Original Article
Overexpression of IL-12 reverses the phenotype and function of M2 macrophages to M1 macrophages

Xiao-Lin Yu, Bi-Tao Wu, Ting-Ting Ma, Yan Lin, Feng Cheng, Hai-Yu Xiong, Chang-Li Xie, Cui-Ying Liu, Qin Wang, Zi-Wei Li, Zhi-Guang Tu

College of Laboratory Medicine, Key Laboratory of Laboratory Medical Diagnostics of Education Ministry, Chongqing Medical University, Chongqing, China

Abstract: The characteristic of M2-polarized macrophage is IL-10^{high}IL-12^{low}. Reports have shown that the combined application of CpG and anti-interleukin-10 receptor antibody could switch M2 to M1, while there are few literatures about the impact of over-expression of IL-12 on M2. After infected with the recombinant adenovirus Ad-IL-12-GFP for 60 h, the generated M2 macrophages were harvested for analysis of PCR/FCM/WB, and the supernatants were collected as conditioned media. HepG2 cells were examined by MTT assay and FCM following incubated with different conditioned media. After overexpression of IL-12, The PCR analysis showed that the M2 macrophages exerted a high level of M1 macrophage specific markers (such as IL-23, IL-1β, et al), a low level of M2 macrophage specific markers (IL-10, TGF-β), the FCM analysis showed that CD86 was up-regulated while CD206 was down-regulated, and the WB showed a similar change trend. The conditioned medium of IL-12 overexpressed macrophages suppressed the proliferation, induced apoptosis, and caused G0/G1 cell cycle arrest in HepG2 cells compared to the control groups (P<0.05). To conclude, these results have indicated that the phenotype and function of M2 macrophages could be reversed by the overexprression of IL-12. It may be beneficial to explore the anti-cancer immunotherapy strategy switching the M2 macrophages to M1 macrophages.

Keywords: Recombinant adenovirus Ad-IL-12, macrophage, M2, M1, polarization

Introduction

Macrophages, the major inflammatory component infiltrating the tumor microenvironment, play an indispensible role in the process of tumor growth and progression. Heterogeneity and plasticity are the remarkable hallmarks of macrophages. The heterogeneity means that macrophages include a variety of activation status or phenotypes, and the plasticity means that the phenotype of macrophages could be reverted responding to its local microenvironment [1-3].

Of the numerous phenotypes, M1- and M2-polarized macrophages are the two extreme groups. Classically activated macrophages (M1), stimulated by IFN-γ alone or with LPS, are characterized by IL-12^{high}IL-23^{high}IL-10^{low}. In contrast, alternatively activated macrophages (M2), promoted by IL-4 and IL-13, and are char-

acterized by IL-10^{high}IL-12^{low}IL-23^{low}. Additionally, the two types of macrophage are apparently differential in production of inflammatory cytokines (including pro- and anti-inflammatory cytokines), chemokines, cytotoxical intermediates, scavenger and mannose receptors, et al [4-7]. Based on these features, M2 are distinguished from M1 macrophages and they are different in functions. For instance, M2 has the capacity of tumor promotion, while M1 is able to suppress tumor through various mechanisms. Tumor-associated macrophages (TAM) are the major inflammatory component of the stroma of solid tumors, and can affect different aspects of the neoplastic tissue. Many researchers have indicated that TAM express several M2-associated phenotype characteristics and pro-tumor functions, represent a unique and distinct polarized M2 subpopulation, and are considered as a potential target for anti-cancer therapy [8].
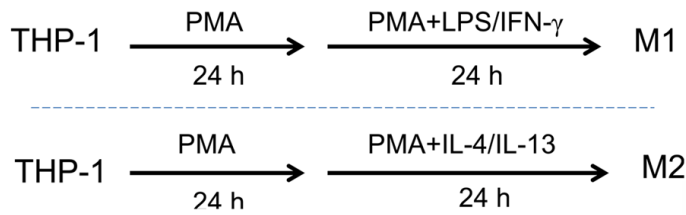
Figure 1. The schematic method to differentiate THP-1 to M1/M1 macrophages. Treated with PMA for 24 h and then cultured with PMA plus LPS/IFN-γ for another 24 h, THP-1 was induced to M1-polarized macrophages ("M1"). Treated with PMA for 24 h and then cultured with PMA plus IL-4/IL-13 for another 24 h, THP-1 was differentiated to M2-polarized macrophages ("M2").

Interleukin-12 (IL-12), linking the innate and adaptive immune responses, belongs to the IL-12 family of cytokines. Consisting of the subunit p35 (a light chain) and p40 (a heavy chain) [9], IL-12 is mainly produced by dendritic cells (DCs), monocytes and macrophages, and exerts various aspects of roles [10-12]. Although a majority of researches have demonstrated that IL-12 is one of the most potential anti-tumor cytokine, but until now IL-12 has not successfully translated into the clinics owing to its non-negligible side-effects when administrated systemically [13, 14].

Based on the pro-tumoral effects of M2, a multitude of researches have focused on the potential anti-tumor strategies targeting on M2 macrophages [15-17]. Guiducci C and his colleagues found that combined application of CpG and anti-interleukin-10 receptor antibody could promptly switch infiltrating macrophages from M2 to M1 within 16 h [18]. Our recent study has proven that IL-12 overexpressed monocytes could directionally polarize to M1-like macrophages [19], and treatment with IL-12 would induce the differentiation of monocytic tumor cells [20]. Considering the characteristic of M2 macrophages (IL-10^{high}IL-12^{low}), it will be of great interest to hypothesize that M2 macrophages could be also reverted after overexpression of IL-12. Therefore, the objective of the current study was to explore whether the phenotype and function of M2 macrophages could be altered by overexprression of IL-12.

