Classically-activated macrophages elimination in tumor-conditioned medium by alternatively-activated macrophages

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Abstract

Cellular interactions are critical during development, tissue fitness and epithelial tumor development. The expression levels of specific genes confer to tumoral cells a survival advantage versus the normal neighboring cells. As a consequence, cells surrounding tumors are eliminated and engulfed by macrophages. We propose a novel scenario in which circulating cells facing a tumor can reproduce these cellular interactions. In vitro cultured macrophages from murine bone marrow were used to investigate this hypothesis. M1 macrophages in tumoral medium upregulated markers of a suboptimal condition, such as Sparc and TyrRS, and undergo apoptosis. However, M2 macrophages display higher Myc expression levels and proliferate at expenses of M1s elimination. Resulting M1 apoptotic debris are engulfed by M2 in a Sparc and TyrRS dependent manner. These findings suggest that tumor-dependent macrophage elimination could deplete immune response against tumors, this possibility could be relevant for macrophage based anti-tumoral strategies.
**Introduction**

Therapeutical strategies in oncology include the adoptive transfer of anti-tumoral classically-activated macrophages (CAMs, also referred as M1) [1]. Ex vivo programmed CAMs have the potential to induce regression of established tumors [2,3]. However, transfer of CAMs for cellular therapy has not reached the expected results so far, exogenous activated macrophages show restricted motility and become rapidly undetectable when facing the tumor microenvironment [2,3,4].

We rationalized that transferred exogenous CAMs used in cellular anti-tumoral therapy might be somehow eliminated, thus, compromising the efficiency of treatment. To study that we use an *in vitro* model of polarized macrophages, M1 which are key effector cells for the elimination of cancer cells and M2 which promote tumoral growth [6], to study their behaviour under tumoral conditions. Our results show that M1 upregulated SPARC and TyrRS, that have been previously shown as markers of compromised cellular fitness[7,8] Concomitantly, M1 macrophages undergo apoptosis and are finally engulfed by M2 macrophages. Based on these observations, we propose that adoptive transfer of macrophages as an anti-tumor therapy might undergo CAMs elimination, and it can have an impact on the effectiveness of the treatment.

**Results**

To characterize whether anti-tumor macrophages are compromised under tumoral conditions, we analyzed SPARC and TyrRS expression. We used an *in vitro* model of murine bone marrow derived M1 and M2 macrophages. Polarization was validated using specific M1 and M2 markers (supplementary Figure 1 [9]). M1 and M2 were separately cultured either in control, B16F10-derived or A-549-derived tumoral media for 24 hours. M1 showed a significant increase of *SPARC* and *TyrRS* expression both at mRNA (Figure 1A-B) and protein levels when cultured in tumor-conditioned medium, compared to M2 (Figure 1C-N, M1 protein levels quantified in Figure 1O).

These results suggest that M1 show a compromised fitness compared to M2. In addition M2 cells upregulated c-Myc expression as compared to M1 [10,11,12,13] (Figure 1P). In line with these results, slightly increased levels of c-Myc confer advantageous properties to *Drosophila* and mammalian epithelial cells [14,15] whereas lower levels of c-Myc determine a suboptimal
state [16]. Altogether, these data indicate that M1 cells are tagged as suboptimal cells in this tumoral context. To determine whether M1 cells are eliminated in a tumoral medium but M2 survive, we studied apoptosis response after culturing M1 and M2 separately in either control, B16F10-derived or A-549-derived tumoral media for 24 hours. There were no differences in TUNEL positive cells between M1 and M2 cells in control medium (Figure 2A). However, we observed that only M1 macrophages underwent apoptosis significantly when cultured in tumoral-conditioned medium (Figure 2A). This observation was further confirmed by active Caspase-3 staining, which showed upregulation specifically in M1 after 24 hours of culture in tumor-conditioned media (Figure 2B-G, quantified in Figure 2I). Then, to rule out the possible apoptotic effect of M2-secreted factors during this time-period, we cultured M1 in M2-derived medium and analyzed the number of apoptotic cells. There were no significant differences in the percentage of apoptotic M1 and M2 macrophages at the different time-points analyzed (Figure 2H and FACS in supplementary Figure 2). These results show that M1 autonomously activate apoptosis in response to tumor-conditioned medium.

