Article

MicroRNA-155 Regulates Inflammatory Cytokine Production in Tumor-associated Macrophages *via* Targeting C/EBPβ

Min He[1], Zhenqun Xu[1], Tong Ding[1], Dong-Ming Kuang[1] and Limin Zheng[1, 2]

Macrophages (Mφ) are prominent components of solid tumors and exhibit distinct phenotypes in different microenvironments. We have recently found that tumors can alter the normal developmental process of Mφ to trigger transient activation of monocytes, but the underlying regulatory mechanisms are incompletely understood. Here, we showed that the protein expression of transcription factor C/EBPβ was markedly elevated in tumor-associated Mφ both *in vitro* and human tumors *in situ*. The expression of C/EBPβ protein correlated with cytokine production in tumor-activated monocytes. Moreover, we found that C/EBPβ expression was regulated at the post-transcriptional level and correlated with sustained reduction of microRNA-155 (miR-155) in tumor-activated monocytes. Bioinformatic analysis revealed that C/EBPβ is a potential target of miR-155 and luciferase assay confirmed that C/EBPβ translation is suppressed by miR-155 through interaction with the 3'UTR of C/EBPβ mRNA. Further analysis showed that induction of miR-155 suppressed C/EBPβ protein expression as well as cytokine production in tumor-activated monocytes, an effect which could be mimicked by silencing of C/EBPβ. These results indicate that tumor environment causes a sustained reduction of miR-155 in monocytes/Mφ, which in turn regulates the functional activities of monocytes/Mφ by releasing the translational inhibition of transcription factor C/EBPβ. *Cellular & Molecular Immunology*. 2009;6(5):343-352.

Key Words: C/EBPβ, inflammatory cytokine, miR-155, TAM, transcription factor

Introduction

Macrophages (Mφ) constitute a major component of immune-cell infiltrates seen in virtually all malignancies, and the tumor-associated Mφ (TAM) are almost entirely derived from circulating monocytes (1, 2). Compelling evidence from human and experimental tumors indicates that TAM release a broad array of cytokines, growth factors and enzymes to promote the proliferation and metastasis of cancer cells (3, 4). Accordingly, clinical studies have shown a significant correlation between TAM density and poor prognosis in most solid tumors (5, 6). In contrast to Mφ in normal or inflamed tissues that possess spontaneous anti-tumor activity, TAM exhibit distinct phenotypes with diverse functional programs in response to different environmental signals (7, 8). We have recently found that soluble tumor-derived factors can alter the normal development of APCs that is intended to induce early activation of monocytes and ultimately impaired their differentiation and activation (9, 10). This temporal preactivation is triggered by a mechanism different from that associated with LPS, but the precise underlying signaling events are not yet clear.

The activation status of monocytes/Mφ is primarily controlled by transcription factors, of which NF-κB is the best-known regulator for TAM dysfunction and can serve as the molecular link between inflammation and tumor progression (11, 12). In addition to NF-κB, C/EBPβ has also been implicated in the regulation of Mφ functional maturation and cytokine production (13). C/EBPβ is a member of C/EBP family of leucine zipper transcription factors, and it plays pivotal roles in coordinating the expression of a wide variety of genes that control immune responses (14, 15). C/EBPβ is transcribed from an intronless gene, and a single transcript is translated into three different isoforms (38 kDa LAP*, 35 kDa LAP and 20 kDa LIP) with the same 3'UTR sequence (16). Although not directly relating

[1]Key Laboratory of Gene Engineering of the Ministry of Education, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou, China;

[2]Correspondence to: Dr. Limin Zheng, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China; Tel: +86-20-8411-2163; Fax: +86-20-8411-2169; E-mail: zhenglm@mail.sysu.edu.cn

Received Sep 13, 2009. Accepted Sep 20, 2009.

Abbreviations: TSN, tumor culture supernatant; TAM, tumor associated macrophages; miRNA, microRNA; siRNA, short interfering RNA; PBMC, peripheral blood mononuclear cell.

to TAM, C/EBPβ was found to regulate the production of inflammatory mediators by Mφ, including cyclooxygenase-2 and various cytokines; and C/EBPβ-deficient mice exhibit defects in Mφ activation and differentiation (17).

MicroRNAs (miRNAs) are noncoding RNAs that can regulate the expression of protein-coding genes at post-transcriptional level through imperfect base-pairing with the 3'UTR of target mRNAs (18). Growing evidence now suggests that miRNAs regulate a wide range of biological processes, including those linked to cancer and immunity (19-22). Because TAM expressed a distinct and unique gene expression profile, the tumor microenvironments may alter the phenotype of monocytes/Mφ through miRNAs. However, it is presently unclear if miRNAs become altered in monocytes after their exposure to tumor environment and how miRNAs regulate the functional activities of these cells.

In this study, we observed an elevated expression of C/EBPβ in tumor-infiltrating Mφ in various human carcinomas as well as in monocytes activated by tumors. The expression of C/EBPβ protein is inversely correlated with miR-155 expression in monocytes after exposure to tumor environment. Moreover, we found that miR-155 could suppress the expression of C/EBPβ by directly targeting its 3'UTR and that introduction of miR-155 significantly attenuated the cytokine production in tumor-activated monocytes. These results suggest that tumor environments may regulate the functional activities of monocytes by decreasing the miR-155 expression to release its translational inhibition of transcript factor C/EBPβ.

Materials and Methods

Cell lines and preparation of tumor culture supernatants

Human hepatoma (SK-Hep-1 and HepG2), cervical (HeLa), transformed embryonic kidney (293T), and acute monocytic leukemia (THP-1) cell lines were obtained from American Type Culture Collection (Manassas, VA); glioma (U251), and lung carcinoma (95D) cell lines were from the cell bank of Chinese Academy of Sciences (Shanghai, China). All cells were proved to be mycoplasma free as routinely tested by a single-step PCR method (23), and they were maintained in complete medium composed of RPMI 1640 (or DMEM, Hyclone, Logan, UT) supplemented with 10% FBS. Tumor culture supernatants (TSNs) were prepared as previously described (9).

Isolation and culture of monocytes

Human peripheral blood mononuclear cell (PBMC) were isolated from buffy coats derived from healthy donors by Ficoll density gradient centrifugation as described previously (24). The cells were incubated in DMEM alone at 4×10^6/well in 24-well plates for 1.5 h, then washed and cultured in DMEM containing 10% human AB serum for 16 h to remove residual lymphocytes. Thereafter, the monocytes in DMEM containing AB serum were incubated in the presence of 15% TSNs, or medium alone for 6 to 8 days to obtain Mφ. Ten ng/ml of LPS was used to activate monocytes, and where indicated, the cells were pretreated with 10 μg/ml polymyxin B before exposure to LPS or TSNs.

