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Original Article

Macrophage functional phenotype can be consecutively and reversibly shifted to adapt to microenvironmental changes

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Abstract: Macrophages are functionally plastic cells, and have developed distinct functional subsets in association with cancer, autoimmune disease, and chronic infections. As an alternative to the concept of subset development, we hypothesized that macrophages, in response to changes in their tissue environment, can reversibly and progressively change the pattern of functions that they express. We examined the reversible and progressive shift of functional phenotypes of macrophages in response to changes in their tissue environment. As demonstrated herein, macrophages can reversibly shift their functional patterns in response to sequential changes in cytokine environment. After treatment alone or alternative with IL-4, MFCS, IL-12, INF-γ or LPS, the cells showed various functional patterns. A progression through multiple functional phenotypes was displayed after sequential treatment of macrophages with multiple cytokines. This ability to adapt to changing cytokine microenvironments has significant relevance in vivo, as evidenced by the fact that developed macrophage functional phenotypes in vivo in aged mice or forestomach carcinoma (MFC) tumor-bearing mice can be shifted by modifying their microenvironment. Therefore, a concept of shifted macrophage functional phenotypes has important implications for therapeutic targeting of macrophages in chronic diseases. The dominance of particular functional phenotypes of macrophages in chronic diseases may play an important role in pathogenesis.

Keywords: Re-polarize, macrophages, functional phenotype, correlate, disease

Introduction

Macrophages are functionally plastic cells. Two nomenclatures have been used to describe macrophage phenotypes [1-3]. Type 1 Macrophages (M1), the so-called Classically Activated macrophages (Ca-MΦ), are induced by type 1 cytokines such as IFN-γ and LPS, TNF-α and are characterized by high interleukin-12 (IL-12) and IL-23 production, abundant production of both inducible nitric oxide synthase (iNOS) and CCL3 but scarce amount of arginase (Arg) CCL22, and found in inflammatory zone 1 (Fizz1), and triggered Th1 response. Macrophages exposed to type 2 cytokines including IL-4, IL-13, immune complexes (IC) and LPS are characterized by an IL-10^{high} and IL-12^{low} phenotype, abundant expression of Arg, CCL22 and Fizz1 but not iNOS or CCL3, which promote type II responses; they have been called Type 2 Macrophages (M2) or Alternatively Activated macrophages (AA-MΦ). Available information [1-3] suggests that M1 participate in polarized Th1 reactions such as proinflammatory activities, immunogenic activities, tissue destructive activities and tumor resistance, whereas M2 are relevant to Th2 reactions such as anti-inflammatory activities, tolerogenic activities, tissue restorative activities and tumor promotion. Furthermore, M1- and M2-inducing signals displayed feelings of hostility in the expression of these signals resulting in transcription of inflammatory chemokines [4-10]. M1 and M2, as fully

polarized type 1 and 2 cells in various versions of activated macrophages, were referred to the two extremes of a spectrum of possible forms of macrophage activation [2].

Tumor-associated macrophages (TAMs), which are derived from circulating monocytes and recruited to the tumor site by chemotactic factors, are polarized M2 subsets and tightly correlating with tumor development [7, 11-13]. It is thought that the tumor microenvironment polarizes macrophages toward a type 2 functional phenotype and does an accentuation in the type 2 functional phenotypes of M2, further, an environment may be established that is likely to skew $CD4^+$ and $CD8^+$ T cell immunity toward a protumor type 2 response [14, 15]. Consequently, TAMs not only suppress acquired and innate anti-tumor immunity but also facilitate tumor growth.

In this study, we used type 1 factors (IL-12 or IFN-γ), type 2 factors (IL-4 or MFCS) and LPS to polarize or repolarize peritoneal peritoneal macrophages in young mouse, aged mouse and forestomach carcinoma (MFC) tumor-bearing mice and investigated whether treatment with type 1 or type 2 cytokines would induce a unique pattern of functions for each cytokine rather than promoting qualitatively similar proinflammatory vs. anti-inflammatory patterns for the type 1 vs. type 2 cytokines, respectively, whether the polarized macrophages could continue to shift their functional patterns in response to sequential changes in the cytokine microenvironment, and whether the anti-inflammatory and pro-tumor macrophage subsets developed during chronic pathologies such as cancer or advanced age could be repolarized to an proinflammatory and anti-tumor phenotype.

Materials and methods

Animals

Female BALB/c mice were obtained from the laboratory animal center of Chongqing Medical University and used at 6-8 wks of age. All animal protocols received prior approval from the Institutional Animal Care and Use Committee and all experiments were performed in accordance with relevant guidelines and regulations.

Murine tumors [16]

Mice were injected s.c. with 0.2 ml of PBS with or without 2×10^6 MFC cells. Four weeks after inoculation, mice with subcutaneous MFC tumor lump were used as a source of tumor-bearing mice peritoneal macrophages (TPM).

Cell preparation and MΦ activation

Mouse gastric cancer MFC cell line was obtained from the Shanghai Cell Bank and was cultured in 1640 supplemented with 10% heat-inactivated FBS.