Materials and methods

Main materials and reagents

Recombinant adenovirus Ad-IL-12-GFP and Ad-CMV-GFP were constructed by our laboratory [18]. Phorbol-12myristate13-acetate (PMA) and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human IL-4 and IFN-γ were obtained from PeproTech (Princeton, NJ, USA). Anti-CD86 and anti-CD206 antibodies conjugated with PE were obtained from eBioscience (San Diego, CA, USA). The other antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA) and BOSTER (Wuhan, China), unless specified. All the primers for PCR were synthesized by Invitrogen (Shanghai, China).

Cell culture

THP-1 (human acute monocytic leukemia cell line) and HepG2 (human hepatocellular carcinoma cell line) were purchased from Chinese Type Culture Collection (Shanghai, China). Cells were cultured in RPMI-1640 and DMEM (Invitrogen, USA), respectively, both supplemented with 10% FBS (Gibco, USA) and 100 mg/ml penicillin-streptomycin, and incubated at 37°C in a humidified atmosphere with 5% CO_2.

For M1 and M2 polarization, the following procedures were performed according to the method described [21]: THP-1 cells were treated with 50 ng/ml PMA for 48 h, and then stimulated with 20 ng/ml IL-4 (added at 24 h later) to generate M2-polarized macrophages (M2), with 20/ng ml IFN-γ+100 ng/ml LPS (added at 24 h later) to generate M1-polarized macrophages (M1) positive control (**Figure 1**). The phenotypes were identified with the expression of surface maker and phenotype-specific cytokines.

Defining experimental groups and collecting conditioned medium

The established M2-polarized macrophages were washed thoroughly 3 times with PBS and then propagated in different media, briefly, in complete medium only (group Blank), complete medium with Ad-CMV-GFP (group Ad-CMV-GFP), or complete medium with Ad-IL-12-GFP (group Ad-IL-12-GFP). After above treatment for 24 h, macrophages of three groups were grown in complete medium for another 48 h, then the supernatants from the three groups were collected, filtered, aliquoted and stored at -20°C until use [22-24]. The cells were harvested for different use (PCR/FCM/WB) described below.

Table 1. Primer sequences for qPCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Amplification size (bp)
TNF-α	CGAGTGACAAGCCTGTAGCC	TGAAGAGGACCTGGGAGTAGAA	171
IFN-β	ATGGCTTATTACAGTGGCA	TAGTGGTGGTCGGAGATT	137
IL-23	TGGAGATGGCTGTGACCC	GAAGCGGAGAAGGAGACG	271
IL-1β	CTGGAGCAATGCAATACAAA	AGGTAGAGGTTGCTGTTGGAA	206
IL-8	GTCTTAGAGGACTGGGTGTA	ACAGTGTGAGCTTGGAGGGA	160
IL-10	AGAACCAAGACCCAGACATCA	GCATTCTTCACCTGCTCCAC	139
TGF-β	ATCCTGTGGAGGAGGAATAC	CTGGAAACGTTGTGATGAGC	174
CD197	GATTACATCGGAGACAACACCA	AGTACATGATAGGGAGGAACCAG	106
IL-12 p35	CTGGACCACCTCAGTTTGG	TCAGAAGTGCAAGGGTAAAA	155
IL-12 p40	GTGGAGTGCCAGGAGGACA	TCTTGGGTGGGTCAGGTTT	148
β-actin	GGAAATCGTGCGTGACATT	CAGGCAGCTCGTAGCTCTT	113

To evaluate the effect of M2 macrophages infected by Ad-IL-12-GFP on cancer cells, HepG2 cells were treated with different conditioned medium described above for indicated time and grouped as HepG2, HepG2/CMV and HepG2/Ad-IL-12, respectively.

RNA isolation, reverse transcription-PCR (RT-PCR) and quantitative real-time PCR (qPCR)

Total RNA was extracted from macrophages using Trizol reagent (TaKaRa, Japan) according to the manufacturer's protocol. Reverse transcription was performed with 1,000 ng total RNA from each sample using PrimeScript RT Reagent Kit (TaKaRa, Japan) in accordance with the manufacturer's protocol. qPCR was carried out following the protocol supplied with TaKaRa kit (TaKaRa, Japan), and the relative expression of target genes was determined using the $2^{-\Delta\Delta ct}$ method by normalizing target gene Ct values to those for reference gene β-actin. The appropriate primers used were shown in **Table 1** and samples were analyzed in triplicates.

Flow cytometry analyses

For cell surface markers of macrophages: After the M2-polarized macrophages were transfected with recombinant adenovirus (Ad-IL-12-GFP, Ad-CMV-GFP) or without for 60 h, cells were collected and washed 3 times with cold PBS, followed by incubation with human CD86-PE, CD206-PE, or isotype IgG2A-PE for 30 min at 4°C. Samples were then analyzed by a flow cytometer (BD Company, NJ, USA).

For cell cycle and apoptosis of HepG2

The protocol was described previously [20, 25], briefly, HepG2 were treated with different conditioned media. Following examinations were performed (1) Cells were washed 3 times with PBS (pH 7.4), and fixed by 75% ethyl alcohol for 1 h, and then immersed in propidium iodide (PI) staining solution for cell cycle analysis by the flow cytometer (BD company, NJ, USA). (2) Cells were washed 3 times with PBS (pH 7.4), and stained by Annexin V/PI staining for cell apoptosis analysis by the flow cytometer (BD company, NJ, USA).

Proliferation assay

The HepG2 cells were seeded into 96-well plates at a density of 1,600 cells per well and cultured with different conditioned media described above for indicated time. The cells were then incubated with complete media plus 10% Cell Counting Kit-8 (CCK-8) solution for additional 1 h. The optical density (OD) was read at 450 nm as recommended by the manufacturer (Dojindo, Kumamoto, Japan). Experiments were performed in triplicate from three independent tests.