RNA extracts, RT-PCR and quantitative real-time PCR

Expression of mRNA was determined by reverse transcription-PCR (RT-PCR). Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and subsequently treated with RNase-free DNase I (Fermentas, Vilnius, Lithuania). For mRNA analysis, aliquot containing 2 μg of total RNA was transcribed reversely using MMLV reverse transcriptase (Fermentas). The specific primers used to amplify *C/EBPβ* and *GAPDH* are as follows: *C/EBPβ*, 5'-TCT CTG CTT CTC CCT CTG CC-3' and 5'-ACA GCA ACA AGC CCG TAG G-3'; *GAPDH*, 5'-CAC CAT CTT CCA GGA GCG AG-3' and 5'-GGG GCC ATC CAC AGT CTT C-3'. *GAPDH* was amplified together with *C/EBPβ* in the same reaction to serve as an internal control. PCR products were resolved in 1.5% agarose gel and visualized by ethidium bromide staining.

Expression of mature miRNAs was determined by miRNA-specific quantitative real-time PCR (qRT-PCR) according to the manufacturer's instruction (Ambion, Austin, TX). cDNA was synthesized from 100 ng of total RNA using gene-specific primers for mature miR-155 and *U6* snRNA. The expression of miR-155 relative to *U6* snRNA was determined with SYBR green real-time quantitative PCR assay performed in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), each in triplicate.

Immunohistochemisty and Immunofluorescence

A total of 34 samples of hepatocellular, lung, cervical and gastric carcinomas (including 14 hepatocellular carcinoma, 6 lung adenocarcinoma, 6 cervical squamous cell carcinoma and 8 gastric adenocarcinoma specimens) were obtained from the Cancer Center of Sun Yat-Sen University and coded anonymously in accordance with local ethical guidelines, as stipulated by the Declaration of Helsinki and a protocol approved by the Review Board of the Cancer Center. Paraffin-embedded and formalin-fixed samples were cut into 5 μm sections, which were then processed for immuno-histochemistry as previously described (25). Following incubation with the Ab against human CD68 (DakoCytomation, Glostrup, Denmark) or C/EBPβ Ab (Santa Cruz Biotech., Santa Cruz, CA), the adjacent sections were stained using the EnVision System (DakoCytomation). Negative controls were performed by replacing the specific primary Ab with isotype matched control Ab at the same concentration.

Fixed cells or frozen tissues were double stained with rabbit anti-human CD68 and mouse anti-human C/EBPβ Ab, followed by incubation with Alexa Fluro 488-conjugated goat anti-rabbit IgG and Alexa Fluro 555-conjuagted goat anti-mouse IgG (Molecular Probes, Carlsbad, CA). Nuclei were then stained with 4'-6-diamidino-2-phenylindole. Images were assessed with a scanning confocal microscope (LSM510 META, ZEISS, Jena, Germany) and analyzed by

LSM510 META software (version 3.2).

ELISA

Cytokine concentrations in the culture supernatants were determined by ELISA kits according to the manufacturer's instructions (eBioscience, San Diego, CA).

Immunoblotting

The cell lysates were extracted as previously described (24). Equal amounts of cellular proteins were separated by 10% SDS-PAGE, immunoblotted with mouse anti-human C/EBPβ mAb, and visualized with a commercial ECL kit (Pierce, Rockford, IL). To confirm that each lane received the same amount of proteins, the blots were stripped and reprobed with rabbit anti-human GAPDH Ab (Abcam, Cambridge, UK).

Vector construction

To construct the miR-155 expression vector (pc3-miR-155), a 351 bp DNA fragment encompassing the mature miR-155 sequence and its 5'- and 3'-flanking regions (135 bp and 194 bp, respectively) was amplified with primers 5'-GCT GAA TTC TAC CTG TCA CCT TGG CTC TCC-3' and 5'-GCT TCT AGA GGT TGA ACA TCC CAG TGA CCA-3', and then cloned into pCDNA3.0 (Invitrogen).

To produce pGL3cm-C/EBPβ-3'-UTR-WT plasmid, a wild-type 3'UTR segment of human C/EBP-β mRNA containing the putative miR-155 binding sequence (1772-1794 nt) was amplified using primers 5'-GAT GAA TTC ACA CGG GAC TGA CGC AAC-3' and 5'-GAT TCT AGA CCC AAA AGG CTT TGT AAC CA-3', and then was inserted downstream of the stop codon of firefly luciferase in the pGL3cm as described previously (26). The pGL3cm-C/EBPβ-3'-UTR-MUT vector, which carried mutated sequence in binding sites of miR-155 (Figure 4A), was generated using fusion PCR method with the following primers 5'-AAA AGT GTT CTT TTA CCT TGA AAC GGA AAA G-3' and 5'-CTT TTC CGT TTC AAG GTA AAA GAA CAC TTT T-3'.

Luciferase Reporter Assay

293T cells were seeded at 4×10^4/well in 48-well plates and co-transfected with 400 ng of pc3-miR-155 or pcDNA3.0, 20 ng of pGL3cm-C/EBPβ-3'-UTR-WT or pGL3cm-C/EBPβ-3'-UTR-MUT, and 4 ng of pRL-TK (Promega) using calcium phosphate precipitation. Luciferase assay was performed as reported (26).

Oligonucleotides and transfection experiments

The hsa-miR-155 mimic was an RNA duplex designed as described previously (27), with the following sequence (sense/antisense): 5'-UUA AUG CUA AUC GUG AUA GGG G-3'/5'-CCU AUC ACG AUU AGC AUU ACU U-3'. The control RNA duplex (named as NC), with the sequence of 5'-UCA CAA CCU CCU AGA AAG AGU AGA-3' /5'-UAC UCU UUC UAG GAG GUU GUU AUU-3', was non-homologous to any human genome sequences. The anti-miR-155 (5'-ACC CCU AUC ACG AUU AGC AUU AA-3') was a 2'-O-methyl-modified oligoribonucleotide designed as an inhibitor of hsa-miR-155. The anti-miR-C (5'-GUG GAU AUU GUU GCC AUC A-3') was used as a negative control for anti-miR-155 in the antagonism experiment. Both miRNA duplexes and miRNA inhibitors were obtained from Genepharma (Shanghai, China). The small interference RNA (siRNA) targeting human C/EBPβ (si-C/EBPβ, identification number 114496 and catalog number 16708) and the negative control siRNA (si-NC, catalog number 4635) were purchased from Ambion (Austin, TX).

Transfection with small RNAs was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol for reverse transfection. HepG2 cells were seeded in antibiotic-free DMEM with 10% FBS and reversed-transfected with indicated RNA oligonucleotides. Fifty nM of RNA duplex and miRNA inhibitor were used for each transfection, unless otherwise noted. For monocytes, cells were pretreated with 15% TSNs for 24 h, followed by reverse transfection with 50 nM of hsa-miR-155 duplex, siRNA or respective control oligonucleotides and then incubated with DMEM with 10% human AB serum. Twenty-four hours after transfection, monoctytes were washed twice and recultured in medium alone or with 15% HepG2-TSN for 8 h, the supernatants were collected for ELISA and the cells were processed for Western blot assay.