Mouse peritoneal macrophages (PM) were obtained from normal mice (PM), or tumor-bearing mice (TPM) or normal control (NC-PM) injected 4 days previously with 1 ml of 3% thioglycollate as described previously [17]. The cells were seeded in a 24-well culture plate (1.0 ml/well) at 5×10^5 cells/well. Purity of > 90% F4/80 was confirmed by flow cytometry for PM.

M1 were prepared by treated PM for 24 h with 100 ng/ml LPS (Sigma) after 24 h pretreatment with 100 U/ml IFN-γ, M2 were prepared by treated PM for 24 h with 20 ng/ml IL-4 (Peprotech) without following LPS stimulation.

Following, TPM, M2 or M1 were treated with LPS after 24 h culture with IFN-γ, IL-12 or MFCS, thoroughly washed, and then stimulated with LPS either immediately or after an additional 24 h culture with MFCS.

Cytokine measurement by ELISA

Measurements of TNF-α, IL-12p70 and IL-10 levels in culture medium of macrophages were performed with commercially available ELISA reagents sets, according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from those macrophages of each group in reverted examinations using TRIzol reagent (Tiangen Biotech, Beijing, China). qRT-PCR was performed with the Applied Biosystems 7500 Sequence Detection System (Applied Biosystems) using SYBR®Green Realtime PCR Master Mix-Plus- (Toyobo Biotech, Shanghai, China) according to the manufacturer's specifications. The mouse GAPDH gene was used as an internal control (Mouse-GAPDH MGB, Applied Biosystems). For data analysis, the comparative threshold cycle (CT) value for GAPDH was used to normalize loading variations in the qPCRs. A $^{\Delta\Delta}$CT value was then

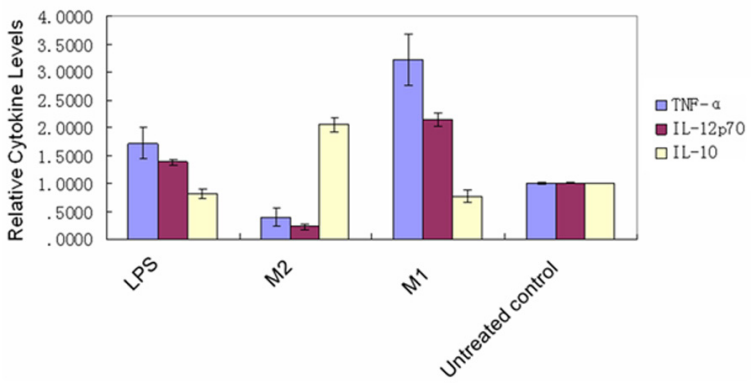

Figure 1. Cytokine production of M1 and M2. PM was purified from peritoneal lavage of 6-8 w normal mice. M1 were prepared by treated PM for 24 h with LPS after IFN-γ pretreatment. M2 were prepared by stimulated PM for 24 h with IL-4 alone without following LPS stimulation. Cytokine production was detected with ELISA. The pattern of cytokine response is displayed as the ratio of the response of treated PM to that of untreated PM. The numerical ratio is displayed above the bars. Representative data are shown from at least three independent experiments. Means ± SD are displayed. *, $P < 0.05$.

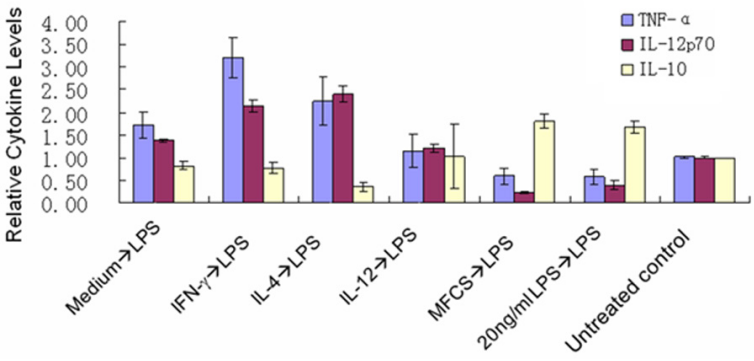

Figure 2. The different factor response patterns activated in Mouse peritoneal macrophages (PM). PM were cultured for 24 h with one factor or alone ligand, washed thoroughly, and then incubated an additional 24 h with LPS. Cytokine production was measured with ELISA. Data is displayed as the ratio of responses by treated vs. untreated PM, as described in the legend for **Figure 1**. The experiment is representative of three to five trials. Means ± SD are displayed. *, $P < 0.05$.

