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# M3 Macrophages Stop Division of Tumor Cells *In Vitro* and Extend Survival of Mice with Ehrlich Ascites Carcinoma

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**Background:** M1 macrophages target tumor cells. However, many tumors produce anti-inflammatory cytokines, which reprogram the anti-tumor M1 macrophages into the pro-tumor M2 macrophages. We have hypothesized that the problem of pro-tumor macrophage reprogramming could be solved by using a special M3 switch phenotype. The M3 macrophages, in contrast to the M1 macrophages, should respond to anti-inflammatory cytokines by increasing production of pro-inflammatory cytokines to retain its anti-tumor properties. Objectives of the study were to form an M3 switch phenotype *in vitro* and to evaluate the effect of M3 macrophages on growth of Ehrlich ascites carcinoma (EAC) *in vitro* and *in vivo*.

**Material/Methods:** Tumor growth was initiated by an intraperitoneal injection of EAC cells into C57BL/6J mice.

**Results:** 1) The M3 switch phenotype can be programed by activation of M1-reprogramming pathways with simultaneous inhibition of the M2 phenotype transcription factors, STAT3, STAT6, and/or SMAD3. 2) M3 macrophages exerted an anti-tumor effect both *in vitro* and *in vivo*, which was superior to anti-tumor effects of cisplatin or M1 macrophages. 3) The anti-tumor effect of M3 macrophages was due to their anti-proliferative effect.

**Conclusions:** Development of new biotechnologies for restriction of tumor growth using *in vitro* reprogrammed M3 macrophages is very promising.

**MeSH Keywords:** Carcinoma, Ehrlich Tumor • Cytokines • Macrophage Activation

**Full-text PDF:** http://www.basic.medscimonit.com/abstract/index/idArt/902285







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## Background

Macrophages play the key role in immune disorders during carcinogenesis [1,2]. A concept put forward by Mills et al. [3,4] and supported by other researchers [5–7], implies that, depending on the microenvironment, macrophages reprogram themselves into either an M1/kill phenotype or an M2/repair phenotype. The M1 phenotype can destroy tumors due to production of nitric oxide [8], pro-inflammatory cytokines [9,10], activation of natural killers [11], and presentation of tumor antigens to lymphocytes [12]. Pro-inflammatory cytokines acting on macrophages shift their phenotype even more towards the M1, which produces more pro-inflammatory cytokines. Thus, the pro-inflammatory mechanism involves positive feedback. However, many tumors produce anti-inflammatory cytokines, such as TGF-β, IL-10, and IL-13 [13,14], which reprogram the anti-tumor M1 phenotype into the pro-tumor M2 phenotype [15]. The M2 phenotype produces large quantities of anti-inflammatory cytokines and has a low capability for presentation of tumor antigens [15–18]. Anti-inflammatory cytokines acting on macrophages shift their phenotype even more towards the M2, which produces more anti-inflammatory cytokines. Thus, the anti-inflammatory mechanism involves positive feedback. The M2 phenotype contributes to suppression of anti-tumor immunity and tumor growth [1,2].

We have hypothesized that tumor growth can be effectively restricted by a special switch phenotype [19]. The switch phenotype, in contrast to the M1 phenotype, should respond to anti-inflammatory pro-tumor cytokines by increasing production of pro-inflammatory anti-tumor cytokines. As a result, macrophages would be able to retain their anti-tumor features in the tumor area.

We suggested this hypothesis after discovering human macrophages with characteristics of the switch phenotype. We showed in pilot experiments that production of pro-inflammatory cytokines by macrophages from patients with chronic obstructive pulmonary disease or bronchial asthma, in contrast to macrophages from healthy subjects, increased in response to anti-inflammatory stimuli [20–22]. The phenotype we discovered was qualitatively different from the M1 and M2 phenotypes in the response to pro- or anti-inflammatory factors; therefore, we named this phenotype the M3 or the switch phenotype [20].

Subsequently, a definition of the M3 phenotype was provided by Jackaman et al. [23], defined as a phenotype with "incomplete polarization into an M1/M2-like phenotype". Despite different definitions of the phenotype, both Jackaman et al. [23] and our group [20] were referring to the same phenotype. The difference in these definitions was that we stressed the internal mechanism for emergence of such a phenotype (switching the signal from anti-inflammatory M2 factors to formation of a pro-inflammatory, M1 phenotype), whereas Jackaman et al. [23] focused on external characteristics of the phenotype (the "M1/M2-like phenotype").

To use the M3 phenotype for restricting tumor growth, it was essential to learn the programming of this phenotype. Previously, we theoretically substantiated this possibility [20]. Briefly, many reprogramming intracellular pathways branch to form an M1 or an M2 phenotype. For example, the TGF-β-dependent signaling can activate the M2 phenotype transcription factor, SMAD3, and the M1 phenotype transcription factors, NF-κB and p38. The JAK-dependent signaling can activate the M2 phenotype transcription factors, STAT3 and STAT6, and the M1 phenotype transcription factor, STAT1. In the tumor area, M1 macrophages activate the M2 phenotype pathway through anti-inflammatory cytokines, which results in activation of STAT3, STAT6, and SMAD3, increased production of anti-inflammatory cytokines, and reprogramming of the pro-tumor M2 phenotype. We suggested that inhibition of the M2 phenotype transcription factors, STAT3, STAT6, and/or SMAD3, could redirect the signal from anti-inflammatory, pro-tumor cytokines to activation of the M1 phenotype transcription factors. In this case, an M3 switch phenotype may form.

The aim of the study was to verify this hypothesis. Objectives of the study were to form an M3 switch phenotype *in vitro* and to evaluate the effect of M3 macrophages on growth of Ehrlich ascites carcinoma (EAC) *in vitro* and *in vivo*. We chose EAC as a tumor model because: 1) many abdominal tumors, such as pancreatic, ovarian, colorectal, and gastric malignancies, are associated with malignant ascites [24–27], and *2*) the murine EAC model is commonly used for evaluation of anti-tumor effects [28,29].

## Material and Methods

### Experimental animals

Experiments were performed on C57BL/6J mice in accordance with the WHO guidelines (*www.cioms.ch/publications/guidelines*). Mice were obtained from the Vivarium Andreevka (Moscow, Russia) (*http://andreevka.msk.ru*). The protocol of experiments was approved by the University Ethics Committee.

### Reagents

We used the following reagents: Stat3 inhibitor (S3I204) (Axon Medchem, USA, cat# 2312), Stat6 inhibitor (As1517499) (Axon Medchem, USA, cat# 1992), IFN-γ (Invitrogen, USA, cat# PMC4034), LPS (Sigma-Aldrich, USA, cat# L3755), SMAD3 inhibitor (SIS3) (Calbiochem, USA, cat# 566405), FBS (Thermo Hyclone, UK, cat# SV30160.03), and cisplatin (TEVA, Israel).