Statistical analysis

All data are represented as means ± SEM from at least three separate experiments. The difference between two groups was analyzed using a two-sided Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

Results

Expression of C/EBPβ protein is upregulated in tumor-associated Mφ

C/EBPβ is known to transcribe a large number of cytokine-encoding genes that are important for inflammatory responses of monocytes/Mφ, such as TNF-α, IL-6 and IL-10 (28-32). To investigate the potential role of C/EBPβ in TAM, we first examined its expression in Mφ by immuno-staining serial sections of human tumor samples with CD68 (marker for monocytes/Mφ) and C/EBPβ. In hepatocellular carcinomas, CD68-positive cells were present in all areas of tumor and non-tumor tissues, but often predominantly in the stroma surrounding the cancer nest. Compared to the Mφ in non-tumor tissues, much higher levels of nuclear C/EBPβ was found in Mφ in peritumor stroma and cancer nests (Figure 1A). C/EBPβ was also abundantly expressed in cancer cells, but was weakly expressed on a fraction of Mφ in the adjacent nontumor tissues. Using confocal microscopy, we confirmed that about 80% of Mφ in the peritumoral stroma and cancer nest showed marked expression of C/EBPβ (Figure 1B). Similar results were obtained in the tissues from human gastric cancer, cervical carcinoma and lung cancer (data not shown), indicating the increased expression of C/EBPβ in TAM is a common phenomenon in

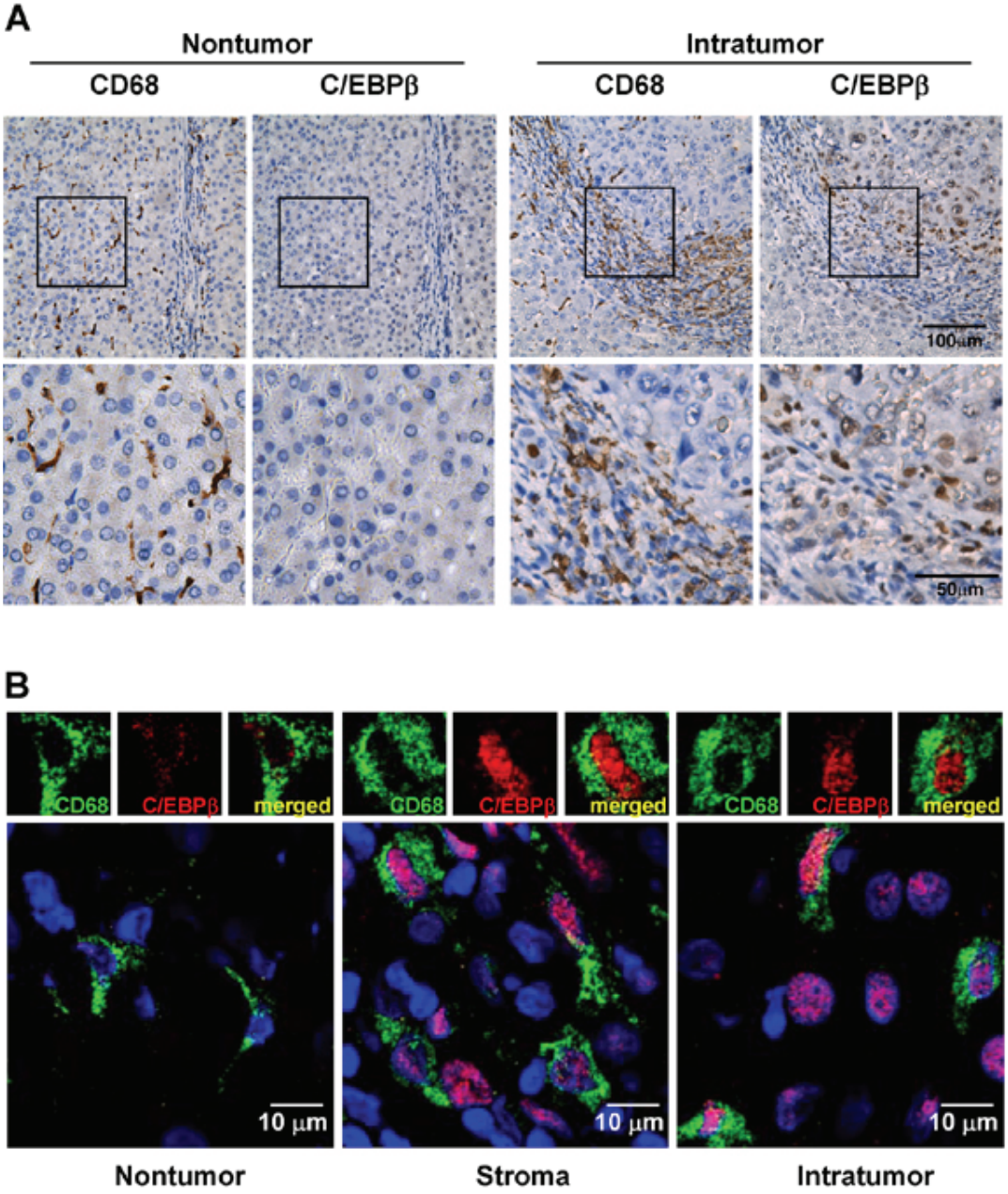

Figure 1. Distinct expression patterns of C/EBPβ in human tumor samples. (A) Adjacent sections of paraffin-embedded hepatocellular carcinoma were stained with an anti-CD68 or anti-C/EBPβ Ab. Positive cells are stained brown. Representative sites show the stained nontumor and stroma region. (B) Multiple staining of CD68 (green), C/EBPβ (red) and DAPI (blue, nuclei) in frozen sections of hepatocellular carcinoma tissue was analyzed by confocal microscopy. Note that most $CD68^+$ cells in peritumor stroma and cancer nest also express C/EBPβ. One of 14 representative patient samples is shown in A, and 1 of 8 representative samples is shown in B.

human tumor tissues.

