RNAs. For the 3′-RACE, cDNA was synthesized using a 3′-RACE adapter. RT-PCR using an lnc-chop-specific primer and a primer binding to the ligated RNA adaptor was performed to amplify the ligated lnc-chop followed by TOPO TA cloning and sequencing to determine the 5′ and 3′ end sequences of the lncRNA. The lnc-chop specific primers are listed in Supplemental Table I.

Northern blot

Lnc-chop was detected by Northern blot based on the reported method with modification (32). Total RNAs harvested with TRIzol reagent were run on 1% agarose-formaldehyde gel. RNA was transferred to a Hybond nylon membrane using the Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad Laboratories). Digoxin-labeled antisense lnc-chop was made using T7 RNA polymerase by in vitro transcription with a biotin labeling kit (Roche). Membrane was prehybridized for 1 h at 42˚C and incubated with the probe overnight at the same temperature. After washing, membrane was blocked and incubated with digoxin Ab conjugated with HRP. The primers used for the digoxin-labeling probe preparation are 5′-GGTCTCGACTCATGTCTTCG-3′ and 5′-TTGGTGTCTGGGTTGATTGTGTTGC-3′ for lnc-chop and 5′-GGTCTCGACTCATGTCTTCG-3′ and 5′-TTATACGACTCTATAGGAAAAATATGGAGCGCTTCAGATT-3′ for U6 RNA.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChiP)–PCR was performed using an EZ-ChIP kit (Millipore) according to the reported method (33). IMDSCs 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) three 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 Bioruptor (Diagenode) on ice. After pelleting debris, protein G–agarose was added and incubated for 1 h at 4˚C with rotation for preclearing. Precleared cell lysate was incubated with the indicated Abs (1 μg/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 the chromatin immune complex was eluted using elution buffer through incubating at room temperature for 15 min. Reverse crosslinks of protein/DNA complexes or free DNA were realized through adding 5 M NaCl and incubating at 65˚C overnight. Quantitative PCR was performed on DNA purified after treatment with RNase (30 min, 37˚C) and proteinase K (2 h, 55˚C) followed by reversal of crosslinks. The ChiP-PCR–specific primers are listed in Supplemental Table I.

Immunostaining and RNA fluorescence in situ hybridization

Immunostaining and RNA fluorescence in situ hybridization (FISH) were performed according to reported protocols (34). Cells were first splitted on sterile and 0.01% polylysine-treated slides in the bottom of a six-well tissue culture dish. After that, the slides were processed sequentially with ice-cold CSK buffer, CSK plus 0.4% Triton X-100 buffer, and CSK buffer for 30 s for cell membrane permeation. The slides were then treated with 4% PFA for 10 min and cold 70% ethanol three times for cell fixation. After rinsing three times with ice-cold PBS, the slides were blocked in prewarmed 5% goat serum for 30 min at 37˚C. Then, the slides were incubated with primary Ab 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 Ab 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 a confocal microscope. The probes used in the RNA-FISH are 5′-TTGGTGTCTGGGTTGATTGTGTTGC-3′ for lnc-chop and 5′-TTGGTGTCTGCATCATACCTC-3′ for the control probe.

RNA immunoprecipitation

RNA immunoprecipitation (IP) was performed according to a previously described protocol (34). Briefly, the cells were harvested, washed, and ice-cold IP lys buffer (Thermo Scientific Pierce) containing 0.5% RNase inhibitor (Invitrogen) was added and the solution was 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, 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 preclearing. The immunoprecipitating Ab was added and incubated over-night at 4˚C with rotation. Protein G-agarose was pelleted by brief centrifugation (3000 × g for 1 min) and then washed sequentially with IP lys buffer (containing 0.5% RNase inhibitor). Finally, RNA was extracted from protein/RNA complexes on the beads using TRIzol reagent and dissolved in diethyl pyrocarbonate–water and quantified by quantitative PCR. The RNA IP-PCR–specific primers are listed in Supplemental Table I.