Previous reports have shown that a population of phagocytic cells contribute to the elimination of apoptotic cells [17]. We therefore decided to characterize whether M2 could participate in the elimination of anti-tumoral M1 once apoptosis is activated. We co-cultivated M1 and M2 macrophages at a 1:1 proportion (previously labeled with different color-cell trackers, see material and methods) in control or B16F10-derived tumoral medium. After 24h, 48h and 5 days of co-culture, we quantified the number of each population; our results show that the ratio M1/M2 remained unaffected when co-cultured in control medium. However, when co-cultured in B16F10-derived tumoral medium M1 population was reduced compared to M2 (Figure 3A-D, supplementary Table 1 and supplementary Figures 3 and 4). To evaluate our hypothesis of an active mechanism to eliminate M1, we blocked TyrRS and SPARC signaling adding specific antibodies to the cell culture medium. TyrRS is secreted by cells to be eliminated and stimulates the recruitment of macrophages that eliminate the apoptotic bodies [7]. When anti-TyrRS was added to the cell culture, M1 elimination was prevented (Figure 3A and supplementary Table 1). This result goes in line with previous reports which postulated the existence of a loser killing signal in a cell competition in vitro model [18]; This result suggests that TyrRS secretion would induce M2 recruitment and therefore might function as a signal to engulf M1 cells. On the other hand, SPARC is a protective signal for suboptimal cells, we incubated the cell culture with anti-SPARC to block
SPARC protective function, consequently M1 cells were eliminated more efficiently (Figure 3A and supplementary Table 1). To validate whether M2 cells are engulfing M1-loser cells we performed live imaging of co-cultured cells. The results showed that M2 engulfed M1 when cultured in the B16F10-derived tumoral medium (supplementary video 1, 2, 3 and 4). Interestingly, engulfment events were observed as early as 6-7 hours of co-culture (see supplementary video 2). According to our previous observations, autonomously tumor-induced M1 apoptosis is not yet induced at this time point. This data suggests that engulfment could be the cause of M1 elimination in the presence of M2. A similar conclusion was previously raised in *Drosophila*, where engulfment genes were shown to be required for apoptosis [19]. Our results might therefore indicate that M1 behave as suboptimal cells in response to tumor signals, activate apoptosis and then are engulfed by M2, which are the tumor-associated macrophages. To discriminate as to whether engulfment is the cause or rather the consequence of apoptosis in the context of a complex tumor microenvironment remains to be elucidated in the future.

Finally, to validate that SPARC works as a protective signal for anti-tumoral macrophages, we tested M1 elimination using bone-marrow derived M1 and M2 from an SPARC KO mouse (Figure 4A-E). M1 cells were unable to up-regulate SPARC, and therefore do not activate the protective signaling dependent on SPARC signaling. Under these conditions, the number of phagocytic events (fragments of M1 inside M2) was significantly higher and M1 macrophages were eliminated more efficiently as compared to a wild type background (Figure 4A-D, quantification in 4E, and supplementary table 2). Consistently, a role for SPARC has been previously associated to an increase in M2 vs M1 ratio in a murine pancreatic cancer model, suggesting that M1 could also be eliminated in vivo [20].

**Discussion**

Although we cannot rule out that the decreased number of injected CAMs in vivo could be partially due to a reprogramming of these CAMs into other macrophage types or rather tumor medium-induced apoptosis, our results indicate that anti-tumoral macrophages can also be engulfed by pro-tumoral associated macrophages, as we have observed in our *in vitro* model. This possibility could explain why CAMs-based antitumoral therapy has not reached the expected efficiency. Expression of specific markers and regulators of compromised cellular fitness such as SPARC and TyrRS identify M1 as suboptimal cells. Consequently, these cells are
eliminated and reduce their anti-tumoral effectiveness. Although still preliminary, these observations might lead to the future prospect of decreasing CAMs suboptimal behavior in a way that their lifetime in the organism would be lengthened, increasing their efficiency in fighting the tumor. Future work with in vivo experiments of adoptively transferred macrophages would be required to address this possibility.