C/EBPβ expression is correlated with cytokine production in TSN-exposed monocytes

TAM are almost entirely derived from peripheral blood monocytes (3, 7). We have recently found that TSNs from different tumor cell lines can mimic the tumor microenvironment to induce the formation of suppressive Mφ and dendritc cells *in vitro* with phenotypic features similar to those isolated from solid tumors (9, 10). Therefore, we used this model to investigate the regulatory mechanisms of C/EBPβ in freshly isolated monocytes after their initial exposure to TSNs. As shown in Figure 2A, exposure of monocytes to TSNs from most solid tumor types used in our

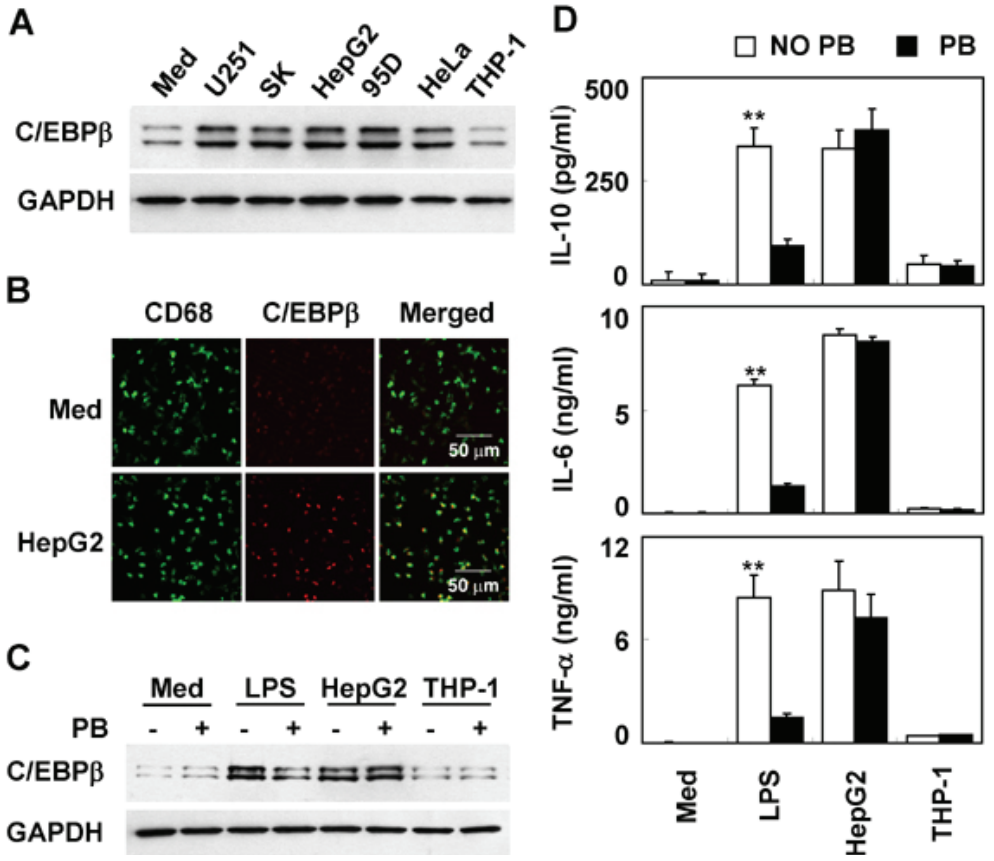

Figure 2. Increased C/EBPβ expression and cytokine production in TSN-exposed monocytes. (A and B) Monocytes were cultured with the indicated TSNs for 24 h. Expression of C/EBPβ in monocytes was determined by Western blotting (A) or confocal microscopic analysis (B). (C and D) Human monocytes were preincubated with (solid bars) or without (open bars) 10 μg/ml polymyxin B (PB) for 10 min, after which the cells were stimulated with LPS or indicated TSNs for 18 h. Expression of C/EBPβ and production of cytokines were determined by Western blot and ELISA, respectively. GAPDH was used for normalization in A and C. The results shown in A-C are representative of 4 experiments. The data in D represent the mean ± SE from 4 separate experiments; *$p < 0.05$ and ** $p < 0.01$, compared with monocytes incubated in medium alone.

study, including U251, SK-Hep-1, hepG2, 95D and HeLa, induced markedly increased expression of C/EBPβ in monocytes at 24 h, which was sustained for at least 144 h (data not shown). In contrast, the TSN from leukemia cells (THP-1), which did not affect the polarization of Mφ (9), failed to affect the expression of C/EBPβ in monocytes (Figure 2A). These results were further confirmed by confocal microscopic analysis, showing that most monocytes exhibited an increased C/EBPβ expression after exposure to TSNs (Figure 2B). The TSNs we used did not contain any measurable levels of TNF-α, IL-10, IL-1β, or IL-12, except for a low concentration of IL-6 (227 ± 53 pg/ml).

C/EBPβ is also knwon to regulate the transcription of cytokine genes in monocytes/Mφ following stimulation by microbial products (16). Indeed, stimulation of monocytes with LPS resulted in a rapid up-regulation of C/EBPβ protein in these cells and accumulation of TNF-α, IL-6, and IL-10 in the culture supernatants (Figure 2C). Pretreatment of monocytes with polymyxin B effectively blocked the activity of LPS, but did not affect the TSN-induced C/EBPβ expression and cytokine production in parallel experiments (Figures 2C and 2D). In addition, TSN from THP-1 cells, which did not affect the C/EBPβ expression in monocytes, had only marginal effect on the cytokine production profiles (Figure 2D). These results suggest that TSNs trigger the C/EBPβ upregulation and cytokine production in monocytes *via* a mechanism different from that associated with LPS-stimulation, and that the expression of C/EBPβ in monocytes coincided with accumulation of cytokine production.

Expression of C/EBPβ protein is inversely correlated with miR-155 expression in TSN-exposed monocytes

To elucidate the underlying mechanism for the induction of

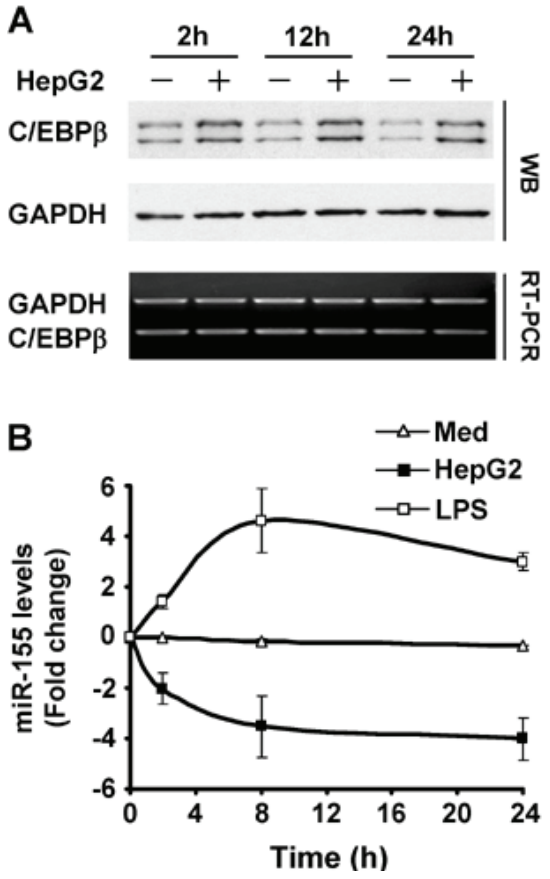

Figure 3. Inversely correlated expression of C/EBPβ protein and miRNA-155 in TSN-exposed monocytes. (A) Kinetic levels of C/EBPβ mRNA and protein in TSN-exposed monocytes. Western blot (Top and middle) and RT-PCR (Bottom) were used to monitor the expression level of C/EBPβ in HepG2-TSN-exposed monocytes for 0 to 24 h. GAPDH was used for normalization. Representative experiments out of 4 are presented. (B) RNA from untreated, LPS-treated or TSN-treated monocytes was analyzed by qRT-PCR to assay the expression of mature miR-155 over a 24-h time course. Results are given as fold changes in mRNA expression with respect to that in untreated monocytes at 0 h. A fold change of 0 indicates no change. Data were normalized to expression of U6 gene and representative of 4 independent experiments, each in triplicate.