RNA-protein pull-down analyses

RNA-protein pull-down analyses were performed using a Pierce magnetic RNA-protein pull-down kit. Cells were harvested and cell lysates were prepared using IP lys buffer (Thermo Scientific Pierce) (6). Lnc-chop was transcribed (New England Biolabs, manual HiScribe T7 in vitro transcription kit) and labeled using an RNA 3′ desethylbiotinylination kit (Thermo Scientific Pierce) in vitro. Fifty microliters of 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-chop 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. One hundred microliters of master mix of RNA-protein binding reaction was added 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 twice with 100 μl of wash buffer, 50 μl of elution buffer was added and incubated 30 min at 37˚C with agitation. The samples were analyzed on a gel.

Statistical analysis

Statistical analyses were performed using a two-tailed Student t test and GraphPad Prism 5 software (GraphPad Software). A paired t test was used most of the time because data were normally distributed. Tumor growth kinetics were assessed in a two-way ANOVA test. A 95% confidence interval was considered significant and was defined as p < 0.05 (*p < 0.05, **p < 0.001, ***p < 0.0001).

Results

Expression of lnc-chop in inflammatory and tumor-associated factor-mediated MDSCs

Previous studies have shown that multiple factors, including cytokines, transcription factors, and multiple signaling pathways, are involved in MDSC differentiation. Cytokines such as GM-CSF, IL-6, and other could in vitro mediate development of MDSCs. To investigate the regulatory mechanisms that control the differentiation and suppressive function of MDSCs in inflammatory and tumor environments, we compared gene expression between GM-CSF plus IL-6 and GM-CSF alone iMDSCs from mouse bone marrow cells using microarrays including lncRNA and protein-coding mRNA. Data showed that a remarkable difference existed not only in the expression of mRNAs but also in lncRNAs (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104718). Because GM16727 was highly expressed in GM-CSF plus IL-6–mediated iMDSCs (Fig. 1A), we further characterized and named it as lnc-chop. Lnc-chop expressed on chromosomal 11 is intron lncRNA (localized in a gene intron region) (Supplemental Fig. 1A). 5′- and 3′-RACE exhibited that...
this lncRNA had a length of ∼1800 bases, and the size was further confirmed by Northern blotting (Fig. 1B, Supplemental Fig. 1C). Lnc-chop was predominately localized to the nucleus and without coding capacity (Fig. 1C, Supplemental Fig. 2). After exposing to IL-6, lnc-chop expression was dose- and time-dependent (Fig. 1D, 1E). The inhibitors of both STAT3 and JAK1, critical factors in the IL-6-mediated signal pathway, could affect IL-6-mediated expression of lnc-chop (Fig. 1F).