obtained by subtracting control $^{\Delta}$CT values from the corresponding experimental $^{\Delta}$CT. The $^{\Delta\Delta}$CT values were converted to fold difference compared with the control by raising two to the $^{\Delta\Delta}$CT power. All assays were performed in triplicate. Primers (Invitrogen, Shanghai, China) used were Arg (168bp), forward 5'-GGT TTT TGT TGT TGC GGT GTT C-3', reverse 5'-CTG GGA TAC TGA TGG TGG GAT GT-3'; iNOS (243bp), forward 5'-AGC GAG GAG CAG GTG GAA GA-3', reverse 5'-GAG GGG GGA ATG ACA TGA GG-3'; CCL3 (283bp), forward 5'-CTG CCC TTG CTG TTC TTC TCT G-3', reverse 5'-CCT CGC TGC CTC CAA GAC TC-3'; CCL22 (198bp), forward 5'-GGG CAT CGC TTT TCC TCT C-3', reverse 5'-GGG AGG GAC CTT CAG GAC AT-3'; Fizz1 (257bp), forward 5'-CAG GAT GCC AAC TTT GAA TAG G-3', reverse 5'-CAC AAG CAC ACC CAG TAG CAG TC-3'; GAPDH (159bp), forward 5'-ACC CAT CAC CAT CTT CCA GGA G-3', reverse 5'-GAA GGG GCG GAG ATG ATG AC-3'.

In vivo experiments

Mice were injected s.c. with 2×10^6 MFC tumor cells mixed or unmixed with 2×10^6 of one of the macrophages (M1, M2, TPM, IL-12-TPM, and MFCS-IL-12-TPM) (n = 20). Control animals were injected with PBS or equal numbers of single macrophages. Tumors were removed 28 days after the inoculation. Tumor size was assessed in two directions with calipers throughout the experiments. Tumor volume = (long axis) × (short axis) × π/6 [16].

Statistical analysis

Data are shown as the mean ± SD. Significant differences were assessed by one-way ANOVA for multiple comparisons and t-test for two groups' comparisons. *P*-values less than 0.05 were considered significant. All of the tests were implemented in SPSS 17.0 (SPSS). All experiments were repeated at least three times.

Results

After evaluation with ELISA, a series of cytokine productions were displayed in **Figures 1**-**5**. For easier visualization, we focused on the patterns of LPS-stimulated cytokine production in those macrophages treated with indicated factors. The cytokine response was normalized to that obtained by LPS stimulation of macrophages without factor treatment (**Figures 1**-**5**). By using

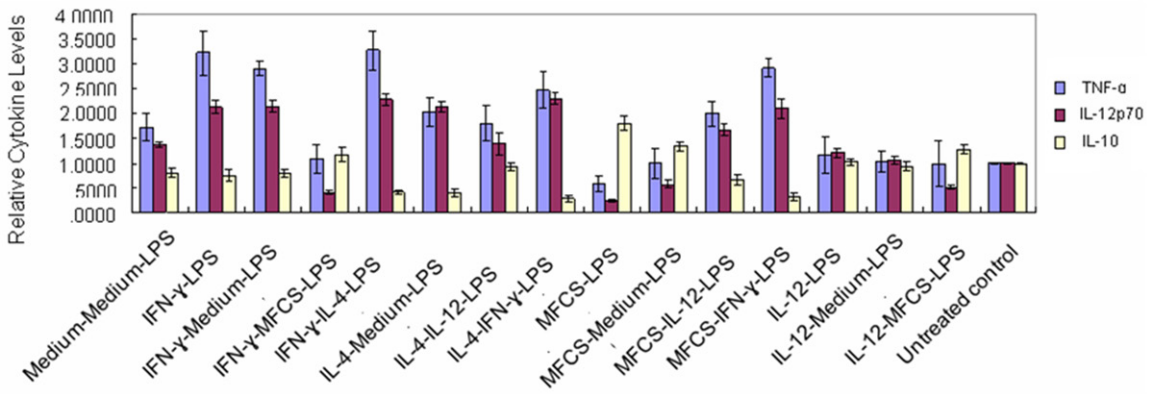

Figure 3. The cytokine response pattern changes of PM after cultured sequentially to different factors. PM was cultured in the one factor for 24 h, thoroughly washed, and then stimulaed with LPS either immediately or after an additional 24 h culture in another factor microenvironment. Cytokine production was assayed with ELISA. Data is displayed as the ratio of responses by treated vs. untreated PM, as described in the legend for **Figure 1**. An experiment representative of three trials is displayed. Means ± SD are displayed. *, $P < 0.05$.

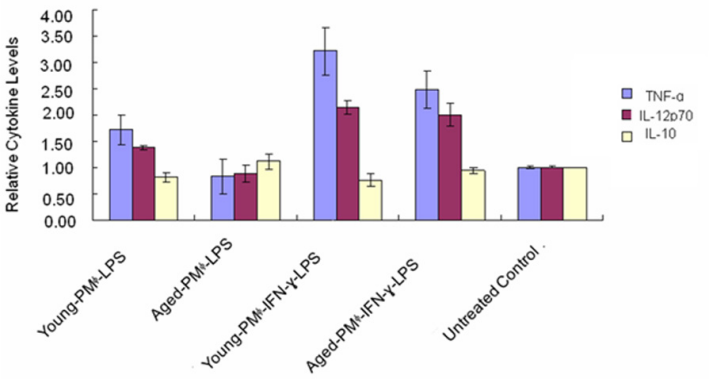

Figure 4. The cytokine production of PM vs. TPM, and young-PM vs. aged-PM in vitro. PM was purified from peritoneal lavage of MFC tumor-bearing mice (TPM), normal controls (NC-PM), young mice and aged mice. These cells were stimulated with LPS either immediately or after an additional 24 h culture in a different factor environment. Cytokine production was detected by ELISA. Blank control was not shown. The ratio of all groups of macrophages to untreated macrophages from normal controls is displayed, as described in the legend for **Figure 1**. Representative data are shown from at least three independent experiments. Mean ± SD are displayed. *, $P < 0.05$.

such a format to observe which cytokines were enhanced, reduced, or unaffected by a particular treatment protocol, it appeared apparent that treatment of PM with indicated factors each induced a unique response pattern.