Western blotting

After transfected with recombinant adenovirus for 60 h, macrophages were treated with lysis buffer containing protease and phosphatase inhibitors. The protein concentrations were detected by BCA protein assay kit (Beyotime, Jiangsu, China). Protein samples were subjected to SDS-PAGE, and the separated proteins
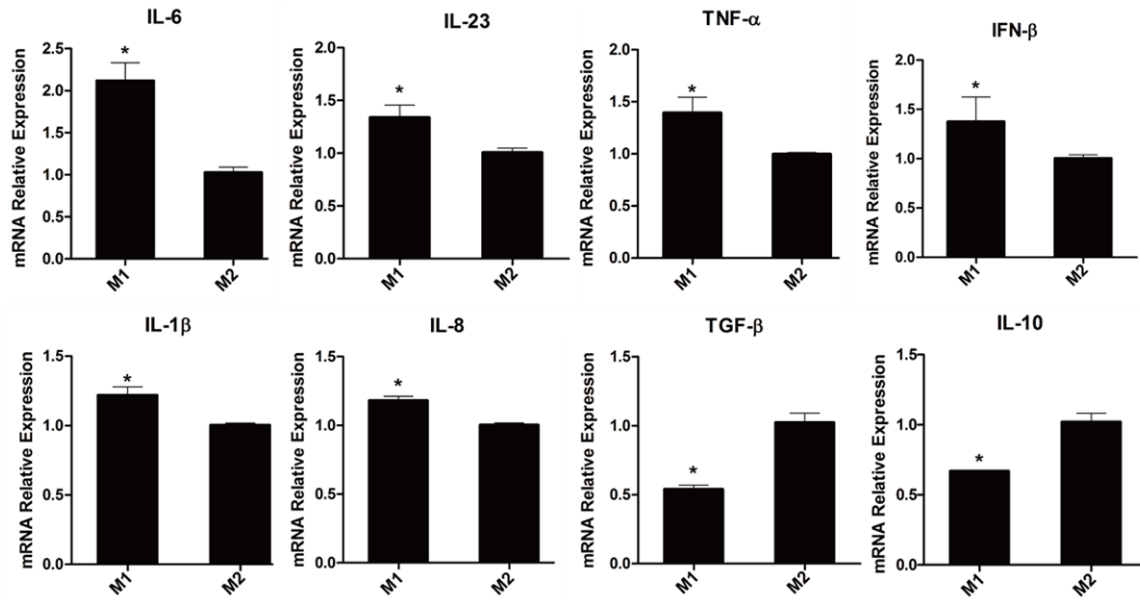
Figure 2. The mRNA expression changes of some pro- and anti-inflammatory factors in THP-1 cells stimulated with IL-4 (M2) or LPS+IFN-γ (M1) for 24 h. Results were represented as mean ± SD of three independent experiments. *P<0.05, compared with group M2.

were transferred onto the PVDF membranes. The membranes were blocked with 5% skim milk, incubated with different primary antibodies (anti-p-stat3, anti-p-stat1, anti-wnt2, anti-β-catenin, anti-c-myc, anti-NF-κ BP50 and anti-NF-κ BP65 antibodies) overnight, followed by incubation with HRP-conjugated secondary antibody, and visualized with the ECL reagents (Millpore, USA). To ascertain equal loading of proteins, house-keeping protein actin was utilized as a loading control.

Statistical analysis

Experimental results were analyzed by Student's t test or One-Way ANOVA with SPSS version 19.0 software, *P<0.05* was considered to be statistically significant. All experiments were repeated at least three times. Values were shown as mean ± SD.

Results

Established M1 and M2 macrophages

In accordance with the method described by Tjiu *et al*, THP-1 was polarized to M1 and M2 macrophages with minor modifications, as shown in **Figure 1**. Some pro- and anti-inflammatory cytokines were analyzed by qPCR, compared with group M1, the mRNA expression of

TNF-α, IFN-β, IL-23, IL-1β, CD197 and IL-8 was significantly decreased, while cytokines IL-10, TGF-β were markedly increased in group M2 (**Figure 2**).

Overexprression of IL-12 in M2 macrophages after transfected with Ad-IL-12-GFP

Recombinant adenovirus transduction was evidenced by GFP expression (**Figure 3A**). The expression of subunits of IL-12 p35 and p40 were examined by RT-PCR and qPCR. As shown in **Figure 3B** and **3C**), M2 macrophages transfected with recombinant adenovirus (Ad-IL-12-GFP) for 36 h had a significantly higher expression of p35 and p40 than those of control groups (Ad-CMV-GFP or M2 blank). These results have indicated that IL-12 over-expressed M2 macrophages were established by Ad-IL-12-GFP transfection.