TyrRS has a dual function, under control conditions it is a Tyrosyl-transferase essential for protein synthesis. However, under certain situations in which the cell integrity is compromised, TyrRS is upregulated, secreted and cleaved to recruit phagocytic cells [21]. Because of these two independent functions, we believe that TyrRS is not an ideal therapeutical target. Although it might be interesting to evaluate the effects of blocking TyrRS in the tumor microenvironment, the side effect on healthy cells make this strategy less attractive. However, SPARC has been shown to protect cells from apoptosis in vitro via activation of integrin-linked kinase and AKT [22] and prevents the elimination of suboptimal cells [8]. We have shown here that initial expression of SPARC in M1 is probably a protective signal and only after a continuous exposure to the apoptotic signal, SPARC endogenous expression is overwhelmed and not enough to impede M1 elimination in vitro. According to these observations, we consider that specifically modulation of SPARC levels could be an interesting strategy to increase/improve CAMs survival rate. Following studies will be aimed to investigate if SPARC expression in CAMs could be upregulated by modulating ligand-mediated intracellular pathways like TGF-β [23], c-Jun [24] and Snail [25] activity. Alternatively, overexpression of SPARC by gene-adoptive transfer could be also a suitable approach to reduce looseness of CAMs.

Overall, we propose that cell-autonomous cell death and the concomitant engulfment could play an important role in regulating tumor progression and should be taking into account when considering the behavior of pro- and anti-tumoral cells in the complex tumor environment.
Material & methods

Murine macrophages and culture conditions

1. Mice and cares.

Wild type (WT) mice (C57BL/6 background) were purchased from The Jackson laboratory, Madison, WI. SPARC-/- femurs and tibiae were a gift from Dr. P.P. López-Casas at CNIO, the corresponding SPARC-/- mice (C57BL/6 background) were sacrificed at CNIO following approved procedures by the CNIO Research Ethics Committee. Mice were maintained on a standard diet (Panlab, Barcelona, Spain). Care of animals was in accordance with institutional guidelines and regulations, and conformed to EU Directive 86/609/EEC and Recommendation 2007/S26/EC regarding the protection of animals used for experimental and other scientific purposes, enacted under Spanish law 1201/2005. All animal procedures have been approved by The Spanish National Cardiovascular Centre (CNIC) or The Spanish National Cancer Centre (CNIO) Research Ethics Committees.

2. Isolation of bone marrow (BM) cells

Bone marrow-derived (BM) cells were harvested from WT and SPARC-/- mice. Briefly, mice were euthanized by carbon dioxide inhalation. 12 mice (8 weeks of age) were culled and femur and tibia were rapidly harvested. Skin, skeletal muscle and fat tissue surrounding the bones were removed. Both bones ends were cut and the BM was flushed with Hank’s Balanced Salt Solution (HBSS) containing 2 mM EDTA using a 1-ml insulin syringe with a 27 G needle. The obtained BM was disaggregated by pipetting and washed with PBS. Erythrocytes were lysed using lysis buffer (KH4Cl 0.15 M, KHCO3 0.01M, EDTA.N2 0.01M, pH7.4). BM cells were cultured (2 × 10⁶ cells/mL) for 7 days with DMEM medium supplemented with inactivated FBS 10% and M-CSF (100ng/ml) to obtain 95%-pure BM-derived non-activated macrophages and finally polarized into M1 or M2 using the following media for 48 hours: For M1 phenotype: complete DMEM (with 10% inactivated FBS) plus lipopolysaccharide, LPS (10ngrs/ml) and interferon gamma, IFNγ (10 ngrs/ml). For M2 phenotype: complete DMEM (with 10% inactivated FBS) plus interleukin-4, IL-4 (20 ngrs/ml).

