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

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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 overexpression 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 indispensable 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 characterized 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, cytotoxic 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].
IL-12 reverses M2 macrophages to M1 macrophages

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 owning to its non-negligible side-effects [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-10highIL-12low), 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 overexpression 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% CO₂.

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.

![Figure 1](image_url)
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Table 1. Primer sequences for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′→3′)</th>
<th>Reverse primer (5′→3′)</th>
<th>Amplification size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>CGAGTGGACAGCCTGTAGGCC</td>
<td>TGAAGAGGACCTGGAGTAGAA</td>
<td>171</td>
</tr>
<tr>
<td>IFN-β</td>
<td>ATGGCTTATTACAGTGCC</td>
<td>TAGTGGTGTCGGAGATT</td>
<td>137</td>
</tr>
<tr>
<td>IL-23</td>
<td>TGGAGATGCGTGGACCC</td>
<td>GAAAGAAGAAGAGGAGCG</td>
<td>271</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CTGGAGCAATGCAAACAAAA</td>
<td>AGGTAAGATTGTCGTGGGAA</td>
<td>206</td>
</tr>
<tr>
<td>IL-8</td>
<td>GTCTTAGAGACTGACGGGTGA</td>
<td>ACAGTGAGCTTGGAGGAA</td>
<td>160</td>
</tr>
<tr>
<td>IL-10</td>
<td>AGAACCAAGACCCACAGACATCA</td>
<td>GCATTCTTACCTGCTACCA</td>
<td>139</td>
</tr>
<tr>
<td>TGF-β</td>
<td>ATCCGTGGAGGAGGAAATAC</td>
<td>CTGAAACGTTGGATGAGC</td>
<td>174</td>
</tr>
<tr>
<td>CD197</td>
<td>GATTACATCGAGACAAACACCA</td>
<td>AGTACATGATGAGGAGGAAACCAG</td>
<td>106</td>
</tr>
<tr>
<td>IL-12 p35</td>
<td>CTGGAGCACCTCATGTGG</td>
<td>TCAGAAGTGCAAGGTTAA</td>
<td>155</td>
</tr>
<tr>
<td>IL-12 p40</td>
<td>GTGGAGTGCCAGGAGGACA</td>
<td>TCTTGCGTGGGTACGTTT</td>
<td>148</td>
</tr>
<tr>
<td>β-actin</td>
<td>GGAAATCGTGTGCAGTCTC</td>
<td>CAGGCAGCTCGTAGCTT</td>
<td>113</td>
</tr>
</tbody>
</table>

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^−ΔΔCt 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 were
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![Graphs showing mRNA expression changes of 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 (Millipore, 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).

Overexpression 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
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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 overexpression 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 conditioned 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 in 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 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.
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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.

Discussion

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 overexpression 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).

Figure 4. The effect of overexpression 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.

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-
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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 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.
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].
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.

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Disclosure of conflict of interest

None.

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