Phenotypical changes of M2 macrophages after transfected with Ad-IL-12-GFP

To explore the effects of overexpression of IL-12 on the phenotype of M2 macrophages, some M1 and M2 macrophage specific markers were assessed by qPCR (**Figure 4A**). Following transfection of recombinant adenovirus for 60 h, M1 macrophage specific markers IL-23, CD197, IL-1β elevated significantly, while M2
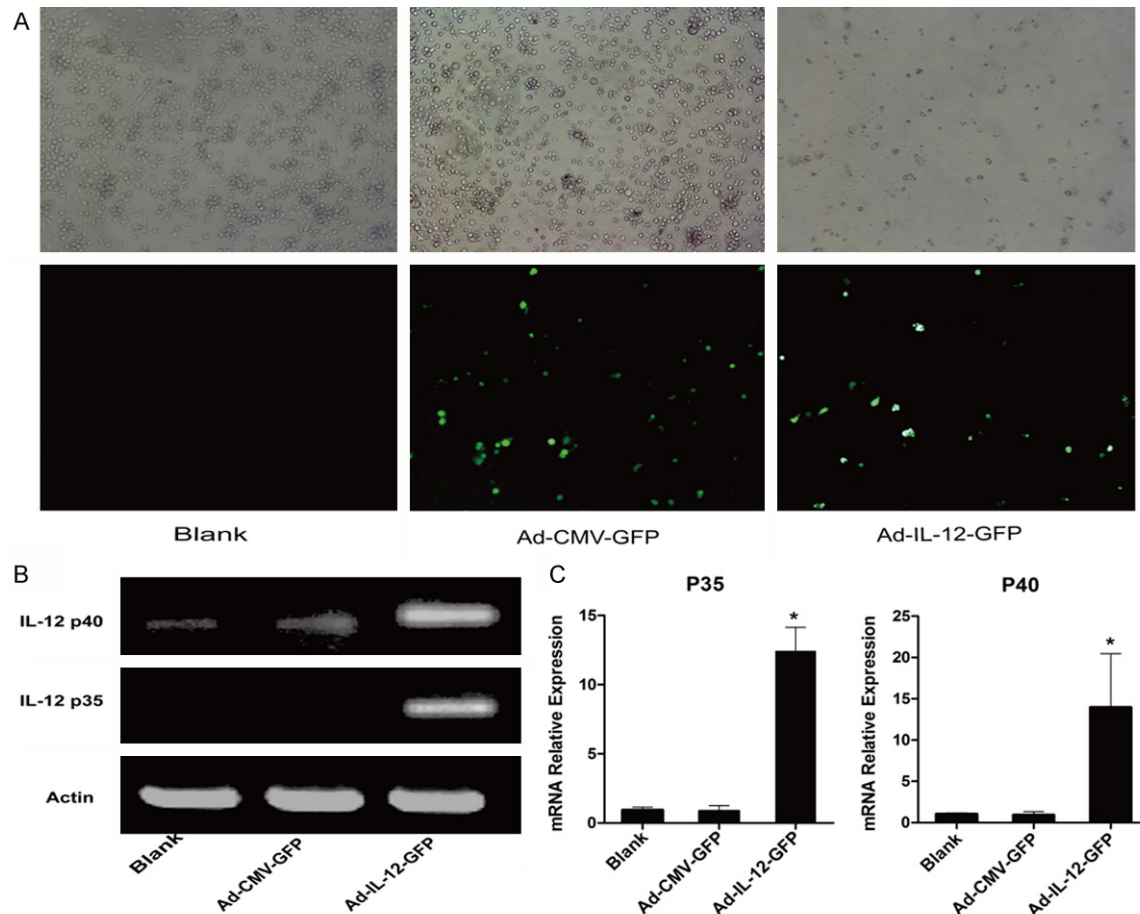
Figure 3. Fluorescence of M2 macrophages infected with recombinant adenovirus for 36 h (A); The mRNA expression of IL-12 subunits p35 and p40 analyzed by RT-PCR (B) and quantitative PCR (C) in M2 macrophages infected with recombinant adenovirus for 36 h. Actin was used as an internal control. Results were represented as mean ± SD of three independent experiments. *P<0.05 compared with control groups.

macrophage specific markers IL-10, TGF-β exhibited markedly reduction in group Ad-IL-12-GFP compared to group Ad-CMV-GFP and group Blank. Furthermore, some cell surface markers were detected by FCM (**Figure 4B**), compared to control groups, group Ad-IL-12-GFP had a higher expression of M1 surface marker CD86 and lower expression of M2 surface marker CD206. These results have suggested that overexprression of IL-12 could reverse the phenotype of M2 macrophages to M1-like macrophages.

Functional changes of M2 macrophages after infected with Ad-IL-12-GFP

To investigate the effects of Ad-IL-12-GFP on the function of M2 macrophages, some biological behaviors of HepG2 were analyzed including the growth, apoptosis and cell cycle distribution. After treatments with different condi-

tioned media, the proliferation of HepG2 was assessed by MTT. AS shown in **Figure 5A**, the proliferation of HepG2 was dramatically inhibited from the day 3 in group HepG2/IL-12 compared with group HepG2 and group HepG2/CMV.