The tumoral medium was obtained as previously described [22]. Briefly, B16-F10 murine melanoma cells or A549 adenocarcinoma human alveolar epithelial cells (ATCC®) were cultured in DMEM supplemented with 10% FCS, L-glutamine and penicillin/streptomycin.
Once grown to 90% confluence, medium was discarded, and flasks were rinsed twice with PBS. Cells were then incubated with fresh complete DMEM for 24 h; the tumor-cell–conditioned medium was collected, filtered (0.20 µm) and stored at −20°C. Control medium was normal DMEM supplemented with 10% FCS, L-glutamine and penicillin/streptomycin or conditioned medium derived from a fibroblast cell line (CCM). Both tumoral and control conditioned media were mixed (3:1) with fresh DMEM to compensate for the possible lack of certain metabolites.

**Quantitative RT-PCR**

Total RNA was isolated from M1 and M2 (Trizol, Invitrogen) and cDNAs were synthetized with M-MLV RT (Invitrogen). The following specific primers were used:

- **c-MYC-Forward**: 5′ GAGCTGGTTTGAAGGCTGGATTT 3′
- **c-MYC-Reverse**: 5′ TCCTGTGGTGTAAGGCTGGATTT 3′
- **SPARC-Forward1**: 5′ TAAACCCTCCACATTCTCG 3′
- **SPARC-Reverse1**: 5′ CACGGTTTCTTCCTCCACTA 3′
- **TyrRS-Forward**: 5′ GCAGGAGGCTAGGAGGAG 3′
- **TyrRS-Reverse**: 5′ GGCTTTCATGTTGTCCAGGT 3′

qRT-PCR was performed using SYBR®-green (Applied Biosystems) using a 7500 Real Time PCR System (Applied Biosystems) with cycling conditions of 95°C for 10 min and 40 cycles of 95°C for 15 s and 55°C for 1 min. Each experimental point was performed with samples from two mice and three replicates per experimental point, and differences were assessed with a 2-tailed Student t test. Results were normalized using the housekeeping GAPDH and the ΔΔ cycle threshold method and are expressed as the relative change (-fold) of the stimulated group over the control group, which was used as a calibrator. qRT-PCR results were analyzed with 7500 v2.0.6 software (Applied Biosystems).

**Immunostaining**

Polarized M1 and M2 cells were co-cultured in a 1:1 ratio for 48 hours in a 60mm petri dish. Cells were fixed with 4% formaldehyde in phosphate-buffered saline for 10 minutes, washed 3 times with 0,1% triton, incubated with primary antibodies: anti TyrRS (1:100, Abnova, Catalog #H00008565-M02, [26]), anti SPARC (1:200, Cell Signaling, Catalog #5420, [27]), or anti caspase 3 (1:100, Cell Signaling, Catalog #9661, [28]), and secondary antibodies Alexa 647
(Life Technologies) and mounted in Vectashield mounting medium with DAPI. Preparations were imaged by confocal microscopy with Leica SP5 microscope. Fluorescence quantification and cell counting was performed with Imaris 6.3.1 (Bitplane).

**Apoptosis and cell viability assays**

Macrophage apoptosis was measured by In situ Cell Death detection kit in M1 and M2 macrophages (TUNEL staining, Roche) and Phosphatidylserine (PS) externalization in M1 macrophages; briefly, M1 were harvested by trypsinization and washed twice with PBS. Washed cells were resuspended in 200 μl binding buffer (PBS containing 1 mM calcium chloride). FITC-conjugated annexin V (0.5 μg ml\(^{-1}\) final concentration) and propidium iodide (PI; 1 μg ml\(^{-1}\) final concentration) were added according to the manufacturer’s instructions (Biosea, China). After incubation for 20 minutes at room temperature, 400 μl binding buffer was added, and samples were immediately analysed on a FACS Calibur flow cytometer (Becton Dickinson, USA) with excitation using a 488 nm argon ion laser. PI was added to samples to distinguish necrotic and late apoptotic events (annexin V-, PI+; annexin V+, PI+) from early apoptotic events (annexin V+, PI-).