C/EBPβ in TAM, we examined the kinetic expression of C/EBPβ mRNA and protein in TSN-exposed monocytes over a 24 h time course. Interestingly, an increased level of C/EBPβ protein was already observed in monocytes at 2 h after TSNs treatment, and maintained for at least 24 h (Figure 3A). However, the level of C/EBPβ mRNA was essentially unchanged in TSN-exposed monocytes, suggesting that the expression of C/EBPβ is posttranscriptionally regulated.

miRNAs have emerged as crucial regulators that suppress gene expression at the translation level. By using target prediction programs TargetScan and miRanda, we found a set of potential miRNAs that may target C/EBPβ, including

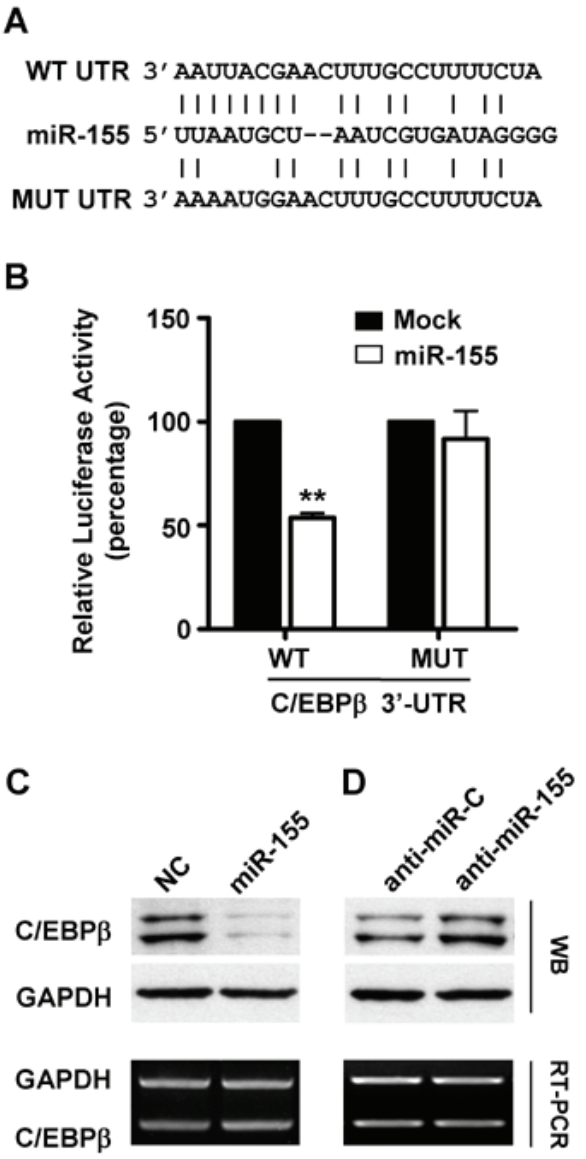

Figure 4. miR-155 regulates C/EBPβ translation. (A) Alignment of *miRNA-155* with C/EBPβ 3'UTR targeted site (nucleotides 1771-1794 of human C/EBPβ 3'UTR). The mutations introduced into the luciferase reporter construct are shown as well. (B) Analysis of luciferase activity. Cells were co-transfected with pc3-miR-155 or pcDNA3.0, firefly luciferase reporter containing either wild-type or mutant 3'UTR (indicated as WT or MUT on the *X* axis), and *Renilla* luciferase expression construct (as an internal control). Luciferase activity was assayed 48 h after transfection. *Firefly* luciferase values, normalized for *Renilla* luciferase, are presented. The data represent the mean ± SE of 4 independent experiments done in duplicates. $^{**}p < 0.01$, compared with cells transfected with empty pCDNA3.0 vector. (C and D) HepG2 cells were transfected with 50 nM of miR-155, anti-miR-155 or their controls (RNA duplex NC and anti-miR-C, respectively) for 48 h. Western blot (Top and middle) and RT-PCR (Bottom) were used to assay the expression level of C/EBPβ in transfected cells at 48 h post-transfection. GAPDH was served as an internal control. Representative experiments out of three are shown for both C and D.

miR-155. Since miRNA-155 has emerged as a key regulator in the homeostasis and function of immune system (33), we hypothesized that this miRNA may suppress post-transcriptionally the expression of C/EBPβ in TSN-exposed monocytes. To test this assumption, we initially examined the kinetic changes of miR-155 expression over a 24 h time course in TSN-treated monocytes. As shown in Figure 3, while the level of C/EBPβ protein but not that of mRNA significantly increases, the miR-155 level displayed a sharp decrease after TSN-treatment. The altered expression levels of both C/EBPβ and miR-155 became evident at 2 h after TSN-exposure and maintained till the end of experiment (Figures 3A and 3B). In contrast, stimulation of monocytes with LPS resulted in a rapid increase in miR-155 accumulation, reaching the peak at 8 h and then gradually declined (Figure 3B). These data support our previous finding that TSNs and LPS regulated Mφ activation *via* different mechanisms (9) and suggest that miR-155 may be involved in control of C/EBPβ expression in TSN-exposed monocytes.

C/EBPβ is a direct target of miR-155

To validate whether C/EBPβ was a direct target of miR-155, dual-luciferase reporter system was first employed. A human C/EBPβ 3'UTR, containing the wild-type or mutant putative binding site for miR-155 (Figure 4A), was cloned downstream of the firefly luciferase reporter gene. As shown in Figure 4B, co-transfection with miR-155 expression vector (pc3-miR-155) significantly decreased the luciferase activity of the reporter vector containing the wild-type 3'UTR of C/EBPβ, but had no effect on the mutant 3'UTR of C/EBPβ (Figure 4B), indicating that miR-155 can regulate gene expression through the putative binding site in the 3'UTR of C/EBPβ mRNA.

