Microenvironmental M1 tumor-associated macrophage polarization influences cancer-related anemia in advanced ovarian cancer: key role of interleukin-6

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Online Supplementary Material for

Microenvironmental M1 tumor-associated macrophage polarization influences cancer-related anemia in advanced ovarian cancer: key role of Interleukin-6.

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Supplementary Patients and Methods

The study was approved by Local Institutional Independent Ethic Committee and was carried out in accordance with Declaration of Helsinki principles. All enrolled patients provided written informed consent for the participation in the study and for the use of their biological samples for the laboratory analyses.

TAMs separation

Immediately after collection, centrifugation of ascites was carried out at 300x *g* for 10 min at room temperature to obtain a cell pellet. Supernatants were then frozen at -80°C until assay. Tumor-associated macrophages (TAMs) were isolated on a Ficoll-Hypaque double density gradient into two distinct layers (Ficoll-Hypaque 100% and 75%) by centrifugation at 300 x *g* for 30 min at room temperature. TAMs formed a clear band on the top of Ficoll-Hypaque 75%. TAMs were collected, washed twice in Hanks solution, and then counted. Their viability was tested by Trypan blue dye exclusion test. TAMs were then kept in complete medium, consisting of RPMI 1640 medium (GIBCO, Carlsbad, CA, USA) with 20% Fetal Calf Serum (Boehringer-Mannheim, Ingelheim, Germany) and 10 mg/mL gentamicin (GIBCO).

Assessment of TAMs polarization and functional status

Immediately after separation, TAMs phenotype was evaluated by CD14 mAb.[17] The assessment of TAM by CD14 staining showed that CD14+ cells were the most represented population in the specific window corresponding to monocyte cells (86.8%), with a contamination by non-lympho-monocyte cells less than 20% and more than 90% of viable cells. Tumor associated macrophages were also gated on a forward scatter versus 90-degrees light scatter or side scatter plot. Data were acquired and analyzed by the flow cytometer (FACScan, Beckton Dickinson, Franklin Lakes, NJ, USA) using the CellQuest software (BD Biosciences, San Jose, CA, USA). To determine the M1

and M2 polarization phenotype, cells were stained with CD14-FITC labeled, plus an anti-human-CD80-PE-labeled (BD Biosciences) or anti-human-CD163-PE-labeled (BD Biosciences), plus an anti-human-Glut-1 receptor (Abcam, Cambridge, MA, USA) or an anti-human HLA-DR (BD Biosciences, San Jose, CA, USA), or an anti-human CD206 (BD Biosciences, San Jose, CA, USA), or an intracellular anti-human Arginase-1 (BD Biosciences, San Jose, CA, USA) APC labeled monoclonal antibody (mAb). Cell suspension of TAMs (100 μL) at a concentration of $1x10^6$ cells/mL were incubated with 5-10 μL of each mAb for 15 min at 4°C, washed twice with PBS, and fixed with 1% paraformaldehyde. For intracellular staining cells were washed, then subjected to surface antigene staining and fixed as described above: then, fixed cells were washed, permeabilized with a permeabilizing solution (BD, Biosciences) according to manufacter instructions and then stained with intracellular mAb in Permeabilization Buffer (BD, Biosciences). Moreover, to confirm the functional activation and the metabolic profile typical of M1 polarized TAM, glucose uptake in CD14+/CD80+ and in CD14+/CD163+ was evaluated by using 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG), a fluorescently-labeled deoxyglucose analogue (Glucose Uptake Cell-Based Assay Kit, Cayman Chemical, Ann Arbor, MI, USA): TAMs a density of at least $5x10^5$ cells/mL were treated in 100 μL glucose-free medium containing 150 μg/mL of 2-NBDG. Cells were incubated for 10 min, then harvested in a plastic tube and centrifuged for 5 min at 400xg at room temperature. The supernatant was aspirated and 1 mL of Assay Buffer was added to each tube. This step was repeated twice and cells were analyzed immediately by flow cytometry. Cells taking up 2-NBDG display fluorescence with excitation and emission at 485 nm and 535 nm, respectively. To assess the functional status TAMs at the time of collection, the cell cycle analysis was performed: cells ($1x10^6$/mL) were washed twice in RPMI 1640, fixed in ice-cold 70% ethanol, and stored at 4°C. After washing, cells were suspended in PBS and 2 mL of propidium iodide (50 μg/mL; Sigma, St. Louis, MO, USA) plus 25 μL of RNAse (1 mg/mL; Sigma) and incubated for 20 min at room temperature. Cells were acquired using a

FACScan cytometer and analyzed by Modfit software (BD Biosciences). Results were calculated as percentage of cells and mean fluorescence intensity (MFI).