**Quantification of M1/M2**

CellTracker Red CMTPx (Molecular Probes\textsuperscript{TM}) and Green CMFDA (Molecular Probes\textsuperscript{TM}) were used to mark M1 and M2, respectively. Quantification was done using the Imaris (Bitplane) software. All the cells from a 6 well plate were counted, each cell was identified as a colour-coded dot. The total number of cells was determined counting the red (M1) or green (M2), then the number of red signals within the green cells was measured to establish engulfment events. The ratio M1/M2 was represented as the average of three independent experiments.

**Live videos**

Cultured M1 or M2 macrophages were mechanically detached from culture plates with scrapers and collected in 15 ml falcons separately. They were then centrifuged at 1200 rpm during 5 minutes, the supernatant was discarded and the pellet resuspended in 1 ml of RPMI medium without serum. M1 macrophages were labeled with 1 ul of CellTracker Red CMTPx (Molecular Probes\textsuperscript{TM}) and M2 macrophages with 1ul of CellTracker Green CMFDA (Molecular Probes\textsuperscript{TM}). After the 30-minute incubation, 10 ml of complete RPMI medium (with 10% FBS)
were added. The cells were centrifuged again at 1200 rpm during 5 minutes and washed with PBS. After counting on a Neubauer chamber the same number of M1 and M2 macrophages were mixed, plated on 8-well plates (Ibidi) and incubated with control medium (DMEM or CCM) or B16F10-derived tumoral medium. Cells were imaged on a time-lapse microscope every 10 minutes during 24 hours.

**Statistical analysis**
Statistical significance was calculated using an ANOVA Bonferroni’s Multiple Comparison Test *(p<0.05) ** (p<0.01) ****(p<0.001), or a t-test, with significant differences between compared groups noted by *P<0.05, when appropriate.

Mean fluorescent intensity was analyzed with ImageJ using CTCF index, which takes into account the Integrated Density – (Area of selected cell X Mean fluorescence of background readings). This index corrects for intensity, background and cell size as it is shown elsewhere [29,30].

**Acknowledgements**
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**Author’s contribution**
FN L and SCT conceived the study, did the experiments, analyze the data and wrote the report. CRL prepared the bone marrow-derived macrophages.

**Competing financial interests**
The authors declare no competing financial interests.
**Abbreviations list**

CAMs, classically-activated macrophages; AAMs, alternatively-activated macrophages; SPARC, Secreted Protein, Acidic, Cysteine-Rich; TyrRS, Tyrosyl-tRNA synthetase.
Bibliography


Figure 1. M1 and M2 macrophages characterization in tumoral-conditioned medium

A and B) qRT-PCR relative expression levels of TyrRS and SPARC in M1 and M2 macrophages cultured in control medium (white bars) or B16F10-derived tumoral medium (black bars) for 24 hours. The analysis was performed twice, with three replicates each time. Average values represent mean±s.e.m. Statistical significance was calculated using a t-test, with significant differences between compared groups noted by *P<0.05.
C-N) Immunostaining against TyrRS or SPARC (both shown in magenta) of M1 (red) and M2 (green) macrophages cultured in control medium (C, D, I and J) or in two different types of tumor-conditioned medium: B16F10-derived tumoral medium (E, F, K and L) or A549-derived tumoral medium (G, H, M and N) for 24 hours. DAPI is shown in blue.

O) Quantification of SPARC and TyrRS mean fluorescent intensity staining under different cultured conditions. 10 to 30 individual cells were analyzed in each case. Statistical significance was calculated using a One-way ANOVA Bonferroni’s Multiple Comparison Test, with significant differences between compared groups noted by *(p<0,05) ** (p<0,01).