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### Isolation of macrophages

Native macrophages (M0 phenotype) were isolated from peritoneal lavage of mice using a standard method [30]. After isolation, macrophages were placed into wells of culture plates containing RPMI-1640 medium containing 10% serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% $CO_2$.

### *In vitro* macrophage reprogramming towards M1 and M3 phenotypes

Macrophages were reprogramed towards the M1 phenotype using 0% FBS [4] and 20 ng/ml IFN-γ [31]. For reprogramming towards the M3 phenotype, IFN-γ and 0% FBS were supplemented with: 1) STAT3 and STAT6 inhibitors ($M3_{STAT3/6}$ phenotype), 2) SMAD3 inhibitor ($M3_{SMAD3}$ phenotype), or 3) STAT3, STAT6, and SMAD3 inhibitors ($M3_{STAT3/6+SMAD3}$ phenotype).

Macrophages were cultured in 10 groups:

Group 1 (unstimulated M0 phenotype): with FBS for 36 h.

Group 2 (stimulated M0 phenotype): with FBS for 12 h and then stimulated with 500 ng/ml lipopolysaccharide (LPS) for 24 h.

Group 3 (unstimulated M1 phenotype): without FBS with IFN-γ for 36 h.

Group 4 (stimulated M1 phenotype): without FBS with IFN-γ for 12 h and then stimulated with 500 ng/ml LPS for 24 h.

Group 5 (unstimulated $M3_{STAT3/6}$ phenotype): without FBS with IFN-γ, 5 μg/ml STAT3 inhibitor, and 10 μg/ml STAT6 inhibitor.

Group 6 (stimulated $M3_{STAT3/6}$ phenotype): without FBS with IFN-γ, 5 μg/ml STAT3 inhibitor, and 10 μg/ml STAT6 inhibitor and stimulated with 500 ng/ml LPS for 24 h.

Group 7 (unstimulated $M3_{SMAD3}$ phenotype): without FBS with IFN-γ, 2 nmol/ml SMAD3 inhibitor.

Group 8 (stimulated $M3_{SMAD3}$ phenotype): without FBS with IFN-γ, 2 nmol/ml SMAD3 inhibitor and stimulated with 500 ng/ml LPS for 24 h.

Group 9 (unstimulated $M3_{STAT3/6+SMAD3}$ phenotype): without FBS with IFN-γ, 5 μg/ml STAT3 inhibitor, and 10 μg/ml STAT6 inhibitor, and 2 nmol/ml SMAD3 inhibitor.

Group 10 (stimulated $M3_{STAT3/6+SMAD3}$ phenotype): without FBS with IFN-γ, 5μg/ml STAT3 inhibitor, and 10 μg/ml STAT6 inhibitor, and 2 nmol/ml SMAD3 inhibitor and stimulated with 500 ng/ml LPS for 24 h.

### Evaluating changes in macrophage phenotype

Macrophage phenotype was determined by cytokine production and content of CD markers. Concentrations of the pro-inflammatory M1 cytokines, IL-1α, IL-1β, IL-2, IL-6, IL-12, IL-17, IL-21, INF-γ, and TNF-α, and the anti-inflammatory M2 cytokines, IL-4, IL-5, IL-10, IL-13, and IL-22 [32,33] were measured in the medium by flow cytometry (Beckman Coulter FC500, USA) using a cytokine test kit (BMS810FF, BenderMedSystems, USA) according to the manufacturer's instructions. CD80 was used as an M1 marker and CD206 was used as an M2 marker [30]. The CD markers were measured by flow cytometry using monoclonal antibodies to CD80 and CD206 (Beckman Coulter, USA, cat# 12-0801-82 and cat# FAB2535P, PE) according to the manufacturer's instructions.

### Evaluating the effect of ascitic fluid from mice with EAC on macrophage activity

The tumor microenvironment was created by addition of ascitic fluid (AF) from mice with EAC to cultured macrophages. Tumor growth was initiated by an intraperitoneal injection of 250 000 EAC cells (from the N.N. Blokhin Cancer Research Center, Moscow, Russia) [34]. AF was collected from the abdominal cavity using a syringe at 11 days after the EAC cell injection. After macrophage reprogramming, the medium was replaced with AF, and macrophage production of cytokines was evaluated.

### Co-culturing of macrophages and EAC cells

After macrophage reprogramming, 25 000 EAC cells were added to activated M0 (Group 2), M1 (Group 4), $M3_{STAT3/6}$ (Group 6), $M3_{SMAD3}$ (Group 8), and $M3_{STAT3/6+SMAD3}$ (Group 10) macrophages at different ratios of macrophage number to EAC cell number, 5: 1, 10: 1, 20: 1, 40: 1, and 80: 1. An anti-tumor drug, cisplatin [35,36], was used at concentrations of 10, 20, and 40 μg/ml as a comparator.

After co-culturing for 24 h, the content of cytokines in the medium was measured and the tumor cells were counted and their number was compared to that of tumor cells cultured without macrophages. Macrophages and tumor cells were separated after co-culturing according to the method described earlier [18]. Briefly, macrophages, as distinct from tumor cells, became firmly fixed to the bottom of the wells. The plate was then vortexed, the content of the wells was pipetted, and all supernatant was collected. Cells of the supernatant were counted. These cells were represented mainly by tumor cells that did not adhere to the plastic. Cell culture experiments were performed in 5 replicates. A group of tumor cells cultured without macrophages was used as a control to tumor cells influenced by macrophages. The plate vortexing procedure left the number of dead cells almost unchanged; at the beginning of co-culturing and after 24 h of culturing followed by vortexing the plate, the number of dead cells did not exceed the range of 5–8% of total cell number.





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**Table 1.** The effect of tumor microenvironment on activities of M1 macrophages and M1 macrophages with inhibited transcription factors, STAT3 and STAT6.

| Cytokines | M1 | | M1+STAT3/6 inhibitor | | M1+AF | | M1+STAT3/6 inhibitor+AF | |
|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | |
| IL-1β (M1) | (83±9) | 100% | (130±15) | 100% | (85±10) | 102.4% | (180±22) | 138.5% |
| IL-21 (M1) | (375±41) | 100% | (410±51) | 100% | (260±31) | 69.3% | (615±28) | 150% |
| IL-23 (M1) | (462±57) | 100% | (515±72) | 100% | (395±42) | 85.5% | (620±71) | 120.4% |
| IL-6 (M1) | (245±29) | 100% | (47±6) | 100% | (44±5) | 17.9% | (162±19) | 344.7% |
| IL-4 (M2) | (60±7) | 100% | (15±2) | 100% | (30±5) | 50% | (0) | 0% |
| IL-5 (M2) | (75±9) | 100% | (50±7) | 100% | (115±13) | 153.3% | (35±4) | 70% |
| IL-10 (M2) | (185±21) | 100% | (71±9) | 100% | (250±29) | 135.1% | (90±11) | 126.8% |
| Shift towards a phenotype | | | | | M2 | | M1 | |

Table () presents absolute values of cytokine concentration in pg/ml. Cytokine concentration in the culture medium without addition of ascitic fluid from mice with EAC to cultured macrophages was taken as 100%. AF – ascitic fluid.