FIGURE 1. Expression of lnc-chop in inflammation and tumor-associated factors induced iMDSCs. (A) LncRNA microarray of iMDSCs. iMDSCs were induced using GM-CSF or GM-CSF plus IL-6, and lncRNA expression was then evaluated to determine their lncRNA profiles using an lncRNA expression microarray. (B) RT-PCR (left) and Northern blot (right) of murine lnc-chop in mouse myeloid-derived iMDSCs. Mouse lnc-chop full length was from RACE and was analyzed by RT-PCR and Northern blot. The primers used for RACE are aligned against the conserved region. 18S and 28S RNA were used as the quality control of total RNA. (C) FISH of lnc-chop in mouse iMDSCs. CtrProb-FAM, control probe; LncProb-FAM, FAM-labeled lnc-chop probe. (D) qRT-PCR of lnc-chop in iMDSCs after exposure to GM-CSF plus IL-6 at different time points. Original magnification ×1000. (E) qRT-PCR of lnc-chop in iMDSCs after exposure to GM-CSF plus different concentrations of IL-6. (F) qRT-PCR of lnc-chop in mouse JAK1 and STAT3 inhibitor-treated iMDSCs. iMDSCs were first treated using JAK1 inhibitor or STAT3 inhibitor, and further cultured for 24 h in the presence of GM-CSF plus IL-6. (G) qRT-PCR of lnc-chop in MDSC subsets isolated from WT and IL-6−/− mice. Total CD11b+, CD11b+Gr1high, CD11b+Gr1int/low, CD11b+Ly6G+Ly6C+, CD11b+Ly6G+Ly6C− cells from tumor tissue in both WT and IL-6−/− mice (n = 5) were sorted, and then the levels of lnc-chop in different subsets were analyzed using qRT-PCR. Wild-type and IL-6−/− mice were s.c. injected with B16 melanoma cells (1 × 106) to establish a tumor model in vivo. (H) qRT-PCR of mouse lnc-chop relative to U6 RNA in MDSCs induced by IL-6, GM-CSF, GM-CSF plus IL-6, GM-CSF plus TNF-α, or 1D8 ovarian cancer, 4T1 breast cancer, B16 melanoma, and Lewis lung cancer cells. Error bars in (D)–(H) represent SDs from three independent measurements. Two-tailed, paired t test was used in (D)–(H). *p < 0.05, **p < 0.01, ***p < 0.001. BMC, CD11b+Gr1+BMC; GM, GM-CSF.
Isolated MDSCs from IL-6-deficient mice bearing B16 tumors also had a reduced lnc-chop (Fig. 1G). Importantly, the levels of lnc-chop were also higher in the iMDSCs, which were induced using GM-CSF, GM-CSF plus TNF-α, or 1D8 ovarian cancer, 4T1 breast cancer, B16 melanoma, and Lewis lung cancer cells (Fig. 1H). Taken together, our data demonstrate that inflammatory and tumor-associated factors such as IL-6 may induce lnc-chop expression in MDSCs.

Lnc-chop promotes immunosuppressive function of MDSCs in vitro

We next determined whether lnc-chop was involved in the regulation of differentiation and immunosuppressive function of iMDSCs. We employed in vitro gain- and loss-of-function studies to investigate the effects of lnc-chop on iMDSCs (Fig. 2A). qRT-PCR and immunoblot showed that Arg-1, CYBB (NOX2), NOS2, and ptgs2 (COX2), which play a critical role in regulating immunosuppressive function of iMDSCs (1, 4), were significantly reduced in lnc-chop knockdown iMDSCs, whereas the expression of these genes was upregulated in the exogenous lnc-chop overexpressed iMDSCs (Fig. 2B, 2C). Their relatively metabolic products such as Arg-1, NO, H2O2, and ROS were much lower in lnc-chop knockdown iMDSCs. However, gain-of-function treatment, lnc-chop promoted Arg-1 activity and production of NO, H2O2, and ROS (Fig. 2D-G). These data are consistent with the low regulatory potential of CHOP (1, 4), which may control the differentiation of MDSCs. MDSCs increased in exogenous lnc-chop–transduced mice injected by exogenous lnc-chop shRNA or exogenous LncRna and exposed to inflammatory and tumor environments. We found that lnc-chop knockdown inhibited BMCs to differentiate into Gr1lowCD11b+ MDSCs, whereas exogenous lnc-chop promoted the differentiation of Gr1lowCD11b+ MDSCs (Fig. 2A). Further analyses showed that lnc-chop knockdown decreased the fraction of the CD11b+Ly6c+Ly6c+MDSC (M-MDSC) subpopulation. However, the percentages of CD11b+Ly6c+Ly6c+MDSCs increased in exogenous lnc-chop–transduced BMCs (Fig. 2A). In mice injected by exogenous lnc-chop–transduced BMCs, the proportion of CD11b+Ly6c+Ly6c+MDSC populations significantly increased in the tumors of mice. Meanwhile, decreased Ly6c+Ly6c+MDSCs could be seen in the tumors of mice injected with lnc-chop knockdown BMCs (Fig. 2B). Thus, lnc-chop may control the differentiation of MDSCs into M-MDSCs. Notably, in CHOβ7–/– (Ddit3–/–) mice, the accumulation of tumor MDSCs did not extend to other myeloid populations, such as dendritic cells (CD11b+Gr11CD11c+) and macrophages (CD11b+Gr11F4/80+) (14), implying that lnc-chop may not exert its function only through CHOP.