In addition, the apoptosis and cell cycles of HepG2 were examined by FCM (**Figure 5B** and **5C**). After incubation with conditioned media for 72 h, the early apoptosis rate of group HepG2/IL-12 was 11.33%, while the early apoptosis rates in group HepG2 and group HepG2/CMV were 2.91% and 3.01%, respectively. The cell cycle distribution of HepG2 was also altered following cultured with different conditioned media. The proportion of G1 phase population was far higher in group HepG2/IL-12 than those in control groups. While compared the proportion of cells in S phase, group
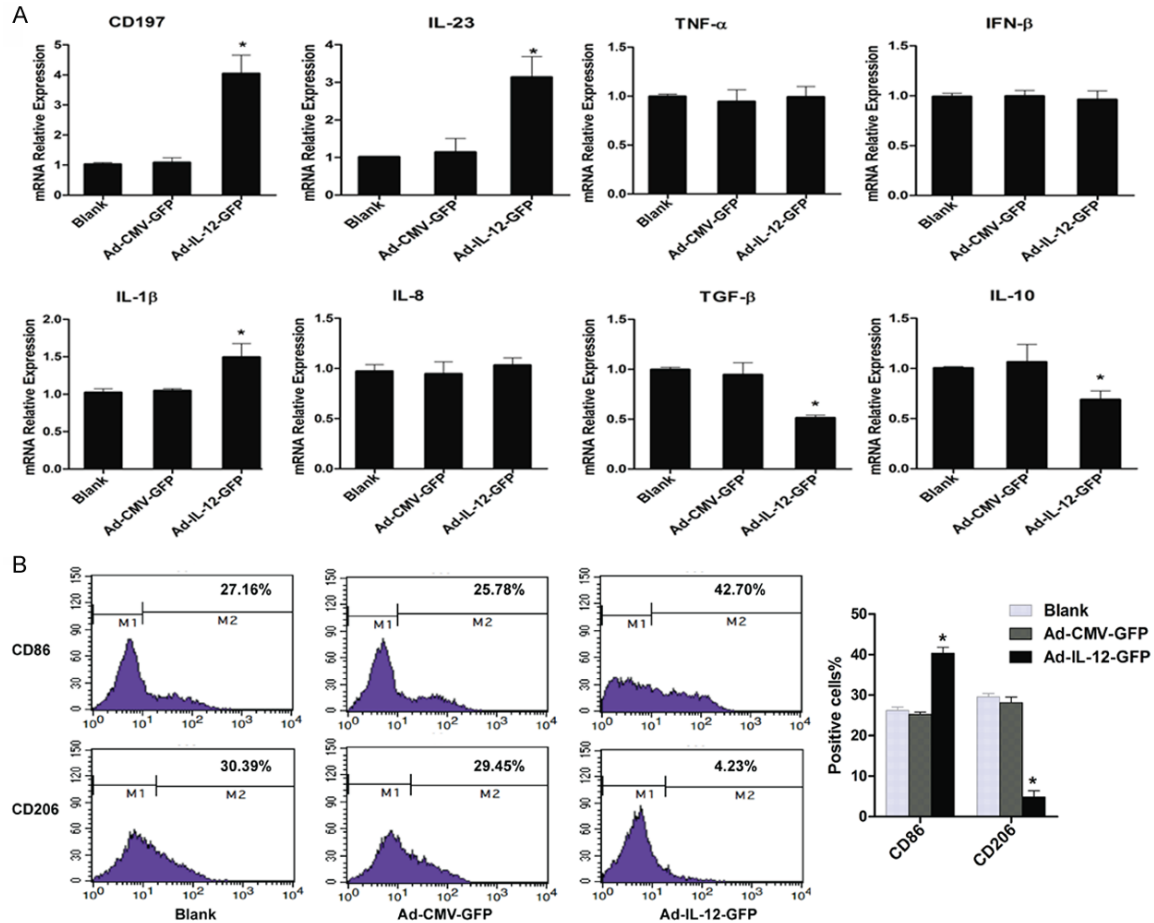
Figure 4. The effect of overexprression of IL-12 on M2 macrophage phenotype. After transferred with recombinant adenovirus for 60 h, the mRNA expression of some M1/M2 macrophage-specific markers was examined by qPCR (A), and the cell surface antigens CD86, CD206 were tested by FCM (B). Results were represented as mean ± SD of three independent experiments. *$P<0.05$ compared with control groups.

HepG2/IL-12 was much lower than the control groups. Similarly G2/M phase was also lower in group HepG2/IL-12 although did not reach the significant level. These findings have revealed that overexprression of IL-12 could change the function of M2 macrophages, which is from tumor promotion to tumor suppression.

Alteration of some signaling molecules related to macrophage polarization

Majorities of signaling molecules were involved in the process of macrophage polarization. Parts of these molecules were analyzed by Western blotting following transduction of recombinant adenovirus. The signaling molecules p-stat3, wnt2, β-catenin, c-myc and NF-κ BP50 were down-regulated in group Ad-IL-12-GFP, while signaling molecules p-stat1 and NF-κ BP65 were up-regulated, compared to

those in group Blank and group Ad-CMV-GFP (**Figure 6**).

Discussion

In the current study we investigated the effects of Ad-IL-12-GFP on the phenotype and function of M2 macrophages. It was found that after over-expression of IL-12, the phenotype of M2 macrophages was re-directed to that of M1-like macrophages, accompanied with alteration of the phenotype characteristic markers and related signaling molecules. And the function of M2 macrophages was also changed from pro-tumor to anti-tumor.

IL-12 is one of the most extensively studied cytokines in the field of anti-tumor immunotherapy [13]. Watkins and his colleagues have found that the functional features of tumor-
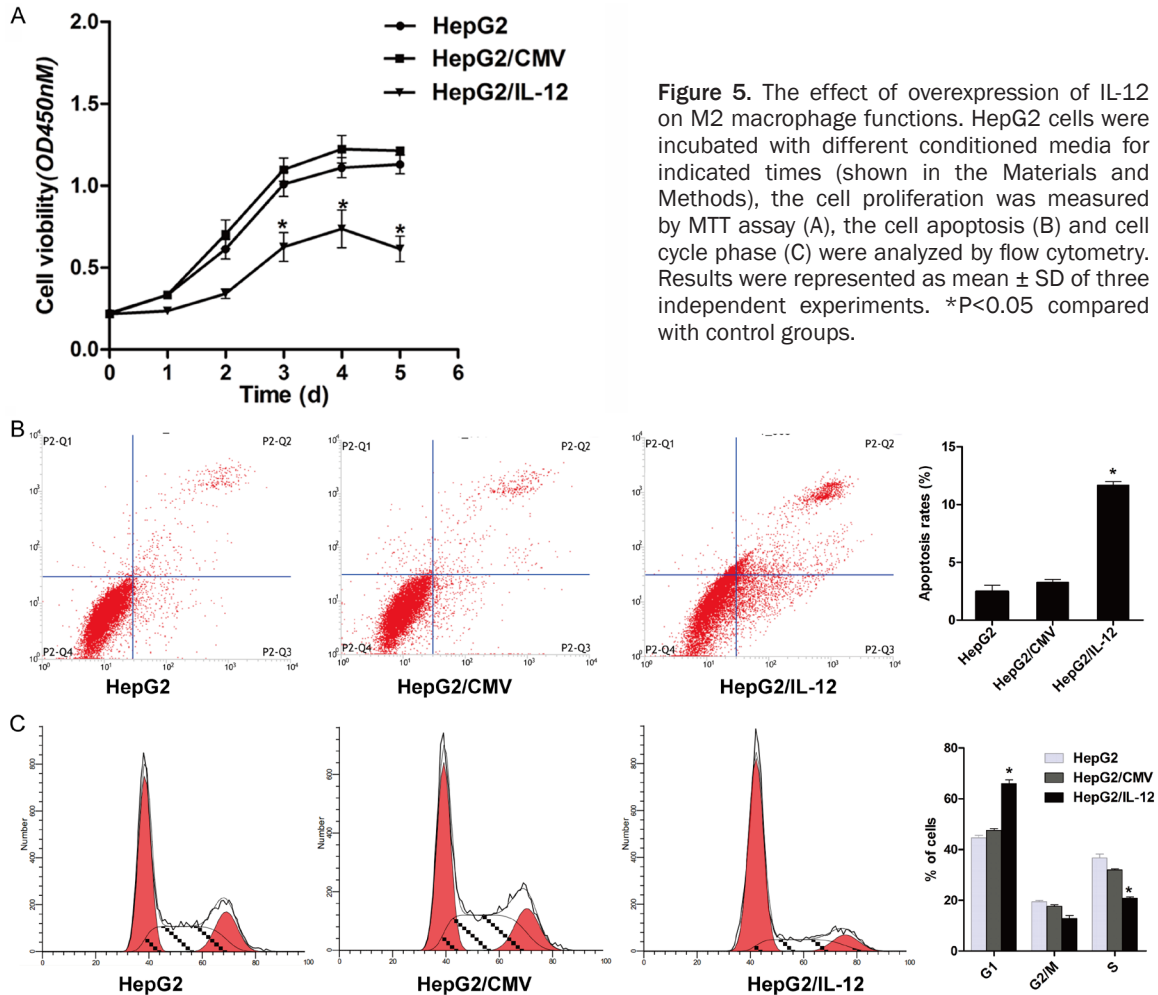
Figure 5. The effect of overexpression of IL-12 on M2 macrophage functions. HepG2 cells were incubated with different conditioned media for indicated times (shown in the Materials and Methods), the cell proliferation was measured by MTT assay (A), the cell apoptosis (B) and cell cycle phase (C) were analyzed by flow cytometry. Results were represented as mean ± SD of three independent experiments. *P<0.05 compared with control groups.