Live and dead macrophages and EAC were counted using a standard method of trypan blue exclusion (*http://www.hyclone.com/pdf/procedure_assay.pdf*).

### Injecting macrophages into the mouse peritoneal cavity

Macrophages from groups 2, 4, 6, 8, and 10 were removed from the bottom of culture wells by incubation at 37°C with PBS containing 5 mM EDTA [5]. The macrophage concentration was adjusted to $8\times10^6$ cells in 0.2 ml PBS. Mice were injected with macrophages intraperitoneally on days 1, 3, 5, and 7 after the EAC cell injection. Six groups of mice were formed:

1. "Tumor" group, mice injected with EAC cells, (n=16);
2. "Tumor+PBS" group, mice injected with EAC cells followed by PBS infusion (n=16);
3. "Tumor+M0-Mac" group, mice injected with EAC cells followed by injections of a suspension of stimulated, non-reprogrammed macrophages (n=16);
4. "Tumor+M1-Mac" group, mice injected with EAC cells followed by injections of a suspension of stimulated M1 macrophages (n=16);
5. "Tumor+$M3_{STAT3/6+SMAD3}$-Mac" group, mice injected with EAC cells followed by injections of a suspension of stimulated $M3_{STAT3/6+SMAD3}$ macrophages (n=16);
6. "Tumor+cisplatin" group, mice injected with EAC cells followed by infusion of 0.05 ml of cisplatin (0.5 mg/ml), an anti-tumor drug [35–37] (n=14).

Effects of injected macrophages and cisplatin were evaluated based on changes in lifespan of mice with EAC.

Statistical analyses were performed using analysis of variance followed by the Student-Newman-Keuls test. Data are presented as mean (M) with standard errors of the mean (±SEM). Differences were considered statistically significant at $p<0.05$.

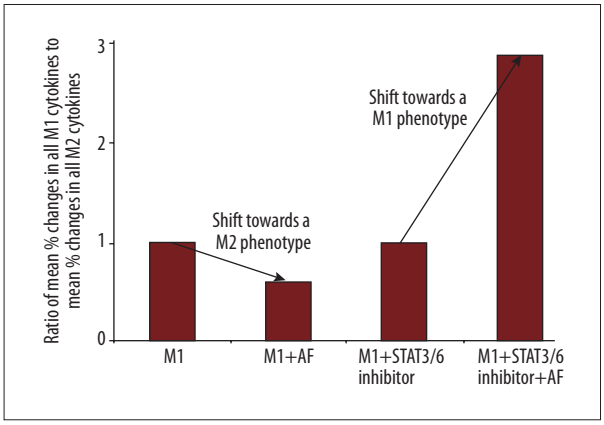


**Figure 1.** Changes in the ratio of mean% changes in all M1 cytokines to mean% changes in all M2 cytokines after placing M1 and M1 macrophages with inhibited STAT3 and STAT6 ($M3_{STAT3/6}$ phenotype) in the pro-tumor environment (constructed by data from Table 1). AF – ascitic fluid.

## Results

### Activation of M1 reprogramming pathways and inhibition of M2 reprogramming pathways programs the M3 phenotype of macrophages

Table 1 and Figure 1 show changes in the cytokine-producing activity of M1 macrophages and the M1 macrophages with simultaneous inhibition of STAT3 and STAT6 (M1+STAT3/6 inhibitor) under the action of AF from mice with EAC. Data demonstrated several important facts.

First, production of the anti-inflammatory cytokines, IL-4, IL-5, and IL-10, decreased and the pro-inflammatory cytokines, IL-1β, IL-21, and IL-23, increased after inhibition of STAT3 and STAT6.





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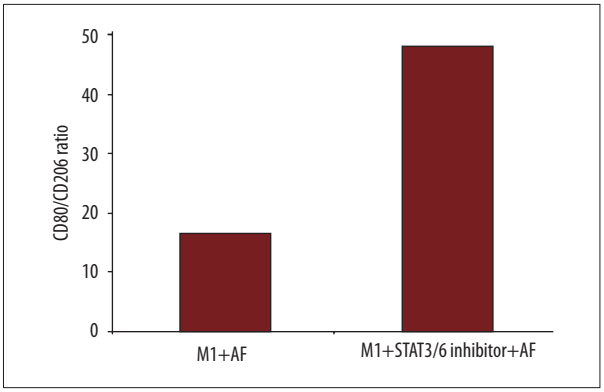


**Figure 2.** The CD80/CD206 ratio in M1 macrophages cultured with AF and in M1 macrophages with inhibited STAT3 and STAT6 (M3 phenotype) cultured with AF.

This fact is consistent with involvement of STAT3 and STAT6 in production of anti-inflammatory cytokines [38,39].

Second, in the tumor microenvironment (AF), M1 macrophages reduced the production of pro-inflammatory cytokines (except for IL-1β). As a result, the mean percent content of all pro-inflammatory cytokines decreased to 68.6%. AF increased concentrations of the anti-inflammatory cytokines, IL-5 and IL-10, and decreased the IL-4 level. As a result, the mean percent concentration of all anti-inflammatory cytokines increased to 112.8%. These changes reflect a shift of the macrophage phenotype towards the M2 phenotype and are consistent with other reports of the pro-tumor, M2-reprogramming effect of the tumor microenvironment [15].

Third, in the tumor microenvironment (AF), M1 macrophages with inhibited STAT3 and STAT6 increased the production of pro-inflammatory cytokines. As a result, the mean percent content of all pro-inflammatory cytokines increased to 188.4%. Under the action of AF on M1 macrophages with inhibited STAT3 and STAT6, mean percent changes in concentrations of all anti-inflammatory cytokines significantly decreased to 65.6%.

Under the action of AF on M1 macrophages, the ratio of pro- to anti-inflammatory cytokines was 0.61, which indicated a phenotype shift towards the anti-inflammatory phenotype. When M1 macrophages with inhibited STAT3 and STAT6 were exposed to the AF, this ratio became 2.90, which indicated a shift of the phenotype towards the pro-inflammatory phenotype (Figure 1).