M1 display type 1 response, whereas M2 show type 2 response

M1 and M2 were regarded as the two extremes of a spectrum of possible forms of macrophage activation [1-3]. Therefore, as an evaluated standardization of macrophage functional phenotypes, we propose M1 and M2 as a basis for the shift of macrophage functional phenotypes in following examinations. To measure cytokine production in M1 and M2, PM were stimulated with LPS after pretreated with IFN-γ in order to induce M1, or directly stimulated with IL-4 but no following LPS treatment so as to produce M2. As expected, analysis (**Figure 1**) showed that M1 produced relatively high levels of both TNF-α and IL-12 but low levels of IL-l0, whereas M2 developed relatively low levels of both TNF-α and IL-12 but high levels of IL-l0 ($P < 0.05$). Further, this observation was extended by qRT-PCR. This analysis ($P < 0.05$) (**Figure 6**) revealed that all of Arg, CCL22 and Fizz1 mRNA were induced robustly in M2, and mRNA levels increased substantially, relative to alone LPS-stimulated PM. Though there was some induction of Arg, CCL22 and Fizz1 mRNA in M1, this induction was fairly modest relative to M2. iNOS and CCL3 mRNA in M1 showed a similar change in Arg, CCL22 and Fizz1 mRNA in M2 ($P < 0.05$) (**Figure 6**). Similar results were also obtained in other studies [18, 19].

Mouse peritoneal macrophages (PM) display distinct functional patterns upon LPS stimulation after treatment with different factors in vitro

To demonstrate that the functional phenotypes of macrophages are able to be polarized with different factors, LPS-inducing cytokine pro-

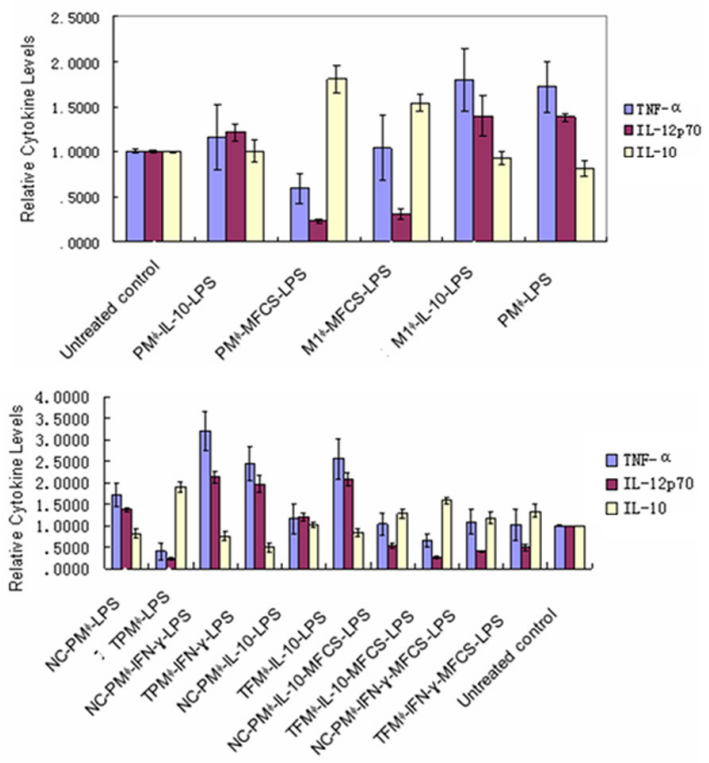

Figure 5. The cytokine response pattern changes of repolarized PM upon LPS stimulation after cultured type 1 or 2 factors. PM with type 1 fuctional pattern were cultured in type 2 factor for 24 h, and PM with type 2 fuctional pattern were cultured in type 1 factor for 24 h, and all groups thoroughly washed, and then stimulaed with LPS either immediately or after an additional 24 h culture in another factor microenvironment. Cytokine production was assayed with ELISA. Data is displayed as the ratio of responses by treated vs untreated PM, as described in the legend for **Figure 1**. An experiment representative of three trials is displayed. Means ± SD are displayed. *, $P < 0.05$.