Lnc-chop binds with both CHOP and C/EBPβ isoform LIP to promote activation of C/EBPβ isoform LAP

We next sought to determine underlying molecular mechanisms by which lnc-chop regulated MDSC immunosuppressive function. Some LncRNAs regulate the expression of neighboring genes (35). However, gain- and loss-of-function studies had no effect on the expression of its nearby coding gene Abr (Supplemental Fig. 3). Because lnc-chop was almost exclusively localized to the chromatin fraction, it might regulate expression of target genes by recruiting accessory factors, similar to other characterized LncRNAs (25, 36, 37). Multiple transcription factors, typically such as C/EBPβ, STAT3, and CHOP, play a central role in the differentiation and function of MDSCs (7, 14, 38). We thus hypothesized that lnc-chop might combine with these transcription factors to regulate MDSC function and differentiation. To test this, we performed RNA IP and pull-down analyses. We found that lnc-chop not only bond with C/EBPβ isoform LAP by binding to both C/EBPβ isoform LAP to promote activation of C/EBPβ isoform LAP.
MDSCs (16, 18). CHOP, a member of the C/EBP family of transcription factors, was thought to lack DNA binding activity and cannot form homodimers (39), but it can form heterodimers with other C/EBP family proteins to affect their activity such as the binding of CHOP with C/EBPβ isoform LIP to promote C/EBPβ isoform LAP activity (40). CHOP-deficient MDSCs also display reduced signaling through C/EBPβ (14). C/EBPβ isoform LIP also lacks DNA activation domains. However, LIP may form heterodimerized forms with other family members to control gene expression (17). Thus, lnc-chop–mediated separation of C/EBPβ LIP and CHOP with the LAP isoform may affect the binding of C/EBPβ LAP on the promoter region of genes (Fig. 5J). To test this, we analyzed the promoter region of lnc-chop–regulated genes Arg-1, NOS2, NOX2, and COX2 and found potential binding sites of C/EBPβ (Fig. 5J). We next employed ChIP-PCR analysis to determine the effects of the binding of lnc-chop to C/EBPβ LIP and CHOP on the C/EBPβ activity on the promoter region of these genes. ChIP-PCR showed that lnc-chop knockdown reduced the enrichment of C/EBPβ, whereas exogenous lnc-chop promoted the enrichment of C/EBPβ to these promoter regions (Fig. 5K), indicating that binding of lnc-chop with both LIP and CHOP promoted the activity of C/EBPβ isoform LAP. C/EBPβ knockdown with exogenous LIP, CHOP, or LAP further confirmed that C/EBPβ LIP, LAP, and CHOP are necessary for the action of lnc-chop (Supplemental Fig. 4).

**FIGURE 2.** Lnc-chop regulates suppressive function of iMDSC in vitro. (A) qRT-PCR of lnc-chop in lnc-chop knockdown and exogenous lnc-chop–treated iMDSCs. (B) qRT-PCR of Arg-1, NOS2, NOX2, and COX2 in lnc-chop knockdown and exogenous lnc-chop–treated iMDSCs. (C) Immunoblotting analyses of Arg-1, NOS2, NOX2, and COX2 in lnc-chop knockdown (left) and exogenous lnc-chop–treated (right) iMDSCs. (D–F) Arg-1, H2O2, and NO in lnc-chop knockdown (left) and exogenous lnc-chop–treated (right) iMDSC. Arg-1, H2O2, and NO were detected according to the described protocol in Materials and Methods. (G) Flow cytometry of ROS in lnc-chop knockdown and exogenous lnc-chop–treated iMDSCs. One representative of three independent experiments is shown. (H and I) IFN-γ analyses in the supernatants of OT-I CD8+ and OT-II CD4+ T cells in the presence of lnc-chop knockdown (left) and exogenous lnc-chop–treated (right) iMDSCs. kdLNC, lentivirus/lnc-chop shRNA; kdNC, empty lentivirus control; oeLNC, lentivirus/lnc-chop; oeNC, empty lentivirus control. Error bars in (A), (B), (D)–(F), (H), and (I) represent SDs from three independent measurements. Two-tailed, paired t test was used in (A), (B), (D)–(F), (H), and (I). *p < 0.05, **p < 0.01, ***p < 0.001.
Taken together, lnc-chop may interact with C/EBPβ isoform LIP and CHOP to encourage the activation of C/EBPβ.