A considerable difference between M1 macrophages and M1 macrophages with inhibited STAT3 and STAT6 in the tumor microenvironment was confirmed by CD markers (Figure 2). When M1 macrophages were cultured without AF, the content of the M1 marker, CD80, was 84.0±10.3%, the content of the M2 marker, CD206, was 5.7±0.9%, and their ratio (CD80/CD206) was 14.7. In M1 macrophages cultured with the AF, the content of CD80 was 57.3±7.5%, the content of CD206 was 10.2±1.7%, and the CD80/CD206 ratio was 5.6. The decrease in CD80/CD206 from 14.7 to 5.6 in M1 macrophages cultured in the tumor microenvironment confirms the shift of macrophage phenotype towards the anti-inflammatory, pro-tumor M2 phenotype.

In macrophages with inhibited STAT3 and STAT6 cultured in the AF, the CD80/CD206 ratio was 45.7 (Figure 2). The higher CD80/CD206 of M1 macrophages with inhibited STAT3 and STAT6 compared to CD80/CD206 of M1 macrophages with uninhibited STAT3 and STAT6 (45.7 *vs.* 5.6) indicates a shift of the phenotype with inhibited STAT3 and STAT6 towards the pro-inflammatory phenotype compared to M1 macrophages with uninhibited STAT3 and STAT6.

Therefore, inhibition of STAT3 and STAT6 changed the response of M1 macrophages to the tumor microenvironment from reprogramming the anti-inflammatory phenotype to reprogramming the pro-inflammatory phenotype. This response of macrophages with inhibited STAT3 and STAT6 is consistent with characteristics of the M3 switch phenotype.

### $M3_{STAT3/6}$ macrophages exerted an anti-tumor effect *in vitro*, which was not inferior to the effect of cisplatin and was superior to the anti-tumor effect of M1 macrophages

Figure 3 shows that for 24 h of culturing in the medium containing 10% FBS, the amount of tumor cells increased 7-fold, from 25 000 to 174 000±7000 cells. Culturing of tumor cells in the macrophage-free media designed for macrophage reprogramming and activation did not influence the tumor cell growth.

Addition of non-reprogramed macrophages (M0) to EAC cells at ratios of macrophages to EAC cells of 5: 1 and 10: 1 slowed, although non-significantly, the EAC growth. However, beginning from the 20: 1 ratio, the M0 phenotype began stimulating growth of EAC cells, although non-significantly. When the ratio was increased to 80: 1, this tendency became significant and the EAC growth increased by 21% (Figure 3). This result provides an additional explanation to a well-known clinical phenomenon that a higher macrophage concentration in the tumor worsens prognosis of the disease [40].

Addition of either M1 or $M3_{STAT3/6}$ macrophages dose-dependently restricted the growth of tumor cells. Furthermore, the anti-tumor effect of $M3_{STAT3/6}$ macrophages was stronger than the effect of M1 macrophages and was not inferior to the effect of cisplatin.





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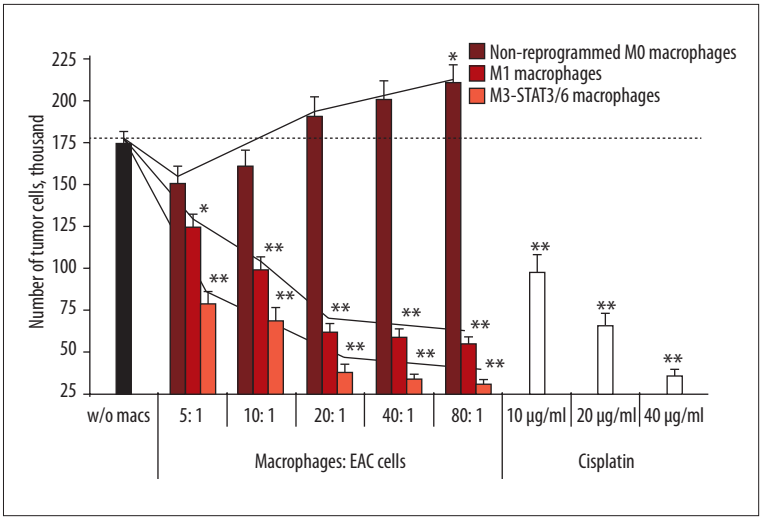


**Figure 3.** Effect of M0, M1, and $M3_{STAT3/6}$ macrophages on the number of tumor cells. Experiment was performed in 5 replicates. w/o macs – without macrophages. Significance of differences from the M0 phenotype: * p<0.05; ** p<0.01

**The anti-tumor effect of $M3_{STAT3/6}$ macrophages *in vitro* was due to their anti-proliferative rather than cytotoxic effect**

After culturing with or without any macrophage phenotype, the proportion of dead tumor cells did not exceed 5–7%. This allows us to conclude that $M3_{STAT3/6}$ macrophages limited only division of EAC cells (Figure 3), but did not kill the tumor cells. In contrast, when tumor cells were cultured with cisplatin, the proportion of dead tumor cells could reach 25%. This suggests that the anti-tumor effect of $M3_{STAT3/6}$ macrophages was due to their anti-proliferative rather than cytotoxic action, while the anti-tumor effect of cisplatin was due to both anti-proliferative and cytotoxic action.

In culturing without EAC cells, co-culturing with EAC cells, and with cisplatin, proportions of dead macrophages of any phenotype were not significantly different, were small, and did not exceed 7–9%.

**The anti-tumor *in vitro* effect of $M3_{STAT3/6}$ macrophages is associated with pro-inflammatory reprogramming of tumor microenvironment**

Tumors suppress the activity of immune cells by releasing various cytokines, which form the pro-tumor microenvironment [41,42]. We suggest that the difference in effects of different macrophage phenotypes (M0, M1, and M3) on tumor growth could depend on the influence on the tumor microenvironment. Analysis of data in Table 2 and Figure 4 confirmed this hypothesis.

*First,* addition of M0 macrophages to EAC cells resulted in decreased concentrations of pro-inflammatory cytokines to 62%, and increased concentrations of anti-inflammatory cytokines by 56%. Therefore, the ratio of mean% changes in M1 cytokines to mean% changes in M2 cytokines decreased from 1.0 in the tumor M0 microenvironment to 0.4 (62/156) in the EAC/macrophage M0 microenvironment. This means that attraction of M0 macrophages to the tumor area shifts the phenotype of the tumor cell microenvironment towards the anti-inflammatory phenotype.

*Second,* addition of M1 macrophages to EAC cells resulted in a 108% increase in concentrations of pro-inflammatory cytokines, as well as slight increases in concentrations of anti-inflammatory cytokines. As a result, the ratio of mean% changes in M1 cytokines to mean% changes in M2 cytokines increased from 1.0 in the tumor M1 microenvironment (Table 2, column 5) to 2.0 in the EAC/M1 macrophage microenvironment (Table 2, column 7). This means that attraction of M1 macrophages into the tumor growth area shifts the phenotype of the tumor cell microenvironment towards the pro-inflammatory one.