P) Relative expression levels of c-Myc in M1 and M2 macrophages cultured in control medium (white bars) or B16F10-derived tumoral medium (black bars) for 24 hours.
Figure 2. M1 macrophages undergo apoptosis when cultured in tumoral medium as compared to M2

A) Percentage of TUNEL positive M1 or M2 macrophages cultured in control medium (white bars) or in B16F10-derived tumoral medium (black bars) for 24 hours. Average values represent mean±s.e.m. Statistical significance was calculated using a t-test, with significant differences between compared groups noted by *P<0.05.

B-G) Immunostaining against Caspase-3 (magenta) of M1 (red) or M2 (green) macrophages cultured in control medium (B and C) or in two different types of tumor-conditioned medium: B16F10-derived tumoral medium (D and E) or A549-derived tumoral medium (F and G) for 24 hours. DAPI is shown in blue.

H) Percentage of AnnexinV+ apoptotic M1 macrophages after being cultured in M2-derived medium for the indicated time points. Average values represent mean±s.d.

I) Percentage of Caspase-3 positive M1 or M2 macrophages in control medium, B16F10-derived tumoral medium or A549-derived tumoral medium.
Figure 3. M1 are engulfed by M2

A) Quantification of the ratio M1/M2 cultured in control medium or B16F10-derived tumoral medium for 1 or 5 days. Antibodies against TyrRS and SPARC were added to B16F10-derived medium for 5 days were indicated. All measurements are relative to M1/M2 ratio cultured in control medium at day 1. Average values represent mean±s.e.m. Statistical significance was calculated using a One-way ANOVA Bonferroni’s Multiple Comparison Test *(p<0.05) ** (p<0.01) *** (p<0.001).

B-D) Representation from IMARIS (Bitplane) of M1 (red dots) and M2 (green dots) after 1 day or 5 days of culture in control or B16F10-derived medium. The representation in B corresponds to both control and tumoral medium as there were no differences after 1 day of incubation between these two conditions.
Figure 4. SPARC-depleted M1 macrophages are more efficiently engulfed by M2 macrophages under tumoral conditions

A-D) Wild type or SPARC-depleted M1 (red) and M2 (green) macrophages cultured in control medium (A and C) or B16F10-derived tumoral medium (B and D).

E) Quantification of the number of engulfed M1 macrophages over the total in control or B16F10-derived tumoral medium comparing co-culture of wild type macrophages (WT) or SPARCKO ones. Average values represent mean±s.e.m. Statistical significance was calculated...
using a One-way ANOVA Bonferroni’s Multiple Comparison Test *(p<0,05) ** (p<0,01) *** (p<0,001).
Supplementary information

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Supplementary figure 1. M1 and M2 polarization-markers

mRNA expression levels measured by qPCR of usual M1 and M2 macrophage-polarization-makers (CD80, mineralocorticoid receptor-MR, Inducible nitric oxide synthase-iNOS, Macrophage arginase 1-Arg1, Resistin-like molecule alpha or found in inflammatory zone protein-Fizz1 and Beta-N-acetylhexosaminidase-Ym1/ChIl3). Each experiment was performed in triplicates. Average values represent mean±s.d. Statistical significance was calculated using a t-test, with significant differences between compared groups noted by *P<0.05. M0-nonpolarized macrophages.
Supplementary figure 2. M1 and M2 FACS analysis after 24h of co-culture in B16F10-derived and control media

M1 (red box) and M2 (green box) macrophages were co-cultured for 24 hours in B16F10-conditioned medium or fibroblast-derived medium (Control) and then stained for AnnexinV (Early apoptosis) and PI (necrosis), and analysed by FACS.
Supplementary figure 3. M1 and M2 viability after 48h of co-culture in B16F10-derived and control media

Left panels. Caspase-3 staining (red or green) of M1 (red) and M2 (green) macrophages cultured for 48h separately in B16F10-derived medium (BCM) or fibroblast-derived medium (CCM, control).