Next, we evaluated the capacity of miR-155 to regulate the endogenous C/EBPβ. The mRNA and protein levels of C/EBPβ were examined in HepG2 cells transfected with miR-155 duplex. In comparison with NC-transfectants, hsa-miR-155-transfected cells revealed a significant reduction (~80%) in the C/EBPβ protein level (Figure 4C, upper panel). Conversely, no fluctuation in C/EBPβ mRNA levels was observed after miR-155 transfection (Figure 4C, lower panel). In particular, all three isoforms of C/EBPβ were downregulated by miR-155, as they share the same 3'UTR (Data not shown). To further confirm these observations, anti-miR-155 was transfected to inhibit the endogenous miR-155 in HepG2 cells. Compared with the anti-miR-C-transfected cells, anti-miR-155-transfectants showed a moderate increase in the C/EBPβ protein but not mRNA level (Figure 4D). All the above results suggest that miR-155 can directly regulate the expression of C/EBPβ protein *in vivo*.

miR-155 regulates the cytokine production in TSN-exposed monocytes through targeting C/EBPβ

Having established the role of miR-155 in regulating C/EBPβ expression in cells, we then examined the possibility that

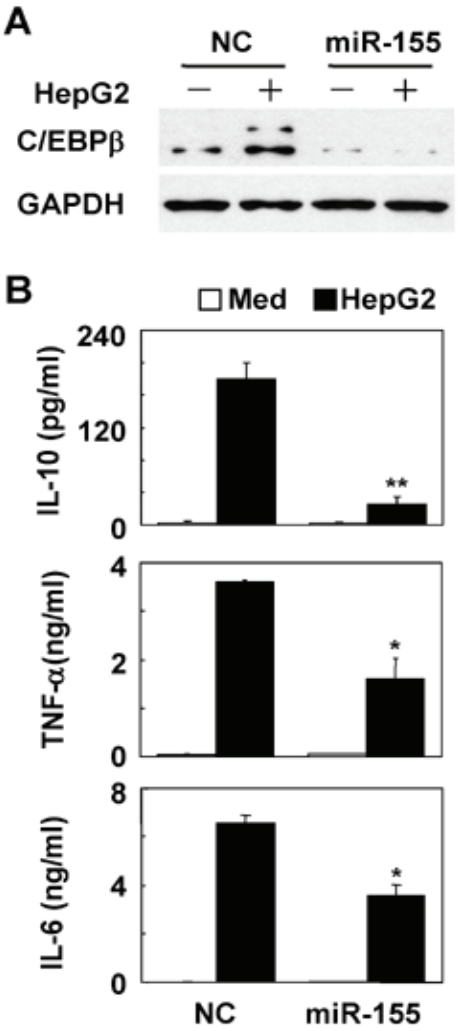

Figure 5. Overexpression of miRNA-155 inhibites C/EBPβ protein expression and cytokine production in TSN-pretreated monocytes. Monocytes were pretreated with HepG2-TSN for 24 h, and then reverse transfected with 50 nM of miR-155 or RNA duplex NC for 24 h. After which the cells were washed twice and recultured in medium alone or with HepG2-TSN for 8 h. (A) Analysis of C/EBPβ protein expression by Western blot in monocytes. GAPDH was used for normalization. A representative experiment out of three is shown. (B) The cytokine levels in the culture supernatants were determined by ELISA. Values represent the mean ± SE of 4 separate experiments. *$p < 0.05$ and **$p < 0.01$, compared with NC-transfected monocytes restimulated with HepG2-TSN.

miR-155 regulates the cytokine production in TSN-exposed monocytes through targeting C/EBPβ. TSN-pretreated monocytes were transfected with control RNA duplex (NC) or miR-155 duplex and then incubated with DMEM with 10% human AB serum. Twenty-four hours after transfection, monocytes were re-exposed to HepG2-TSN or medium alone for another 8 h. Interestingly, although restimulation with TSNs increased the level of C/EBPβ in NC-transfectants,

TSNs were unable to induce the expression of C/EBPβ in miR-155-transfected cells (Figure 5A). Furthermore, the level of C/EBPβ in monocytes transfected with miR-155 was obviously lower than those with NC duplex, in the case of re-exposure to either TSNs or medium alone (Figure 5A). Consistent with these results, miR-155-transfectants showed an attenuated cytokine production in monocytes after TSN-restimulation (Figure 5B). These results suggest that miR-155 may confer a major impact on the cytokine production of TSN-exposed monocytes *via* targeting the C/EBPβ.

To gain further evidence that miR-155 regulates the cytokine production in TSN-exposed monocytes through targeting C/EBPβ, we used siRNA against C/EBPβ to assay its function. As shown in Figure 6A, transfection of siRNA into HepG2 cells effectively downregulated C/EBPβ in both mRNA and protein levels. When the same siRNA was used to transfect TSN-exposed monocytes (Figure 6B), we found that the introduction of si-C/EBPβ significantly blocked the cytokine production in monocytes after TSNs re-exposure (Figure 6C). These results indicate that TSN-treatment may trigger the cytokine production in monocytes by downregulating the miR-155 expression to release its translational inhibition of transcript factors C/EBPβ.

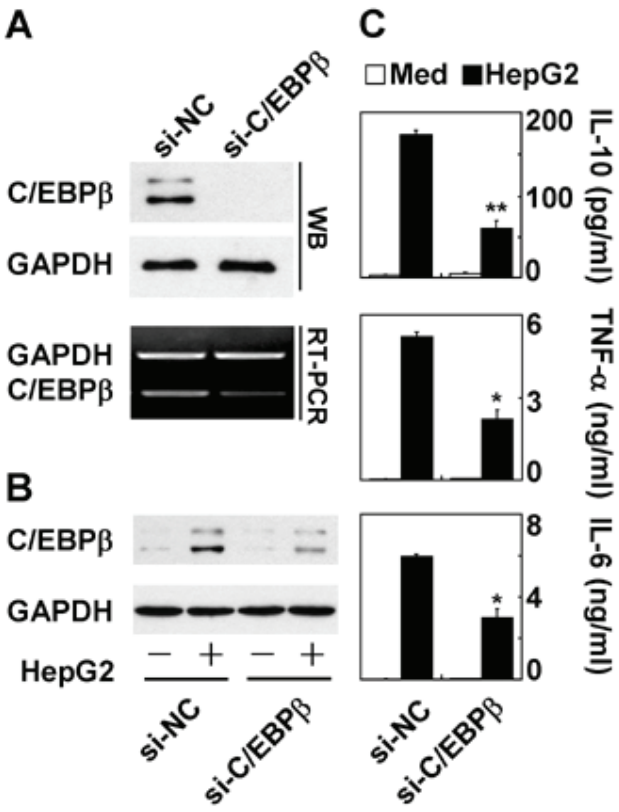

Figure 6. C/EBPβ silencing inhibits the cytokine production in TSN-exposed monocytes. (A) HepG2 cells were transfected with 50 nM of si-C/EBPβ or Mock, and then collected for subsequently analysis at 48 h post-transfection. Top and middle, C/EBPβ protein expression by Western blot in transfected cells and GAPDH was used for normalization. Bottom, C/EBPβ mRNA expression by RT-PCR in transfected cells. The GAPDH gene served as an internal control. (B and C) Monocytes were pretreated with HepG2-TSN for 24 h, and then reversed transfected with 50 nM of si-C/EBPβ or Mock. After incubation for 24 h, the cells were washed twice and recultured in medium alone or with HepG2-TSN for 8 h. (B) Analysis of C/EBPβ protein expression by Western blot. A representative experiment out of three is shown. (C) The cytokine levels in the supernatants were determined by ELISA. Values represent the mean ± SE of 4 separate experiments. *$p < 0.05$ and **$p < 0.01$, compared with Mock-transfected monocytes restimulated with HepG2-TSN.