The fact that the anti-inflammatory microenvironment facilitates while the pro-inflammatory microenvironment limits its tumor growth [41,42] suggests that the pro-tumor effect of M0 macrophages and the anti-tumor effect of M1 macrophages (Figure 3) are probably due to alternative reprogramming actions of M0 and M1 macrophages on the tumor microenvironment.

*Third,* addition of $M3_{STAT3/6}$ macrophages to EAC cells resulted in a 94% increase in concentrations of pro-inflammatory cytokines and in a 56% increase in concentrations of anti-inflammatory cytokines. As a result, the ratio of M1 to M2 cytokines concentration increased from 1.0 in the tumor $M3_{STAT3/6}$-microenvironment (Table 2, column 8) to 1.2 in the EAC/macrophage $M3_{STAT3/6}$-microenvironment (Table 2, columns 10). This means that attraction of $M3_{STAT3/6}$ macrophages into the tumor growth area, as distinct from M0 macrophages, shifts the microenvironment phenotype towards the pro-inflammatory, anti-tumor phenotype. This effect apparently contributes to the anti-tumor effect in $M3_{STAT3/6}$ macrophages (Figure 3).





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**Table 2.** Effects of M0, M1, and $M3_{STAT3/6}$ macrophages on cytokine microenvironment of tumor cells.

| Cytokines | M0 medium | EAC cells in M0 medium | M0 Mϕ | M0 Mϕs+ EAC cells | EAC cells in M1 medium | M1 Mϕs | M1 Mϕs+ EAC cells | EAC cells in $M3_{STAT3/6}$ medium | $M3_{STAT3/6}$ Mϕs | $M_{3STAT3/6}$ Mϕs + EAC cells |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| IL-1α (M1) | 48.1 | 67.1 | 101.5 | 60.7 | 92.8 | 86.3 | 138.4 | 76.8 | 102.3 | 131.7 |
| IL-1β (M1) | 0 | 0 | 86.1 | 95.6 | 17.2 | 117.1 | 280.0 | 62.9 | 113.9 | 215.3 |
| IL-2 (M1) | 4.0 | 4.0 | 20.5 | 25.9 | 0 | 152.6 | 98.0 | 0 | 196.9 | 150.7 |
| IL-6 (M1) | 77.1 | 129.7 | 111.7 | 125.9 | 144.1 | 65.4 | 136.7 | 375.7 | 110.7 | 458.4 |
| IL-12 (M1) | 0 | 54.5 | 45.2 | 36.7 | 64.9 | 137.8 | 161.9 | 179.4 | 157.5 | 577.7 |
| IL-17 (M1) | 0 | 48.5 | 90.9 | 69.4 | 102.2 | 105.9 | 129.8 | 180.2 | 95.6 | 125.1 |
| IL-21 (M1) | 36.0 | 379.3 | 42.3 | 2.3 | 94.2 | 238.6 | 6.9 | 97.9 | 303.1 | 309.4 |
| INF-γ (M1) | 37.1 | 85.2 | 17.1 | 30.5 | 97.6 | 96.1 | 61.3 | 198.2 | 159.1 | 112.0 |
| TNF-α (M1) | 6.3 | 19.2 | 22.4 | 42.0 | 38.1 | 55.2 | 343.2 | 113.0 | 133.3 | 403.1 |
| IL-4 (M2) | 0 | 0 | 72.3 | 32.3 | 0 | 35.6 | 21.4 | 51.2 | 96.9 | 25.8 |
| IL-5 (M2) | 0 | 0.2 | 31.8 | 29.1 | 61.8 | 22.7 | 8.7 | 9.5 | 7.0 | 13.8 |
| IL-10 (M2) | 56.0 | 59.1 | 61.3 | 25.6 | 156.8 | 48.1 | 50.3 | 122.4 | 58.5 | 5.9 |
| IL-13 (M2) | 59.7 | 110.4 | 138.8 | 71.9 | 155.9 | 142.5 | 273.8 | 72.4 | 124.5 | 344.1 |
| IL-22 (M2) | 0 | 38.5 | 147.1 | 166.9 | 64.7 | 271.2 | 101.9 | 55.8 | 117.4 | 97.8 |
| Shift towards a microinvironment phenotype | | | | AIP as compared to column 2 | | | PIP as compared to column 5 | | | PIP as compared to column 8 |

In the serum-free culture medium, cytokines were not detected or were below the detection limit. M0 medium is a normal medium (10% FBS); M1 medium is a medium, which reprograms macrophages towards the M1 phenotype (0% FBS+IFNγ+LPS); and M3 medium is a medium which reprograms macrophages towards the M3 phenotype (0% FBS+IFN-γ+inhibitor STAT3/6+LPS).
AIP – anti-inflammatory phenotype; PIP – proinflammatory phenotype; [] – concentration; Mϕs – macrophages.

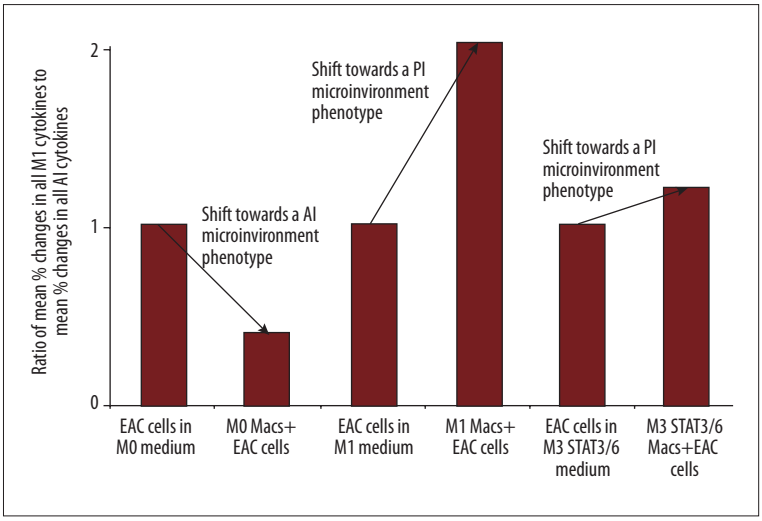


**Figure 4.** Changes in the ratio of mean% changes in all PI cytokines to mean% changes in all AI cytokines after addition of macrophages with M0, M1, and $M3_{STAT3/6}$ phenotypes (constructed by data from Table 2). The ratio of PI-to-AI cytokines in the macrophage-free tumor microenvironment was taken as 1.0. A decrease in the ratio to below 1.0 means a shift of the microenvironment phenotype towards the anti-inflammatory phenotype, whereas an increase above 1.0 means a shift towards the pro-inflammatory phenotype. AI – anti-inflammatory; PI – pro-inflammatory.