Lnc-chop promotes enrichment of H3K4me3 on the promoter region of Arg-1, NOS2, NOX2, and COX2

Epigenetic modification also plays a critical role in the gene expression such that lysine methylation regulates the activation and repression of transcription by methylation of specific residues in the histone (41). Because the H3K4me3 mark represents active genes, we mainly focused on the changes of epigenetic components, which are related to H3K4me3. We thus examined the H3K4me3 mark on the 5′ regions of active genes in MDSCs. A Genome Browser image showed an H3K4me3 mark on the 5′ regions of Arg-1, NOS2, NOX2, and COX2 (Fig. 6A). ChiP-PCR demonstrated that lnc-chop...
FIGURE 4. Lnc-chop affects the differentiation of M-MDSCs. (A) Flow cytometry of Gr1^CD11b^, Gr1^{high}CD11b^, and Gr1^{low}CD11b^ MDSCs in lnc-chop knockdown or exogenous lnc-chop–treated iMDSCs (upper). Flow cytometry of CD11b^-Ly6G^-Ly6C^- and CD11b^-Ly6G^-Ly6C^ MDSCs in lnc-chop knockdown or exogenous lnc-chop–treated iMDSCs (lower). Percentage changes of iMDSC subsets were compared (n = 6). (B) Flow cytometry of CD45.1^ Gr1^CD11b^, Gr1^{high}CD11b^, and Gr1^{low}CD11b^ MDSCs in tumor tissues of mice bearing OVA-B16 tumor after infusing gain- and loss-of-function of CD45.1^ iMDSCs (upper). Flow cytometry of CD45.1^ Ly6G/Ly6C MDSC subsets in tumor tissues of mice bearing OVA-B16 tumor after infusing gain- and loss-of-function of CD45.1^ iMDSCs (lower). Percentage changes of MDSC subsets in tumor site were compared (n = 6). A two-tailed, paired t test was used in (A) and (B). *p < 0.05, **p < 0.05, ***p < 0.001. kdLNC, lentivirus/lnc-chop shRNA; kdNC, empty lentivirus control; oeLNC, lentivirus/lnc-chop; oeNC, empty lentivirus control.
FIGURE 5. *lnc-chop* binds to CHOP to promote the dissociation of LAP/LIP and improve the activation of LAP. (A) RNA IP (RIP) in iMDSCs. RIP was performed in GM-CSF alone or GM-CSF plus IL-6–induced iMDSCs using anti-C/EBPβ, and then PCR for *lnc-chop*. GM, GM-CSF; NC, negative control (water); PC, positive control. (B) RIP in V5-tagged LAP or LIP and *lnc-chop* cotransfected HEK293T cells. RIP was performed using anti-V5 Ab, and then PCR for *lnc-chop*. pcDNA3.1 represents the control plasmid. NC, negative control; PC, positive control. (C) RNA-protein pull-down in V5-tagged LIP-transfected HEK293T cells. RNA pull-down was performed using 3′ biotin–linked RNA in V5-tagged LIP-transfected HEK293T cells. No RNA and antisense RNA were used as controls. (D) RIP in MDSCs. RIP was performed in GM-CSF alone or GM-CSF plus IL-6–induced iMDSCs using anti-CHOP, and then PCR for *lnc-chop*. GM, GM-CSF; NC, negative control; PC, positive control. (E) RIP in V5-tagged CHOP and *lnc-chop*–cotransfected HEK293T cells. RIP was performed using anti-V5 Ab, and then PCR for *lnc-chop*. pcDNA3.1 represents the control. (Figure legend continues)
knockdown reduced the enrichment of the H3K4me3 mark to the promoter region, whereas lnc-chop promoted the enrichment of the H3K4me3 mark to the 5′ regions of these genes (Figs. 6B, 7). Thus, lnc-chop may promote the expression of Arg-1 and production of NO, H₂O₂, and ROS through promoting the enrichment of the H3K4me3 mark to the promoter region of these genes.