duction was determined in pretreated PM with type 1 (IFN-γ, IL-12) or type 2 (IL-4, MFCS) factors. Compared with medium-pretreated PM, stimulating IFN-γ-primed PM with LPS resulted in a noticeable increase in IL-12 and TNF-α production and an equally noticeable decrease in IL-10 production ($P < 0.05$) (**Figure 2**). Similar results were obtained when IL-4-pretreated PM were stimulated with LPS ($P < 0.05$) (**Figure 2**), and identical result was also reported by another group [20]. However, the response pattern to LPS stimulation resulting after MFCS pretreatment, characterized by profoundly reduced IL-12 and TNF-α production whereas elevated IL-10 production ($P < 0.05$) (**Figure 2**). There was only an insignificant difference in the cytokines production between PM exposed to LPS after cultivation with culture media and PM pretreated with IL-12 before LPS stimulation ($P < 0.05$) (**Figure 2**). The increase in IL-12 production ($P < 0.05$) did not result from an addition of rIL-12 as evidenced by the absence of detectable IL-12 in supernatant fluid from PM cultures treated with IL-12 but unactivated with LPS (data not shown). Thereby, the LPS stimulation of pretreated PM with different factors in vitro gave rise to distinct functional phenotypes.

Chronic infection being relevant to tumor is frequently tolerant to pathogen due to organism adaptability, and the macrophages play an important role in the induction of tolerance to pathogen. As a result, LPS tolerance examination of macrophages in vitro was performed. The PM was cultured with a low dose (20 ng/ml) of LPS for 24 h before 100 ng/ml LPS stimulation. Contrast with LPS stimulation without LPS tolerance in PM, LPS tolerance followed by the stimulation with LPS significantly inhibited IL-12 and TNF-α production but enhanced IL-10 production in PM (**Figure 2**).

The pattern of mouse peritoneal macrophage functions continues to shift in response to the changes in factor microenvironment from one factor to another in vitro

To examine the functional adaptivity of macrophages whose phenotype had been established in vitro, PM were treated with IFN-γ, IL-12, IL-4 or MFCS for 24 h, thoroughly washed free of the cytokine, and then incubated for another 24 h with an alternate cytokine or medium before washing and stimulation with LPS. Cytokine production by these cells was detected by ELISA. The results are shown in **Figure 3**. PM treated with IFN-γ→Medium→LPS displayed enhanced production of IL-12 and TNF-α and reduced production of IL-10 and the treatment of IFN-γ→IL-4→LPS interestingly induced a more remarkable increase in IL-12 and TNF-α production and an more marked decrease in IL-10 production, whereas, IFN-γ→MFCS→LPS dramatically resulted in an inversion in the production between type 1 and type 2 cytokine ($P < 0.05$) (**Figure 3**). An alternative of IL-12 and MFCS led to similar changes to the alternative