*Forth*, tumor cells alone respond to changing the culture medium from M0 to M1 and $M3_{STAT3/6}$ media (Table 2, columns 2, 5, and 8). However, in neither M1 nor $M3_{STAT3/6}$ culture medium without macrophages, growth of tumor cells decreased compared with the M0 medium. This means that in M1 and M3 media, anti-tumor effects of M1 and $M3_{STAT3/6}$ macrophages were due primarily to the activity of macrophages themselves.





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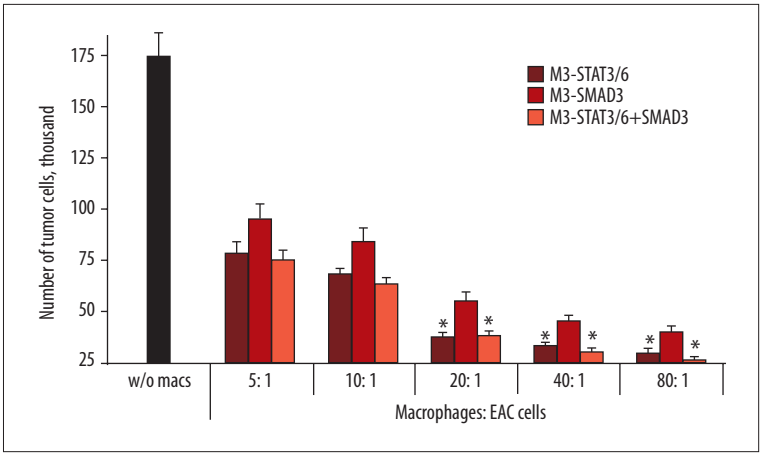


**Figure 5.** Effect of $M3_{SMAD3}$, $M3_{STAT3/6+SMAD3}$, and $M3_{STAT3/6}$ macrophages on the number of tumor cells. Experiment was performed in 5 replicates. w/o macs – without macrophages. Significance of differences from $M3_{SMAD3}$ macrophages: * $p<0.05$.

### $M3_{STAT3/6+SMAD3}$ macrophages exert a more pronounced *in vitro* anti-tumor effect than $M3_{STAT3/6}$ and $M3_{SMAD3}$ macrophages

Figure 5 shows that $M3_{SMAD3}$ and $M3_{STAT3/6+SMAD3}$ phenotype macrophages, like $M3_{STAT3/6}$, possessed a pronounced anti-tumor effect. Furthermore, $M3_{STAT3/6+SMAD3}$ macrophages exerted the strongest anti-tumor effect.

We revealed an interesting fact. There are 2 segments in the dose-response curve for dependence of the anti-tumor effect on M1 and M3 macrophages (Figures 3, 5), a segment of proportional depression of EAC cell growth (in the ratio of M1 and M3 macrophages to EAC cells from 5: 1 to 20: 1), and a segment of relative absence of dose-dependence (in the ratio of M1 and M3 macrophages to EAC cells from 20: 1 to 80: 1). This fact allowed us to suggest the existence of 2 pools of tumor cells: those with high-sensitivity and those with low-sensitivity to the anti-proliferative effect of macrophages. Division of the highly sensitive pool stops when the macrophage-to-EAC cell ratio increases from 5: 1 to 20: 1. After that, despite the increase in the macrophage to EAC cell ratio from 20: 1 to 80: 1, division of EAC cells from the low-sensitivity pool continues. We calculated the pools of EAC cells that had high-sensitivity and low-sensitivity to M1 macrophages and to $M3_{Stat3/6}$ macrophages (Figure 3) using the formulas:

EAC cells with **high-sensitivity** to macrophages = 100% – (EAC cells low sensitive to macrophages)%.

EAC cells with **low-sensitivity** to macrophages = (the number of EAC cells with macrophages in the ratio of macrophages to EAC cells 20: 1/the number of EAC cells without macrophages) ×100%.

We found that the pool of EAC cells highly sensitive to M1 macrophages accounts for approximately 65% and the pool of EAC cells with low-sensitivity accounts for approximately 35%. The pool of tumor cells with high-sensitivity to $M3_{Stat3/6}$ macrophages accounts for 79% and those with low-sensitivity account for only 21%.

### Anti-tumor effects of $M3_{SMAD3}$ and $M3_{STAT3/6+SMAD3}$ macrophages, like the effects of $M3_{STAT3/6}$ macrophages, were associated with pro-inflammatory reprogramming of tumor microenvironment

Table 3 (columns 3, 5, 6, 8, 9, and 11) compares effects of adding different M3 macrophage phenotypes on the tumor microenvironment.

Addition of $M3_{SMAD}$ macrophages to EAC cells led to a 12% increase in pro-inflammatory cytokine concentration (from 75.5 to 85.5 pg/ml) and to a 22% decrease in concentration of anti-inflammatory cytokines (from 64.7 to 51.7 pg/ml). The resultant ratio of mean% changes in M1 cytokines to mean% changes in M2 cytokines increased from 1.0 (conventional units) in the tumor $M3_{SMAD}$-microenvironment to 1.43 (112/78) in the EAC/macrophage $M3_{SMAD}$-microenvironment (Figure 6.) This means that attraction of $M3_{SMAD}$ macrophages into the tumor growth area, like attraction of $M3_{STAT3/6}$, shifts the microenvironment phenotype towards a pro-inflammatory phenotype.

Further, addition of $M3_{STAT3/6+SMAD}$ macrophages to EAC cells resulted in a 186% increase (from 45.4 to 175.1 pg/ml) in concentration of pro-inflammatory cytokines, and a 94% increase in concentration of anti-inflammatory cytokines (from 29.2 to 56.6 pg/ml). Thus, the ratio of mean% changes in M1 cytokines to mean% changes in M2 cytokines increased from 1.0 (conventional units) in the tumor $M3_{STAT3/6+SMAD}$ microenvironment to 1.47 (286/194) in the EAC/macrophage $M3_{STAT3/6+SMAD}$ microenvironment (Figure 6.). This means that attraction of $M3_{STAT3/6+SMAD}$ macrophages into the tumor growth area, like attraction of $M3_{STAT3/6}$ and $M3_{SMAD}$ macrophages, shifted the microenvironment phenotype towards a pro-inflammatory phenotype.