Discussion
In this study, we found that the interaction of lnc-chop with both CHOP and C/EBPβ isoform LIP encourages the activity of C/EBPβ and enrichment of H3K4me3, improves the immunosuppressive function of MDSCs, and meanwhile induces differentiation of M-MDSCs. We also demonstrate that lnc-chop expression may be induced in inflammatory and tumor environments. Thus, our data suggest an autologously regulating minicircuit to promote the immunosuppressive function and control the differentiation of MDSCs in inflammatory and tumor environments (Fig. 7). The identification of lnc-chop provides a specific regulatory locus that allows for rapid improvement of MDSC differentiation and suppressive function in response to extracellular inflammatory and tumor-associated signals.

We demonstrate that binding of lnc-chop with both LIP and CHOP may promote LAP isolation from C/EBPβ LAP/LIP and CHOP complexes to cause the activation of C/EBPβ LAP. CHOP was originally isolated as the gene induced in response to DNA plasmid. NC, negative control; PC, positive control. (F) RNA-protein pull-down in V5-tagged CHOP-transfected HEK293T cells. RNA pull-down was performed using 3′ biotin–linked RNA in V5-tagged CHOP-transfected HEK293T cell. No RNA and antisense RNA were used as controls. (G) Immunoblot of CHOP and LIP in HEK293T cells. LIP/pDNA3.1 (without V5 tagged), V5-tagged CHOP/pDNA3.1, and different concentration of lnc-chop/pDNA3.1 plasmids were cotransfected into HEK293T cells. IP was performed using V5 Ab, and then immunoblot using anti-C/EBPβ (specific for both LAP and LIP) to determine the interaction of CHOP and LIP. (H and I) Immunostaining and RNA-FISH of C/EBPβ and lnc-chop (H) as well as CHOP and lnc-chop (I) in mouse iMDSCs. Red shows C/EBPβ or CHOP, green shows lnc-chop, and blue shows nuclei. Original magnification ×800. (J) Schematic diagram showing possible mechanism of lnc-chop at C/EBPβ binding site on the Arg-1, NOS2, NOX2, and COX2 promoter region. The binding of lnc-chop with LIP could promote the interaction of LAP with C/EBPβ binding site on the promoter regions of Arg-1, NOS2, NOX2, and COX2. The bindings of C/EBPβ in the promoter regions of Arg-1, NOS2, NOX2, and COX2 were analyzed using the University of California, Santa Cruz Genome Browser. (K) ChIP-PCR of C/EBPβ binding regions on the promoter of Arg-1, NOS2, NOX2, and COX2. ChIP assays were performed using anti-C/EBPβ and then qRT-PCR. The data in all panels are a representative of at least three independent experiments. Comparisons between knockdown and control, as well as overexpression and control, are based on a two-tailed t test. *p < 0.05, **p < 0.05. kdLNC, lentivirus/lnc-chop shRNA; kdNC, empty lentivirus control; oeLNC, lentivirus/lnc-chop; oeNC, empty lentivirus control.
FIGURE 7. Mechanism of lnc-chop to promote immunosuppressive function of MDSCs in tumor and inflammatory environments. Tumor and inflammatory factors such as IL-6 may induce the expression of lnc-chop. lnc-chop may interact with CHOP and C/EBPB isoform LIP to promote activity of C/EBPB isoform LAP, which may cause the expression of immunosuppressive genes such as Arg-1, NOS2, NOX2, and COX2. Meanwhile, lnc-chop also promotes the accumulation of H3K4me3 in the promoter region of these immunosuppressive genes. As a result, IL-6-mediated lnc-chop promotes immunosuppressive function of MDSCs.
Luc-chop REGULATES THE IMMUNOSUPPRESSIVE FUNCTION OF MDCs