associated and tumor-infiltrating macrophages could be rapidly altered by IL-12 carried through microcapsules [26]. Our recent researches have also revealed that IL-12 overexpressed monocytes would directionally differentiate to M1-like macrophages through downregulation of p-stat-3 and result in the inhibition of HCC growth in vitro and vivo [20]. Furthermore, IL-12 treatment could induce monocytic tumor cells differentiation and its possible mechanism is related with the up-regulation of c-fms expression and the phosphorylation of CSF-1R Tyr-809 site [21].

THP-1 was the widely utilized cell lines to explore the features of monocytes and macrophages in vitro [25]. In accordance with a variety of documents [21, 27, 28], especially the report by Tjiu et al [21], we induced THP-1 to M1 and M2 macrophages, respectively. Based on the characteristics of polarized macrophages, some inflammation factors and cell surface antigens were applied as markers to distinguish M1 and M2 macrophages in our research. The results showed that "M2" exhibited a high level of IL-10, TGF-β, whereas a low level of CD197 TNF-α, IFN-β, IL-23, IL-1β, and IL-8, suggesting that the M2 macrophage model was successfully established, which was also in line with the reports [21].

Overexpression of target gene is usually accomplished with viral-vector to transfer target genes to interesting cells. Frequently, the viral-vector carries target genes as well as green fluorescent protein (GFP) gene. The expression of GFP was used as a visible indicator of the viral-vector successful transfection. In our study, the transfection of Ad-IL-12-GFP vector was identified by fluorescence microscopy, and the expression of target gene IL-12 was proved by the high expression of IL-12 subunits P35 and
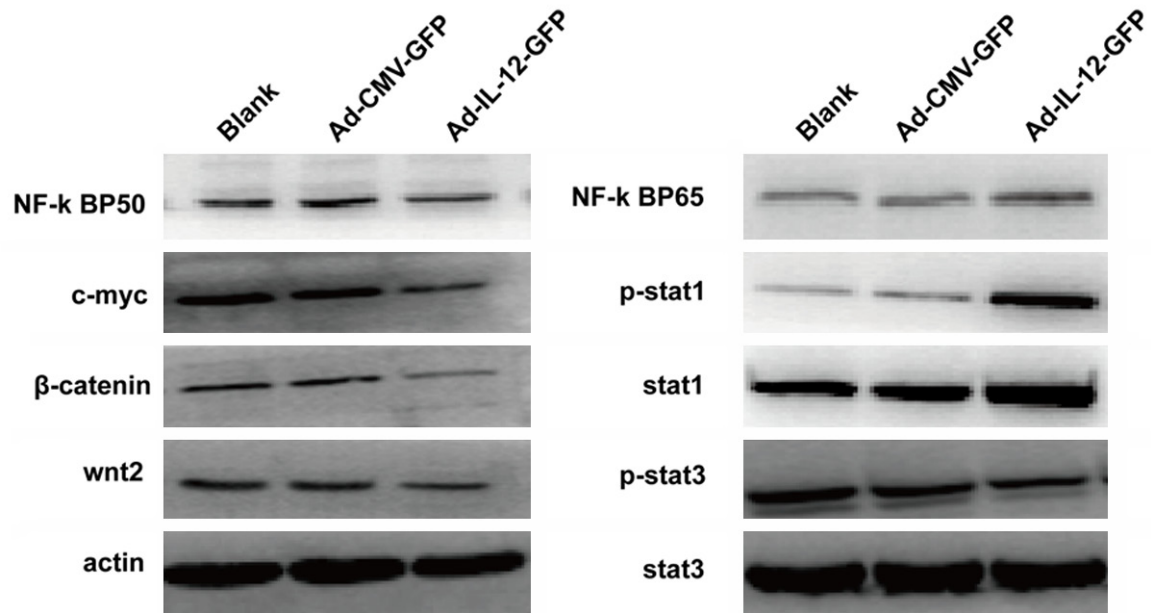
Figure 6. The changes of some signaling molecules related to macrophage polarization. The M2 macrophages were infected with recombinant adenovirus for 60 h and the protein levels of the signaling molecules were analyzed by Western blotting. The actin served as a loading control.