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**Table 3.** Effects of $M3_{STAT3/6}$, $M3_{SMAD3}$ and $M3_{STAT3/6+SMAD3}$ macrophages on cytokine microenvironment of tumor cells.

| Cytokines | M0 medium | EAC cells in M0 medium | EAC cells in $M_{3STAT3/6}$ medium | $M3_{STAT3/6}$ Mϕs | $M3_{STAT3/6}$ Mϕs + EAC cells | EAC cells in $M3_{SMAD3}$ medium | $M3_{SMAD3}$ Mϕs | $M3_{SMAD3}$ Mϕs + EAC cells | EAC cells in $M3_{STAT3/6+SMAD}$ medium | $M3_{STAT3/6+SMAD}$ Mϕs | $M3_{STAT3/6+SMAD}$ Mϕs + EAC cells |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| IL-1α (M1) | 48.1 | 67.1 | 76.8 | 102.3 | 131.7 | 68.9 | 53.7 | 77.0 | 24.1 | 346.5 | 222.7 |
| IL-1β (M1) | 0 | 0 | 62.9 | 113.9 | 215.3 | 67.0 | 44.1 | 64.1 | 96.0 | 951.5 | 284.2 |
| IL-2 (M1) | 4.0 | 4.0 | 0 | 196.9 | 150.7 | 0 | 94.9 | 107.4 | 0 | 44.7 | 303.9 |
| IL-6 (M1) | 77.1 | 129.7 | 375.7 | 110.7 | 458.4 | 143.6 | 46.5 | 83.9 | 12.9 | 150.8 | 268.4 |
| IL-12 (M1) | 0 | 54.5 | 179.4 | 157.5 | 577.7 | 54.5 | 24.1 | 91.4 | 59.9 | 371.6 | 82.8 |
| IL-17 (M1) | 0 | 48.5 | 180.2 | 95.6 | 125.1 | 66.4 | 52.6 | 118.0 | 62.3 | 146.9 | 93.2 |
| IL-21 (M1) | 36.0 | 379.3 | 97.9 | 303.1 | 309.4 | 116.0 | 0 | 92.6 | 0 | 330.5 | 83.6 |
| INF-γ (M1) | 37.1 | 85.2 | 198.2 | 159.1 | 112.0 | 39.1 | 77.5 | 33.5 | 45.7 | 145.9 | 121.2 |
| TNF-α (M1) | 6.3 | 19.2 | 113.0 | 133.3 | 403.1 | 124.4 | 62.7 | 97.5 | 107.5 | 199.9 | 115.5 |
| IL-4 (M2) | 0 | 0 | 51.2 | 96.9 | 25.8 | 34.0 | 66.1 | 68.3 | 56.2 | 32.4 | 35.1 |
| IL-5 (M2) | 0 | 0.2 | 9.5 | 7.0 | 13.8 | 4.7 | 18.6 | 19.9 | 8.6 | 67.7 | 0 |
| IL-10 (M2) | 56.0 | 59.1 | 122.4 | 58.5 | 5.9 | 129.2 | 0 | 0 | 2.6 | 45.6 | 2.8 |
| IL-13 (M2) | 59.7 | 110.4 | 72.4 | 124.5 | 344.1 | 106.9 | 0 | 80.1 | 78.7 | 877.0 | 202.4 |
| IL-22 (M2) | 0 | 38.5 | 55.8 | 117.4 | 97.8 | 48.8 | 0 | 90.3 | 0 | 140.3 | 42.8 |
| Shift towards a microi-nvironment phenotype | | | | | PIP as compared to column 3 | | | PIP as compared to column 6 | | | PIP as compared to column 9 |

Abbreviations are as in Tables 1 and 2.

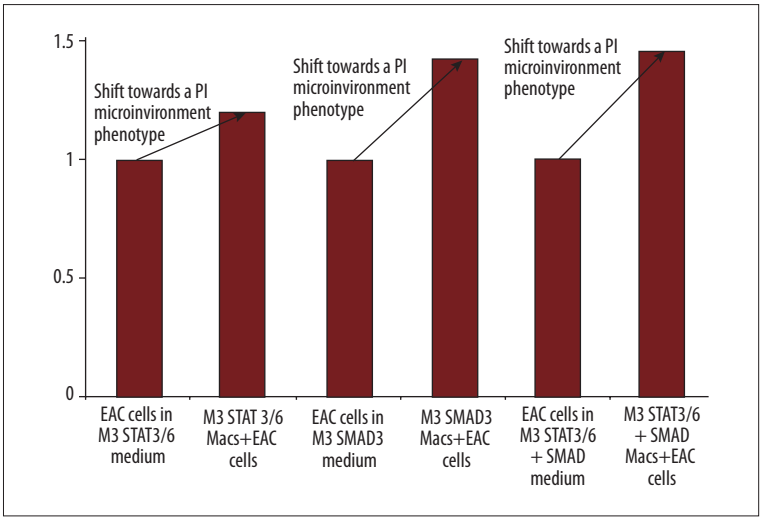


**Figure 6.** Changes in the ratio of mean% changes in all PI cytokines to mean% changes in all AI cytokines after addition of macrophages with $M3_{STAT3/6}$, $M3_{SMAD}$ and $M3_{STAT3/6+SMAD}$ phenotypes (constructed by data from Table 3). The ratio of PI-to-AI cytokines in the macrophage-free tumor microenvironment was taken as 1.0. A decrease in the ratio to below 1.0 means a shift of the microenvironment phenotype towards the anti-inflammatory phenotype, whereas an increase above 1.0 means a shift towards the pro-inflammatory phenotype. AI – anti-inflammatory; PI – pro-inflammatory.





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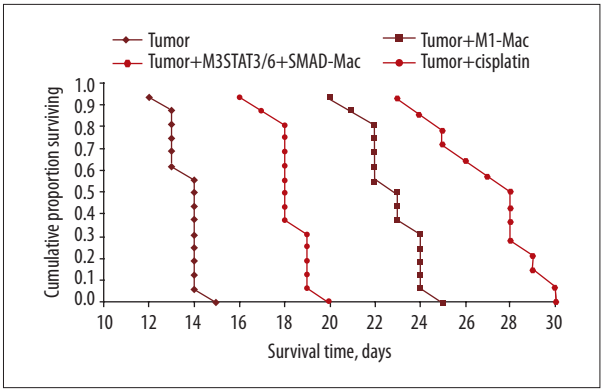


**Figure 7.** Effect of injected M1 and $M3_{STAT3/6+SMAD}$ macrophages and cisplatin on lifespan of mice, with EAC in Kaplan-Meier survival plots.

We suggest that the anti-tumor effect of $M3_{STAT3/6+SMAD}$ macrophages and $M3_{SMAD}$ (Figure 5), like effects of $M3_{STAT3/6}$ macrophages, was due to pro-inflammatory reprogramming of the tumor microenvironment.