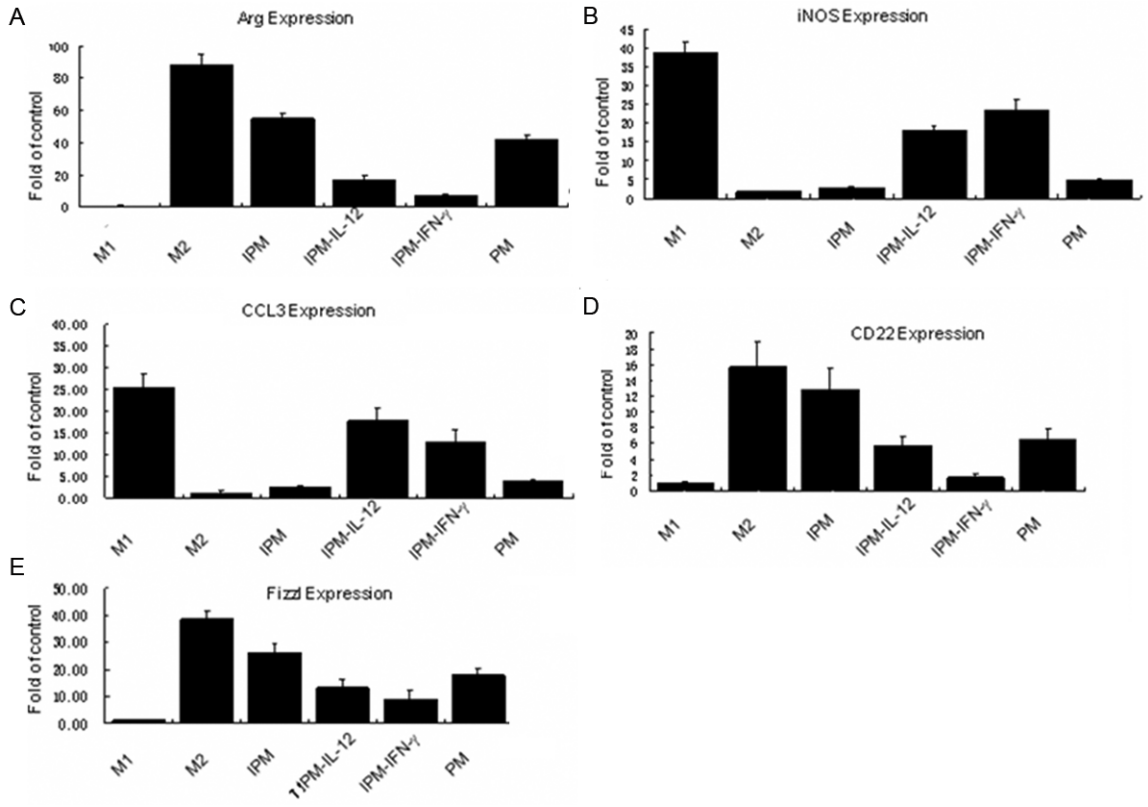

Figure 6. The patterns of iNOS, CCL3, CCL22, Fizz1 and arginase (Arg) mRNA expression in different groups. TPM from MFC tumor-bearing mice (TPM) were incubated in IL-12 or IFN-γ for 24 h, thoroughly washed, then immediately stimulated with LPS for 24 h. Total RNA was harvested at several time points and then mRNA expression was assayed with qRT-PCR. GAPDH mRNA was used to normalize loading. A representative experiment of three trials is displayed. A: Arg gene expression in macrophages of different groups by qRT-PCR. B: iNOS gene expression in macrophages of different groups by qRT-PCR. C: CCL3 gene expression in macrophages of different groups by qRT-PCR. D: CCL22 gene expression in macrophages of different groups by qRT-PCR. E: Fizz1 gene expression in macrophages of different groups by qRT-PCR.

of IFN-γ and MFCS (**Figure 3**). PM with the incubation of IL-4→Medium→LPS showed an enhanced IL-12 and TNF-α production but an inhibited IL-10 production ($P < 0.05$) (**Figure 3**), furthermore, both IL-4→IFN-γ→LPS and IL-4→IL-12→LPS treatments led PM into a response pattern as IL-12 and TNF-α production was more distinguished enhanced whereas IL-10 production was more noticeable inhibited ($P < 0.05$) (**Figure 3**). The treatment of MFCS→Medium→LPS in PM resulted in a decrease in IL-12 and TNF-α production and an increase in IL-10 production while both MFCS→IFN-γ→LPS and MFCS→IL-12→LPS treatment dramatically enhanced the production of IL-12 and TNF-α and equally dramatically reduced the production of IL-10 in PM ($P < 0.05$) (**Figure 3**).

Functional phenotypes of mouse peritoneal macrophages established in vivo for a prolonged period of time could still retain their ability to functionally adapt to changes in their factor microenvironment in vitro

It has been reported that macrophages from aged mice (> 20 months) displayed reduced functional activity in response to stimulation [21-23], and macrophages from animals bearing metastatic tumors developed a functional phenotype that accumulate in the tumor mass [7, 11, 13, 15, 20, 21]. Thereby, after the verification that the functional phenotypes of polarized macrophages in vitro could be re-polarized, we further investigated whether the stability of the macrophage functional phenotypes had been established for a prolonged period of time in immunosenescent and MFC tumor-bearing

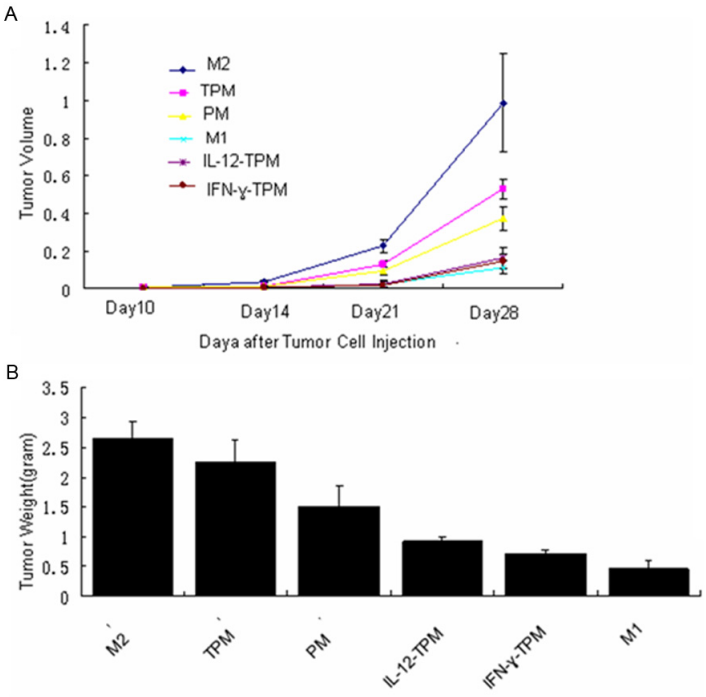

Figure 7. Inhibition of MFC tumor growth by repolarized TPM. MFC cells were mixed with different types of macrophages and injected into BALB/c mice subcutaneously. The tumor size was followed continuously as described in Materials and Methods, and tumors were allowed to grow for 28 days before mice were euthanized to measure tumor weights. A: Tumor growth curve over the period of 28 days. B: Final tumor weight after 28 days. Tumors from mice injected with IL-12- and IFN-γ-repolarized TPM (IL-12-TPM and IFN-γ-TPM) were much smaller than those from mice with TPM.