Discussion

Tumor microenvironment is known to dynamically educate the recruited monocytes to take on distinct phenotype at different locations. The present study shows that the expression of C/EBPβ, a key transcription factor to modulate inflammatory cytokine expression, was increased in TAM both *in vitro* and human tumors *in situ*. Notably, tumor microenvironment elicit upregulation of C/EBPβ *via* a mechanism different from that associated with LPS, which was correlated with sustained reduction of miR-155. Moreover, we provided evidence that *miR-155* directly targeted C/EBPβ mRNA and regulated its expression *via* post-transcriptional mechanisms, which in turn interrupted the function and differentiation of TAMs by producing distinct cytokine profiles.

The casual relationship between inflammation, immunity and cancer is now widely accepted (34, 35). In neoplasia, Mφ constitute a major component of the inflammatory infiltrates and produce a number of inflammatory mediators to potentiate tumor progression (3). Accordingly, activation of the master inflammatory transcription factor NF-κB has been shown to promote tumor progression in inflammation-associated cancers (36). Here, we showed a marked expression of nuclear C/EBPβ in intratumoral monocytes/Mφ from several types of human cancers. Data from *in vitro* study showed that exposure to tumor environment resulted in a rapid increased C/EBPβ expression in monocytes, an effect which is correlated to their cytokine production. Moreover, silencing of C/EBPβ efficiently attenuated the cytokine production in TSN-exposed monocytes. In addition, We recently observed that autocrine inflammatory cytokines (TNF-α and IL-10) released from TSN-treated monocytes stimulated expression of PD-L1 (also termed B7-H1) which could effectively suppress tumor-specific T cell immunity and contribute to the growth of human tumors *in vivo* (37). These results indicate that C/EBPβ is an important transcription factor involved in modulating the function of monocytes/Mφ in human tumors. This notion is supported by previous reports showing that C/EBPβ is a crucial regulator for cytokine production in Mφ upon stimulation with a variety of stimuli, including LPS and IL-6 (28, 29).

Previous studies have shown that the expression of C/EBPβ could be regulated by several different mechanisms, including transcriptional up-regulation, post-translational modifications and nuclear translocation (13, 16). In this context, LPS is known to upregulate C/EBPβ expression at both mRNA and protein levels (16). Apparently, the tumor environment induces C/EBPβ expression in monocytes/Mφ *via* a mechanism different from that associated with LPS, which is not affected by polymyxin B. Moreover, we found that exposure to TSNs led to an increased expression of C/EBPβ protein, but did not affect its mRNA, suggesting that the expression of C/EBPβ protein in TAM is regulated at post-transcriptional level.

Mammalian miRNAs have emerged as the key post-transcriptional regulators of gene expression in various biological processes, including those linked to cancer and immunity (19-21, 38). The present study provides evidence that miR-155 regulates inflammatory cytokine production *via* C/EBPβ targeting. The results of 4 sets of experiments support this conclusion. First, bioinformatic analysis revealed that C/EBPβ is a potential target of miR-155, accordingly, the kinetics of C/EBPβ protein upregulation is closely correlated with that of miR-155 reduction in TSN-exposed monocytes. Second, the results from luciferase reporter assay demonstrated that miR-155 could regulate C/EBPβ protein expression through the conserved miR-155 binding site in the 3'UTR of the C/EBPβ mRNA. Third, the level of C/EBPβ protein is downregulated by ectopic expression of miR-155, but is upregulated by inhibition of endogenous miR-155 with synthetic inhibitor. However, both miR-155 duplex and anti-miR-155 oligonucleotides did not affect the expression of C/EBPβ mRNA, which further support the post-transcriptional regulation of C/EBPβ expression by miR-155. Forth, the forced expression of miR-155 resulted in an attenuated C/EBPβ protein expression as well as the cytokines production in monocytes after TSN-stimulation. These results clearly indicate that C/EBPβ is a *bona fide* target for miR-155 and that miR-155 controls cytokine production in TSN-exposed monocytes by releasing its translational inhibition of C/EBPβ.

In our experiments, ectopic expression of miR-155 is more potent than si-C/EBPβ in inhibiting IL-10 production, which indicates that other regulatory pathway may contribute to the miR-155 mediated cytokine production in TSN-exposed monocytes. Rodriguez and colleagues have recently shown that c-Maf is also the putative target of miR-155 (33), and ectopically expressed c-Maf is sufficient to trigger IL-10 production by immune cells (39, 40). In addition to miR-155, several other miRNAs, including miR-146 and miR-150 have been shown to play pivotal roles in the inflammatory responses and hematopoiesis of immune cells (21, 41). MiR-146 can attenuate expression of the innate signaling proteins IRAK1 and TRAF6, suggesting a possible regulatory role for miR-146 in human monocytes during Toll-like receptor signaling (41). Therefore, characterization of the physiological roles of miR-155 as well as other miRNAs in myeloid cells may provide new avenues for better understanding the regulation and function of immune system in tumor environments.

Aberrant miRNAs expressions have been observed in different types of cancer and their expression signatures can be extremely informative for cancer diagnosis (42, 43). However, the pathophysiologic roles of miRNAs as well as the molecular mechanisms by which miRNAs regulate the tumor progression are still largely unknown. The present study showed that tumor environment causes a sustained reduction of miR-155 in monocytes/Mφ, which in turn regulates the functional activities of monocytes/Mφ by releasing its translational inhibition of transcription factor C/EBPβ. Interestingly, several recent studies have shown that miR-155 increases substantially after exposure of monocytes/Mφ and myeloid dendritic cells to a variety of inflammatory stimuli (44, 45). Therefore, further elucidation of miR-155 targets and their roles in regulating Mφ activities in different microenvironments might provide a novel molecular basis for selective modulating Mφ activities in tumors.

Acknowledgements

This work was supported by the Outstanding Young Scientist Fund and project grants from the NSFC, China (30425025 and 30730086), and The Ministry of Health of China (2008ZX10002-019).