Right graph. Percentage of apoptotic M1 and M2 macrophages cultured for 48h separately or mixed (mix) in B16F10-derived medium (BCM) or fibroblast-derived medium (CCM, control). Boxplots include medians, tails indicate 25% and 75% quartiles. Statistical significance was calculated using a One-way ANOVA Bonferroni’s Multiple Comparison Test *(p<0.05) ** (p<0.01) *** (p<0.001), n.s.: not significant.
Supplementary figure 4. M1 and M2 viability after 5 days of co-culture in B16F10-derived and control media

Left panels. Caspase-3 staining (magenta) of M1 (red) and M2 (green) macrophages co-cultured for 5 days in B16F10-derived medium (BCM) or fibroblast-derived medium (CCM, control).

Right graph. Ratio M1 or M2 macrophages over total number of cells after 5 days of co-culture in B16F10-derived medium (BCM) or fibroblast-derived medium (CCM, control). Boxplots include medians, tails indicate 25% and 75% quartiles. Statistical significance was calculated using a One-way ANOVA Bonferroni’s Multiple Comparison Test *(p<0.05) ** (p<0.01) *** (p<0.001).
Movies

Supplementary video 1. M1 vs M2 in control medium

Time-lapse video of M1 (red) and M2 (green) macrophages co-culture in control medium. Images were taken every 10 minutes during 24 hours.

Supplementary video 2. M1 vs M2 in B16F10-derived tumoral medium

Time-lapse video of M1 (red) and M2 (green) macrophages co-culture in B16F10-derived tumoral medium. Images were taken every 10 minutes during 24 hours. Arrows indicate M2 macrophages engulfing M1 macrophages.
Supplementary video 3. M1 vs M2 in control medium

Time-lapse video of M1 (red) and M2 (green) macrophages co-culture in fibroblast-derived medium (control). Images were taken every 20 minutes.

Supplementary video 4. M1 vs M2 in tumoral medium

Time-lapse video of M1 (red) and M2 (green) macrophages co-culture in B16F10-derived tumoral medium. Some M2 macrophages carry M1-leftovers inside. Images were taken every 20 minutes.
Supplementary table 1 (related to figure 3). M1 are engulfed by M2

Quantification of the ratio M1/M2 cultured in control medium or B16F10-derived tumoral medium for 1 or 5 days. Statistical significance was calculated using a One-way ANOVA Bonferroni’s Multiple Comparison Test *(p<0.05) ** (p<0.01) *** (p<0.001).

Supp. Table 1

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<tr>
<td>Day5 N vs Day 5 T anti-Sparc</td>
<td>73,25</td>
<td>20,42</td>
<td>Yes</td>
<td>***</td>
<td>61.46 to 85.04</td>
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<tr>
<td>Day 5 T vs Day 5 T anti-TyrRS</td>
<td>-52,00</td>
<td>14,50</td>
<td>Yes</td>
<td>***</td>
<td>-63.79 to -40.21</td>
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<tr>
<td>Day 5 T vs Day 5 T anti-Sparc</td>
<td>16,75</td>
<td>4,670</td>
<td>Yes</td>
<td>**</td>
<td>4.963 to 28.54</td>
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<tr>
<td>Day 5 T anti-TyrRS vs Day 5 T anti-Sparc</td>
<td>68,75</td>
<td>19,17</td>
<td>Yes</td>
<td>***</td>
<td>56.96 to 80.54</td>
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</tbody>
</table>
Supplementary table 2 (related to figure 4). SPARC-depleted M1 macrophages are more efficiently engulfed by M2 macrophages under tumoral conditions.

Quantification of the number of engulfed CAMs macrophages over the total in control or B16F10-derived tumoral medium comparing co-culture of wild type macrophages (WT) or SPARCKO ones. Statistical significance was calculated using a One-way ANOVA Bonferroni’s Multiple Comparison Test *(p<0,05) ** (p<0,01) *** (p<0,001).