P40 through RT-PCR and qPCR. These results have indicated that M2 macrophage model of over-expression of IL-12 is well constructed.

In the aspect of M2 macrophage phenotype influenced by over-expression of IL-12, some related markers including cytokines, chemokines and cell surface antigens were measured. It was found that the expression of genes related to M1 specific markers IL-23, CD197 and IL-1β were significantly elevated, while M2 macrophage specific markers IL-10 and TGF-β exhibited markedly reduction (**Figure 4**). In consistent with above results, the cell surface antigens CD86, a marker of M1 macrophages [29], was up-expressed, whereas M2 macrophages marker CD206 [30] showed a down-expression. Furthermore, in the aspect of M2 macrophage function, some biological behaviors of HepG2, such as HepG2 cell proliferation, cell apoptosis and cell cycle were examined. It was found that the HepG2 cells exhibited proliferation suppression, apoptosis induction and G0/G1 cell cycle arrest following treatment with conditioned medium of over-expression IL-12 macrophages culture compared with those of two control groups. The reason that we used conditioned medium rather than the Transwell co-culture model was due to the M2 macrophage redirected in phenotype until infected with recombinant adenovirus for as long as 60

h, and at this time point, the cell state of M2 macrophages was so poor that it could not afford to continue co-culturing with HepG2 for an extra long time. The conditioned medium was extensively applied in similar researches [22, 23]. However, our results could prove that M2 macrophages with pro-tumor action could be re-educated to M1-like macrophages with anti-tumor effect by over-expression IL-12.

It is well known that a majority of signaling molecules are involved in the macrophage polarization. For instance, p-stat3 [31-33], wnt2 [34], β-catenin [34], c-myc [35], NF-κ BP50 [36], p-stat1 [36] and NF-κ BP65 [36] are all involved in the M1/M2 macrophage polarization. Yu H at al [31] demonstrated that p-stat3 was constitutively activated in a multitude of carcinoma cells and TAM. The activated p-stat3 in TAM could promote tumor immune tolerance in the tumor microenvironment [37, 38]. Base on the research by us [20] and Zhang X at al [39], p-stat3 was suppressed following the polarization of monocytes and TAM "re-education" to M1-like macrophages. In present study, it was shown that signaling molecules p-stat3, wnt2, β-catenin, c-myc and NF-κ BP50 were all down-regulated in group Ad-IL-12-GFP, while signaling molecules p-stat1 and NF-κ BP65 were up-regulated, and the change trends were consistent with other reports [31-39]. These

findings have suggested that over-expression of IL-12 inducing the phenotype and function of M2 macrophages into M1-like macrophages involve above signaling molecules. Nevertheless, further analysis with the effect of each individual signal molecule and their interactions in the regulation mechanism of macrophage re-direction by overexpression IL-12 is needed.

In conclusion, present study reveals that over-expression of IL-12 could reverse the phenotype and function of M2 macrophages into M1-like macrophages; it means from tumor promotion action conversion to tumor suppression effect. This may contribute to the understanding of anti-tumor immune therapy strategies targeting the polarization of M2 macrophages and TAM to M1 macrophages with IL-12 over-expression.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Zhi-Guang Tu, College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, China. Tel: 86-23-68485759; Fax: 86-23-68485239; E-mail: tuzhiguang@aliyun.com

References

[1] Sica A and Mantovani A. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 2012; 122: 787-795.

[2] Mosser DM and Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008; 8: 958-969.

[3] Mantovani A, Sozzani S, Locati M, Allavena P and Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002; 23: 549-555.

[4] Adams DO and Hamilton TA. The cell biology of macrophage activation. Annu Rev Immunol 1984; 2: 283-318.

[5] Stein M, Keshav S, Harris N and Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med 1992; 176: 287-292.

[6] Martinez FO, Gordon S, Locati M and Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J Immunol 2006; 177: 7303-7311.

[7] Martinez FO, Sica A, Mantovani A and Locati M. Macrophage activation and polarization. Front Biosci 2008; 13: 453-461.

[8] Sica A, Schioppa T, Mantovani A and Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: Potential targets of anti-cancer therapy. Eur J Cancer 2006; 42: 717-727.

[9] Jalah R, Rosati M, Ganneru B, Pilkington GR, Valentin A, Kulkarni V, Bergamaschi C, Chowdhury B, Zhang GM, Beach RK, Alicea C, Broderick KE, Sardesai NY, Pavlakis GN and Felber BK. The p40 Subunit of Interleukin (IL)-12 Promotes Stabilization and Export of the p35 Subunit. J Biol Chem 2013; 288: 6763-76.

[10] Gee K, Guzzo C, Che Mat NF, Ma W and Kumar A. The IL-12 Family of Cytokines in Infection, Inflammation and Autoimmune Disorders. Inflamm Allergy Drug Targets 2009; 8: 40-52.

[11] Colombo MP and TrinchieriG. Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev 2002; 13: 155-168.

[12] Xu M, Mizoguchi I, Morishima N, Chiba Y, Mizuguchi J and Yoshimoto T. Regulation of Antitumor Immune Responses by the IL-12 Family Cytokines, IL-12, IL-23, and IL-27. Clin Dev Immunol 2010; 1-9.

[13] Tugues S, Burkhard SH, Ohs I, Vrohlings M, Nussbaum K, Vom Berg J, Kulig P and Becher B. New insights into IL-12-mediated tumor suppression. Cell Death Differ 2015; 22:237-246.

[14] Marshall E. Cancer trial of interleukin-12 halted. Science 1995; 268: 1555.

[15] Wahl L and Kleinman HK. Tumor-associated macrophages as targets for cancer therapy. J Natl Cancer Inst 1998; 90: 1583-1584.

[16] Mantovani A and Locati M. Tumor-Associated Macrophages as a Paradigm of Macrophage Plasticity, Diversity, and Polarization. Arterioscler Thromb Vasc Biol 2013; 33: 1478-1483.