mice. To do so, PM were purified from peritoneal lavage of young mice (6-8 w) (Young-PM) and aged mice (20 w) (Aged-PM), or MFC tumor-bearing mice (TPM) and normal controls (NC-PM). These cells were stimulated with LPS after 24 h of culture either with indicated factors or immediately. Cytokine production was detected by ELISA (**Figures 4** and **5**), and the expression of iNOS, CCL3, Arg, CCL22 and Fizz1 mRNA was detected by RT-PCR and qRT-PCR. LPS-stimulated Aged-PM without any factors pretreatment showed a decreased TNF-α and IL-12 production and an increased IL-10 production. Compared with Aged-PM activated with LPS without any factors treatment, LPS stimulation after pretreatment with IFN-γ profoundly increased the production of IL-12 and TNF-α, but reduced the production of IL-10 in Aged-PM ($P < 0.05$) (**Figure 4**). Similar results were obtained in TPM with LPS stimulation with or without IFN-γ or IL-12 pretreatment ($P < 0.05$) (**Figure 5**). LPS-stimulated TPM showed reduced TNF-α and IL-12 production but enhanced IL-10 production, whereas the pretreatment with IFN-γ or IL-12 before LPS stimulation significantly increased the production of TNF-α and IL-12 and decreased the production of IL-10 in TPM ($P < 0.05$) (**Figure 5**). TPM stimulated with LPS profusely induced Arg, CCL22 and Fizz1 mRNA but failed to those of both iNOS and CCL3 (**Figure 6**). By contrast, LPS stimulation after IFN-γ or IL-12 pretreatment resulted in copious mRNA expressions of iNOS and CCL3 whereas all of Arg, CCL22 and Fizz1 mRNA were poorly expressed in TPM (**Figure 6**).

Type 2 functional patterns of macrophages could be repolarized into type 1 functional patterns, and macrophages with type 1 functional patterns might be developed to display type 2 functional patterns

The above studies had given a demonstration of macrophages activated in vivo or in vitro having the nature of being polarized and repolarized their functional phenotypes in vitro. We follow to verify that macrophage functional phenotypes were capable of being reversed to opposite functional phenotypes in vitro.

Initially, we investigated whether both M1 and M2, representing two extremes in the spectrum of the different macrophage phenotypes, could be inverted into opposite to their functional phenotypes or not. Both M1 and M2 were prepared with above methods, and then M1 were treated with LPS after 24 h of culture with MFCS, and M2 were simultaneously activated with LPS after 24 h of incubation with IL-12 or INF-γ. Subsequently, the cytokines in supernatants were measured by ELISA. The results showed that, M1 exhibited an relatively IL-12^{high}, TNF-α^{high} but IL-10^{low} cytokine response pattern ($P < 0.05$) (**Figure 5**) with the mRNA expression of both of iNOS and CCL3 instead of all Arg, CCL22 and Fizz1 (**Figure 6**), whereas M2 produced relatively low levels of TNF-α and IL-12 but high levels of IL-l0 ($P < 0.05$) (**Figure 5**) and

plenteously expressed all of Arg, CCL22 and Fizz1 mRNA but neither iNOS nor CCL3 mRNA (**Figure 6**). After an exposure to MFCS, M1 secreted significantly reduced TNF-α and IL-12 production but increased IL-10 production ($P < 0.05$) (**Figure 5**), followed by an opposite gene expressed pattern to M1. In contrast, the treatment with IL-12 or INF-γ (IL-12-M2 and INF-γ-M2) remarkably enhanced production of TNF-α and IL-12 while reducing production of IL-10 in M2 ($P < 0.05$) (**Figure 5**) and resulted in a similar gene expressed pattern to M1 (**Figure 6**). Accordingly, we obtained a successful inversion in functional phenotypes between M1 and M2.

Next, we researched whether TPM from MFC tumor-bearing mice were also able to be induced into the macrophages showing type 1 functional phenotypes. Macrophages were purified from peritoneal lavage of MFC tumor-bearing mice (TPM) and normal controls (NC-PM). TPM and NC-PM were incubated in IL-12 or INF-γ for 24 h, thoroughly washed, and then stimulated with LPS either immediately or after an additional 24 h culture with MFCS. Cytokine production was detected by ELISA (**Figure 5**). The expression of iNOS, CCL3, Arg, CCL22 and Fizz1 mRNA were evaluated by qRT-PCR. The results demonstrated that TPM exposed to IL-12 or INF-γ (IL-12-TPM, INF-γ-TPM) abundantly produced mRNA for both iNOS and CCL3 but not one of Arg, CCL22 and Fizz1 mRNA ($P < 0.05$) (**Figure 6**), and displayed type 1 functional phenotypes that TNF-α and IL-12 production was profoundly increased whereas IL-10 production was decreased (**Figure 5**). Conversely, MFCS treatment dramatically induced a generous expression of Arg, CCL22 and Fizz1 mRNA but failed to iNOS and CCL3 mRNA ($P < 0.05$) (**Figure 6**), and dramatically reduced the production of TNF-α and IL-12 while enhanced the production of IL-10 in both IL-12-TPM and INF-γ-TPM (MFCS-IL-12-TPM, or MFCS-INF-γ-TPM) ($P < 0.05$) (**Figure 5**).