Supp. Table 2

<table>
<thead>
<tr>
<th></th>
<th>Engulfed/Total</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Tumoral</td>
<td>Normal SPA</td>
<td>Tumoral SPA</td>
</tr>
<tr>
<td></td>
<td>0.16190476</td>
<td>0.2006079</td>
<td>0.28571429</td>
<td>0.28301887</td>
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<td>0.125</td>
<td>0.18575851</td>
<td>0.27272727</td>
<td>0.34736842</td>
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<td>0.06521739</td>
<td>0.18832891</td>
<td>0.20879121</td>
<td>0.4</td>
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<td>0.14285714</td>
<td>0.16796875</td>
<td>0.27142857</td>
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<td>0.096465</td>
<td>0.210876</td>
<td>0.11764706</td>
<td>0.31034483</td>
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<td>0.107213</td>
<td>0.205483</td>
<td>0.16363636</td>
<td>0.32352941</td>
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</tbody>
</table>

Table Analyzed

One-way analysis of variance

<p>| | | | |</p>
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<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>P value</td>
<td>P&lt;0.0001</td>
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<tr>
<td>P value summary</td>
<td>***</td>
<td></td>
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<tr>
<td>Are means signif. different? (P &lt; 0.05)</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Number of groups</td>
<td>4</td>
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<tr>
<td>F</td>
<td>24.11</td>
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<tr>
<td>R squared</td>
<td>0.7834</td>
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</tbody>
</table>

Bartlett’s test for equal variances

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<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Bartlett’s statistic (corrected)</td>
<td>11.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.0080</td>
<td></td>
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<tr>
<td>P value summary</td>
<td>**</td>
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<tr>
<td>Do the variances differ signif. (P &lt; 0.05)</td>
<td>Yes</td>
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</tbody>
</table>

ANOVA Table

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>df</th>
<th>MS</th>
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<tbody>
<tr>
<td>Treatment (between columns)</td>
<td>0.1384</td>
<td>3</td>
<td>0.04613</td>
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<tr>
<td>Residual (within columns)</td>
<td>0.03828</td>
<td>20</td>
<td>0.001914</td>
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<tr>
<td>Total</td>
<td>0.1767</td>
<td>23</td>
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Bonferroni’s Multiple Comparison Test

<table>
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<tr>
<th></th>
<th>Mean Diff.</th>
<th>t</th>
<th>Significant?</th>
<th>FSummary</th>
<th>95% Cl of diff</th>
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<tbody>
<tr>
<td>Normal vs Tumoral</td>
<td>-0.08006</td>
<td>3.170</td>
<td>Yes</td>
<td>*</td>
<td>-0.1540 to -0.006129</td>
</tr>
<tr>
<td>Normal vs Normal SPARCKO</td>
<td>-0.1035</td>
<td>4.100</td>
<td>Yes</td>
<td>**</td>
<td>-0.1775 to -0.02962</td>
</tr>
<tr>
<td>Normal vs Tumoral SPARCKO</td>
<td>-0.2125</td>
<td>8.414</td>
<td>Yes</td>
<td>***</td>
<td>-0.2865 to -0.1386</td>
</tr>
<tr>
<td>Tumoral vs Normal SPARCKO</td>
<td>-0.02349</td>
<td>0.929</td>
<td>No</td>
<td>ns</td>
<td>-0.09742 to 0.05044</td>
</tr>
<tr>
<td>Tumoral vs Tumoral SPARCKO</td>
<td>-0.1325</td>
<td>5.244</td>
<td>Yes</td>
<td>***</td>
<td>-0.2064 to -0.05853</td>
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<tr>
<td>Normal SPARCKO vs Tumoral SPARCKO</td>
<td>-0.1090</td>
<td>4.314</td>
<td>Yes</td>
<td>**</td>
<td>-0.1829 to -0.03504</td>
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</tbody>
</table>