[17] Guiducci C, Vicari AP, Sangaletti S, Trinchieri G and Colombo MP. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. Cancer Res 2005; 65: 3437-3446.

[18] Cheng F, Kuang WB, Wang Q, Qin XL, Fan XQ, Dong JY, Liang QD, Li P and Tu ZG. Construction of adenovirus vector containing hIL-12 gene and its effects on the proliferation and TGF-β expression of Hep3B cells. Chinese J Immunol 2012; 5: 402-406.

[19] Wang Q, Cheng F, Ma TT, Xiong HY, Li ZW, Xie CL, Liu CY, Tu ZG. Interleukin-12 inhibits the hepatocellular carcinoma growth by inducing macrophage polarization to the M1-like phenotype through downregulation of Stat-3. Mol Cell Biochem 2016; 415: 157-168.

[20] Ma TT, Wu BT, Lin Y, Xiong HY, Wang Q, Li ZW, Cheng F and Tu ZG. IL-12 could induce monocytic tumor cells directional differentiation. Mol Cell Biochem 2015; 402: 157-69.

[21] Tjiu JW, Chen JS, Shun CT, Lin SJ, Liao YH, Chu CY, Tsai TF, Chiu HC, Dai YS, Inoue H, Yang PC, Kuo ML and Jee SH. Tumor-Associated Macrophage-Induced Invasion and Angiogenesis of Human Basal Cell Carcinoma Cells by Cyclooxygenase-2 Induction. J Invest Dermatol 2009; 129: 1016-1025.

[22] Engström A, Erlandsson A, Delbro D, Wijkander J. Conditioned media from macrophages of M1, but not M2 phenotype, inhibit the proliferation of the colon cancer cell lines HT-29 and CACO-2. Int J Oncol 2014; 44: 385-392.

[23] Hedbrant A, Erlandsson A, Delbro D and Wijkander J. Conditioned media from human macrophages of M1 phenotype attenuate the cytotoxic effect of 5-fluorouracil on the HT-29 colon cancer cell line. Int J Oncol 2015; 46: 37-46.

[24] Guihard P, Danger Y, Brounais B, David E, Brion R, Delecrin J, Richards CD, Chevalier S, Rédini F, Heymann D, Gascan H and Blanchard F. Induction of Osteogenesis in Mesenchymal Stem Cells by Activated Monocytes/Macrophages Depends on Oncostatin M Signaling. Stem Cells 2012; 30: 762-772.

[25] Qin Z. The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. Atherosclerosis 2012; 221: 2-11.

[26] Watkins SK, Egilmez NK, Suttles J and Stout RD. IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages in vitro and in vivo. J Immunol 2007; 178: 1357-1362.

[27] Laskar A, Eilertsen J, Li W and Yuan XM. SPION primes THP1 derived M2 macrophages towards M1-like Macrophages. Bicchem Biophy Res Commun 2013; 441: 737-742.

[28] Jeong SK, Yang K, Park YS, Choi YJ, Oh SJ, Lee CW, Lee KY, Jeong MH and Jo WS. Interferon gamma induced by resveratrol analog, HS-1793, reverses the properties of tumor associated macrophages. Int Immunopharmacol 2014; 22: 303-310.

[29] Mantovani A, Sozzani S, Locati M, Allavena P and Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002; 23: 549-555.

[30] Hamilton TA, Zhao C, Pavicic PG Jr and Datta S. Myeloid colony-stimulating factors as regulators of macrophage polarization. Front Immunol 2014; 5: 554.

[31] Takaishi K, Komohara Y, Tashiro H, Ohtake H, Nakagawa T, Katabuchi H and Takeya M. Involvement of M2-polarized macrophages in the ascites from advanced epithelial ovarian carcinoma in tumor progression via Stat3 activation. Cancer Sci 2010; 101: 2128-2136.

[32] Yu H, Kortylewski M and Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat Rev Immunol 2007; 7: 41-51.

[33] Lang R. Tuning of macrophage responses by Stat3-inducing cytokines: molecular mechanisms and consequences in infection. Immunobiology 2005; 210: 63-76.

[34] Siapati EK, Papadaki M, Kozaou Z, Rouka E, Michali E, Savvidou I, Gogos D, Kyriakou D, Anagnostopoulos NI and Vassilopoulos G. Proliferation and bone marrow engraftment of AML blasts is dependent on β-catenin signaling. Br J Haematol 2011; 152: 164-174.

[35] Pello OM, De Pizzol M, Mirolo M, Soucek L, Zammataro L, Amabile A, Doni A, Nebuloni M, Swigart LB, Evan GI, Mantovani A and Locati M. Role of c-MYC in alternative activation of human macrophages and tumor-associated macrophage biology. Blood 2012; 119: 411-421.

[36] Porta C, Rimoldi M, Raes G, Brys L, Ghezzi P, Di Liberto D, Dieli F, Ghisletti S, Natoli G, De Baetselier P, Mantovani A and Sica A. Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor B. Proc Natl Acad Sci U S A 2009; 106: 14978-14983.

[37] Cheng F, Wang HW, Cuenca A, Huang M, Ghansah T, Brayer J, Kerr WG, Takeda K, Akira S, Schoenberger SP, Yu H, Jove R and Sotomayor EM. A critical role for Stat3 Signaling in immune tolerance. Immunity 2003; 19: 425-436.

[38] Williams L, Bradley L, Smith A and Foxwell B. Signal transducer and activator of transcription 3 is dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. J Immunol 2004; 172: 567-576.

[39] Zhang X, Tian W, Cai X, Wang X, Dang W, Tang H, Cao H, Wang L and Chen T. Hydrazinocurcumin Encapsuled Nanoparticles "Re-Educate" Tumor-Associated Macrophages and Exhibit Anti-Tumor Effects on Breast Cancer Following STAT3 Suppression. PLoS One 2013; 8: e65896.