References

1. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002;23:549-555.
2. Sica A, Saccani A, Mantovani A. Tumor-associated macrophages: a molecular perspective. Int Immunopharmacol. 2002;2:1045-1054.
3. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer. 2004;4:71-78.
4. Mantovani A, Romero P, Palucka AK, Marincola FM. Tumour immunity: effector response to tumour and role of the microenvironment. Lancet. 2008;371:771-783.
5. Chen JJ, Lin YC, Yao PL, et al. Tumor-associated macrophages: the double-edged sword in cancer progression. J Clin Oncol. 2005;23:953-964.
6. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol. 2002;196:254-265.
7. Murdoch C, Giannoudis A, Lewis CE. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. Blood. 2004;104:2224-2234.
8. Maeda H, Kuwahara H, Ichimura Y, Ohtsuki M, Kurakata S, Shiraishi A. TGF-β enhances macrophage ability to produce IL-10 in normal and tumor-bearing mice. J Immunol. 1995; 155:4926-4932.
9. Kuang DM, Wu Y, Chen N, Cheng J, Zhuang SM, Zheng L. Tumor-derived hyaluronan induces formation of immuno-suppressive macrophages through transient early activation of monocytes. Blood. 2007;110:587-595.
10. Kuang DM, Zhao Q, Xu J, Yun JP, Wu C, Zheng L.

Tumor-educated tolerogenic dendritic cells induce CD3epsilon down-regulation and apoptosis of T cells through oxygen-dependent pathways. J Immunol. 2008;181:3089-3098.
11. Biswas SK, Gangi L, Paul S, et al. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-κB and enhanced IRF-3/STAT1 activation). Blood. 2006;107:2112-2122.
12. Li Q, Withoff S, Verma IM. Inflammation-associated cancer: NF-kappaB is the lynchpin. Trends Immunol. 2005;26:318-325.
13. Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. J Biochem. 2002;365:561-575.
14. Friedman AD. Transcriptional regulation of granulocyte and monocyte development. Oncogene. 2002;21:3377-3390.
15. Li H, Gade P, Xiao W, Kalvakolanu DV. The interferon signaling network and transcription factor C/EBP-beta. Cell Mol Immunol. 2007;4:407-418.
16. Poli V. The role of C/EBP isoforms in the control of inflammatory and native immunity functions. J Biol Chem. 1998;273:29279-29282.
17. Pizarro-Cerda J, Desjardins M, Moreno E, Akira S, Gorvel JP. Modulation of endocytosis in nuclear factor IL-6$^{-/-}$ macrophages is responsible for a high susceptibility to intracellular bacterial infection. J Immunol. 1999;162:3519- 3526.
18. Ambros V. The functions of animal microRNAs. Nature. 2004; 431:350-355.
19. Li QJ, Chau J, Ebert PJ, et al. miR-181a is an intrinsic modulator of T cell sensitivity and selection. Cell. 2007;129: 147-161.
20. Zhou B, Wang S, Mayr C, Bartel DP, Lodish HF. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. Proc Natl Acad Sci U S A. 2007;104:7080-7085.
21. Xiao C, Calado DP, Galler G, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell. 2007;131:146-159.
22. Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD. MicroRNAs: new regulators of immune cell development and function. Nat Immunol. 2008;9:839-845.
23. Uphoff CC, Drexler HG. Detection of mycoplasma in leukemia-lymphoma cell lines using polymerase chain reaction. Leukemia. 2002;16:289-293.
24. Zheng L, He M, Long M, Blomgran R, Stendahl O. Pathogen-induced apoptotic neutrophils express heat shock proteins and elicit activation of human macrophages. J Immunol. 2004;173:6319-6326.
25. Ding T, Xu J, Wang F, et al. High tumor-infiltrating macrophage density predicts poor prognosis in patients with primary hepatocellular carcinoma after resection. Human pathology. 2009;40:381-389.
26. Su H, Yang JR, Xu T, et al. MicroRNA-101, downregulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. Cancer Res. 2009;69:1135-1142.
27. Lim LP, Lau NC, Garrett-Engele P, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature. 2005; 433:769-773.
28. Pope R, Mungre S, Liu H, Thimmapaya B. Regulation of TNF-α expression in normal macrophages: the role of C/EBPβ. Cytokine. 2000;12:1171-1181.
29. Brenner S, Prosch S, Schenke-Layland K, Riese U, Gausmann U, Platzer C. cAMP-induced Interleukin-10 promoter activation depends on CCAAT/enhancer-binding protein expression and monocytic differentiation. *J Biol Chem* 2003; 278:5597-5604.
30. Bretz JD, Williams SC, Baer M, Johnson PF, Schwartz RC. C/EBP-related protein 2 confers lipopolysaccharide-inducible expression of interleukin 6 and monocyte chemoattractant protein 1 to a lymphoblastic cell line. Proc Natl Acad Sci U S A. 1994;91:7306-7310.
31. Cho YH, Lee CH, Kim SG. Potentiation of lipopolysaccharide-inducible cyclooxygenase 2 expression by C2-ceramide *via* c-Jun N-terminal kinase-mediated activation of CCAAT/ enhancer binding protein beta in macrophages. Mol Pharmacol. 2003;63:512-523.
32. Matsumoto M, Sakao Y, Akira S. Inducible expression of nuclear factor IL-6 increases endogenous gene expression of macrophage inflammatory protein-1 alpha, osteopontin and CD14 in a monocytic leukemia cell line. Int Immunol. 1998; 10:1825-1835.
33. Rodriguez A, Vigorito E, Clare S, et al. Requirement of bic/microRNA-155 for normal immune function. Science. 2007;316:608-611.
34. Greten FR, Eckmann L, Greten TF, et al. IKKβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell. 2004;118:285-296.
35. Pikarsky E, Porat RM, Stein I, et al. NF-κB functions as a tumour promoter in inflammation-associated cancer. Nature. 2004;431:461-466.
36. Karin M, Greten FR. NF-κB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol. 2005;5:749-759.
37. Kuang DM, Zhao Q, Peng C, et al. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. J Exp Med. 2009;206:1327-1337.
38. Fontana L, Pelosi E, Greco P, et al. MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. Nat Cell Biol. 2007;9:775-787.
39. Cao S, Liu J, Song L, Ma X. The protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. J Immunol. 2005;174:3484-3492.
40. Ho IC, Lo D, Glimcher LH. c-maf promotes T helper cell type 2 (Th2) and attenuates Th1 differentiation by both interleukin 4-dependent and -independent mechanisms. J Exp Med. 1998; 188:1859-1866.
41. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A. 2006;103:12481-12486.
42. Kloosterman WP, Plasterk RH. The diverse functions of microRNAs in animal development and disease. Dev Cell. 2006; 11:441-450.
43. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435:834-838.
44. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A. 2007; 104:1604-1609.
45. Brown BD, Gentner B, Cantore A, et al. Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. Nat Biotechnol. 2007;25:1457-1467.