### $M3_{STAT3/6+SMAD3}$ macrophages exerted an anti-tumor effect *in vivo*, which was superior to anti-tumor effects of cisplatin and M1 macrophages

To understand whether the *in vitro* anti-tumor effect of M3 macrophages can be reproduced *in vivo,* we evaluated the effect of $M3_{STAT3/6+SMAD3}$ macrophages on the life span of mice with EAC. The $M3_{STAT3/6+SMAD3}$ phenotype was selected for its strongest *in vitro* anti-tumor effect among all M3 phenotypes (Figure 5).

Figure 7 shows the effect of injected macrophages and cisplatin on lifespans of mice with EAC in Kaplan-Meier survival plots. The survival duration of mice injected with EAC cells was 13.4±0.4 days ("Tumor" group). The survival duration of mice injected with M1 macrophages ("Tumor+M1-Mac" group) was 22.8±0.8 days ($p<0.01$), which is 70% longer than in the "Tumor" group. The survival duration of mice injected with the $M3_{STAT3/6+SMAD}$ macrophages ("Tumor+M3-Mac" group) was 27.1±0.5 days ($p<0.01$), which is 102% longer than in the "Tumor" group. The anti-tumor effect of $M3_{STAT3/6+SMAD}$ macrophages was greater than the effect of the anti-tumor drug, cisplatin ("Tumor + cisplatin" group).

Infusion of PBS ("Tumor + PBS" group) or M0 macrophages ("Tumor + M0-Mac" group) did not significantly influence the survival duration in mice with EAC.

Therefore, injection of the $M3_{STAT3/6+SMAD3}$ macrophages significantly increased the resistance of mice to development of EAC.

## Discussion

We used S3I204, As1517499, and SIS3 for inhibiting transcription factors STAT3, STAT6, and SMAD3, respectively. The inhibitory effects of S3I204, As1517499, and SIS3 on these transcription factors has been repeatedly proven [18]. The data in Table 1 show that addition of S3I204, As1517499, and SIS3 to macrophages actually decreased the production of anti-inflammatory cytokines.

The results of the present study confirmed our hypothesis that the switch phenotype can be programmed by activation of M1-reprogramming pathways with simultaneous inhibition of the M2 phenotype transcription factors, STAT3, STAT6, and/or SMAD3, and also that tumor growth could be effectively limited by the switch phenotype. Is it justified to isolate this phenotype as an independent M3 phenotype and what does distinguish the M3 from M1 and M2 phenotypes? An important feature of programming the M1 phenotype is that the inflammatory cytokines produced by the M1 phenotype shift the macrophage phenotype even further towards M1. As a result, a pro-inflammatory positive feedback mechanism forms to enable fast programming of the antimicrobial and antitumoral M1 phenotype. Similarly, anti-inflammatory cytokines such as IL-10 or TGF-β produced by the M2 phenotype shift the macrophage phenotype even further towards the M2 phenotype, thereby forming an anti-inflammatory positive feedback mechanism, which enables fast programming of the M2 macrophage phenotype [43,44].

In the pro-tumor environment, the M1 phenotype becomes reprogrammed towards the pro-tumor M2 phenotype [15] and begins producing more anti-inflammatory cytokines. As distinct from the M1 phenotype, the switch phenotype produces even more anti-tumor pro-inflammatory cytokines in the tumor microenvironment and, thereby, provides negative feedback.

The M3 hypothesis requires substantial verification and many questions need to be answered. The first of them is whether the concept of a steady-state M3 phenotype, as well as M1 and M2, is consistent with the data on extremal plasticity of macrophage function and phenotype [6,45].

The model of M1-M2 macrophage activation categorizes macrophages in a functional, output-specific fashion: M1/kill/inflammatory phenotype that promotes Th1 response and antimicrobial and tumoricidal properties, or M2/repair/anti-inflammatory phenotype that promotes Th2 response and antiparasitic properties [46]. This model has received support [47–50]. However, it also became obvious that the M1-M2 model insufficiently described macrophage activation [51].





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For these reasons, a multidimensional model of macrophage activation was recently suggested. This model, as distinct from the M1–M2 dichotomy model, includes multiple phenotypes formed in an input-specific fashion, with sufficient accounting for effects of the entire array of ontogenetic, tissue-specific, and microenvironmental factors [51].

The notion of the M3 phenotype can "reconcile" 2 concepts and suggests "a multidimensional input – multifunctional output" model of macrophage activation. In this model, as in the multidimensional model, a huge number of microenvironmental and internal signals may influence macrophages and determine the resultant functional phenotype, such as M1/kill, or M2/repair, or antigen-presenting, or Th-stimulating. M3, via the negative feedback, probably represents a component of macrophage plasticity to provide the multifunctional activation. For instance, in the inflammatory environment, the M3 probably reprograms inflammatory to anti-inflammatory macrophages to terminate inflammation.

Among immune cells, the Treg lymphocytes are cells that behave similarly to the switch phenotype, producing anti-inflammatory cytokines in response to the inflammatory environment [52,53]. This similarity suggests that innate and adaptive responses have comparable cell and functional organization. As in the adaptive response, in which a great variety of antigens program numerous effectors such as Th1 or Th2 lymphocyte phenotypes, in the innate response, various microenvironmental factors program various effector macrophage phenotypes. Similar to Treg, which regulates functional activity of effector lymphocytes by the negative feedback mechanism, M3 macrophages can regulate activity of macrophage phenotypes. Other researchers also came to similar conclusions [54,55].

Macrophages have long attracted attention of researchers as a target for anti-tumor therapy. Thus, anti-tumor macrophage activity can be increased by stimulating Toll-like receptors and inhibiting TGF-β receptors [56]; culturing in a serum-free medium [5]; enhancing activities of IFN-γ and IL-12 genes [57]; binding pro-tumor factors or their receptors on macrophages [58,59]; depressing macrophage pro-tumor properties with antisense oligonucleotides [60], small molecules [61]; and other approaches. Essentially, all these approaches are aimed at formation of an anti-tumor M1 phenotype. However, the tumor generally reprograms the M1 phenotype into the pro-tumor M2 phenotype [15].

The qualitative difference between the M3 and M1 phenotypes apparently underlies the quantitatively greater anti-tumor effect of M3 compared to M1. The M3 phenotype exerted an anti-proliferative anti-tumor effect *in vitro* and prolonged survival time of mice with EAC. The more pronounced anti-tumor effect of M3 macrophages, as compared with M1 macrophages, may be due to the greater inflammatory activity of M3 and its ability to switch the signal of the anti-inflammatory environment to production of pro-inflammatory cytokines.

## Conclusions

We reported the sensitivity limits on the cytokine test used, the artifacts of the co-culture methods, and the need for additional assays to validate the findings. The fact that M3 macrophages considerably depress tumor cell growth *in vitro* and prolong survival time of mice with EAC, shows that development of new biotechnologies for restriction of tumor growth using *in vitro* reprogrammed M3 macrophages is very promising.

### Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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