Macrophages with type 2 functional patterns after repolarized into type 1 functional patterns in vitro would display a significant delay in tumor growth in vivo, whereas those with type 1 functional patterns after activated into type 1 functional patterns in vitro would remarkably favored tumor progression in vivo

To investigate the effectiveness on tumor growth by those macrophages repolarized from type 2 functional phenotypes into type 1 or from type 1 into type 2 functional phenotypes, mice were injected s.c. with MFC tumor cells with or without one of those macrophages including untreated PM, M1, M2, TPM, IL-12-TPM, INF-γ-TPM (n = 20). Compared with MFC tumor mass in mice vaccinated with untreated PM mixed with MFC tumor cells, the tumor size and weight were significantly greater in all of mice vaccinated with MFC tumor cells mixed in one of M2, or TPM ($P < 0.05$), whereas MFC tumor mass from mice vaccinated with M1, INF-γ-TPM or IL-12-TPM mixed in MFC tumor cells displayed a striking delay growth at the experiment end point ($P < 0.05$) (**Figure 7**). All groups vaccinated with single macrophages failed to developed MFC tumors (data not shown).

Discussion

A number of studies [1, 2, 7, 13, 17, 18] have demonstrated that macrophages display various functional phenotype in chronic disease pathologies such as cancer, inflammatory disease, autoimmune, tissue remodeling and angiogenesis. Our studies indicate that macrophages display different patterns of function in different microenvironments. In the current study [19], macrophages displayed many distinct patterns of expression of those proteins when exposed to different activated agents. Nonetheless, the patterns did not display a strict dichotomy between type 1 and type 2 responses. For example, treatment of macrophages with the type 1 factor, IL-12, before LPS stimulation did not display a significant type 1 functional phenotype. Moreover, treatment of macrophages with the type 2 factor, IL-4, before LPS stimulation strongly enhanced inflammatory activity (TNF-α and IL-12 production, iNOS and CCL3 expression) and inhibited anti-inflammatory activity (IL-10 production). This suggests that pretreatment with type 2 factor, IL-4, results in the enhanced proinflammatory activity in macrophages. After LPS stimulation in vitro, the macrophages from young mice display a type 1 functional phenotype, whereas the macrophages from aged mice a type 2 functional phenotype. This suggests that macrophages from aged mice display reduced inflammatory activity in response to stimulus, possibly as a result of the impaired respiratory burst and reactive nitrogen intermediates by

aged macrophages and neutrophils [20]. However, the pre-treatment with IFN-γ results in macrophages from aged and young mice that displayed nearly identical type 1 functional patterns. These data further suggest that macrophage functional phenotype could be a compromise to settle the differences between type 1 cytokines and type 2 cytokines.

Our results demonstrated that activated macrophages in vitro with different cytokines can distinctly alter their functional patterns. The functional plasticity of macrophages whose phenotype had been established in vivo for a prolonged period of time is a vitally important issue in the current study. The functional reversibility of macrophages would provide a fundamental therapeutic importance in numbers of chronic pathological processes such as cancer, inflammatory disease, autoimmune, tissue remodeling and angiogenesis, which lead to the dominance of particular functional phenotypes of macrophages that play an important role in disease pathogenesis. Our data demonstrate that both polarized populations of macrophages in aged mice and MFC tumor-bearing mice steadily retained their instinct to adapt to a different cytokine environment, in other words, the functional phenotype of these activated macrophages in vivo can be shifted and progress from one functional phenotype to another even the opposite in response to changes in the microenvironmental influences. Therefore, as an alternative to the concept of stable macrophage subsets, we propose a model of functional adaptivity as the basis for understanding macrophage function in physiologically dynamic responses. The model of functional adaptivity predicts not only the adaption of macrophages to microenvironmental signals via differential regulation of function, but also the progressive change of their functional phenotypes in response to microenvironmental signals.

A critical point of this study is that macrophages retain their functional adaptivity and then their functional phenotype can be changed in response to an appropriate stimulus. The concept of functional adaptivity provides a potential therapeutic approach on macrophage biology and macrophage function in many diseases such as inflammation and tumor. For example, tumor involves dramatic changes in tissue microenvironment, as a result, macrophage polarize into TAMs displaying pro-tumor type 2 functional phenotype in response to tumor microenvironment. Further, TAMs produce a number of pro-tumor cytokines so as to strengthen tumor microenvironment. In turn, more macrophages are induced to polarize into TAMs in strengthened tumor microenvironment. So a vicious cycle is created. If changing the tumor microenvironmental influences, it would be possible that TAMs may adaptively shift into type 1 functional phenotype and thus interrupt the vicious cycle and inhibit tumor. Based on the results of the present study, therapeutic approaches to chronic disease such as tumor could be targeted toward changing the functional phenotype of the macrophages, i.e., changing the pattern of cytokines, metabolites, and enzymes produced by the macrophages. Based on the results of our study, therapeutic approaches to chronic disease such as tumor should be targeted toward changing the functional phenotype of the macrophages but not to inhibit the biological activity of cytokines and metabolites produced by the macrophages in chronic pathological conditions.

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Disclosure of conflict of interest

None.

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