Furthermore, the synthesis of IL-6 and hepcidin by cultured TAMs has been assessed and compared to that of CD14+ macrophages isolated from peripheral blood and cultured with complete medium with or without the addition of PHA as TAMs. TAMs (>85% CD14 +cells) were cultured at $1x10^6$ cells/mL in complete medium at 37°C in a humidified atmosphere with 5% CO2 without or with addition of phytohemoagglutinin (PHA) (5 µg/mL). After 72 h of culture with PHA, the cell cycle analysis was re-assessed as described above. Moreover, after 24 hours non-stimulated and PHA-stimulated cultured TAMs were gently detached from the well and the cell suspension was collected and centrifuged at 1500xg for 10 min to spin down the cells, supernatants were frozen at -80°C until assayed. Levels of IL-6 and hepcidin in culture supernatants were assessed using commercially available enzyme-linked immunosorbent assays (ELISA) kits (DRG, Marburg, Germany). The ability of cultured TAMs to release hepcidin and IL-6 was compared to that of CD14+ macrophages isolated from peripheral blood and cultured with complete medium with or without the addition of PHA as described above for TAMs.

Isolation, phenotype analysis and hepcidin synthesis of CD14+macrophages from peripheral blood of ovarian cancer patients at baseline and after culture in presence of ascites +/- anti-IL6 mAb.

Heparinized peripheral blood samples from 20 patients with primary ovarian cancer were collected at enrolment after overnight fasting. Peripheral blood macrophages were isolated by centrifugation on a discontinuous density gradient (Ficoll-Hypaque 75%) and assessed by the evaluation of CD14 mAb staining. The purity of CD14+ cells was >90%. To determine the M1 and M2 polarization phenotype the same monoclonal antibodies described above for TAMs have been used. To test whether ascites was able to modulate macrophage polarization, as well as the macrophages ability to release hepcidin, CD14+ macrophages isolated from peripheral blood of each patient were

cultured at 1×10^6 cells/mL in separate wells for 24 and 72 hours with medium (RPMI 1640 medium with 10 mg/mL gentamicin) containing 20% of fetal calf serum without (control well) and with medium containing 20% of cell-free ascites obtained from the same patient and incubated at 37°C in a humidified atmosphere with 5% CO_2. After 24 hours, cell cultures supernatants were collected to assess the levels of hepcidin by ELISA assay (DRG): results are expressed as percentages of depletion of hepcidin levels, defined as follows: % = (untreated supernatants-treated supernatants)/(untreated supernatants-complete medium) x100. After 72 hours, the cells were collected and their phenotype was analyzed. Viability of cells by Trypan blue dye exclusion test was assessed at both timing points (24 and 72 hours) and we verified that more than 90% of cells were viable. To assess the potential role of ascitic IL-6, in separate wells CD14+ peripheral blood macrophages (at a concentration of 1×10^6 cells/mL) were cultured for the same time (24 and 72 hours) in medium containing ascites with the addition of the anti-IL-6R antibody, tocilizumab, or an equal dose of the isotype control for the anti-IL-6 antibody (IgG1 mAb purchased from R&D Systems (Minneapolis, MN), at increasing doses starting from a concentration of 1 μg/mL compatible to those reported to be found in plasma of arthritis patients treated with such drug (i.e., 1, 10, 100 and 1000 μg/mL).[12] The same evaluations (analysis of hepcidin synthesis after 24 hours and macrophages phenotype after 72 hours) were performed after the addition of tocilizumab, or an equal dose of the isotype control mAb.

Circulating and ascitic levels of inflammatory, reactive oxygen species, and iron metabolism parameters

At enrollment, circulating levels of inflammatory markers (i.e., Interelukin (IL)-6, C-reactive protein, CRP, fibrinogen), reactive oxygen species (ROS), and iron metabolism parameters (i.e., hepcidin, ferritin, transferrin, and free iron) were assessed. Interleukin-6, ROS, hepcidin, ferritin, transferrin, and free iron were measured also in the ascites. Hemoglobin (Hb), iron, ferritin, transferrin, CRP, and fibrinogen were analyzed by standard laboratory procedures in accordance

with internal quality control. IL-6 and hepcidin were assessed by enzyme-linked immunosorbent assays (ELISA) kits (DRG, Marburg, Germany). ROS levels were evaluated by a colorimetric method (FORT test, Callegari Spa, Parma, Italy).

Statistical analysis

Considering a probability of 90% to detect a relationship between Hb (dependent variable) and the other laboratory parameters (independent variables) at a two-sided significance level of 0.01, at least 80 patients should be enrolled. Data are reported as mean±SD. All tests have been performed on all 125 patients. Differences between means were tested by Student's t-test. To determine the correlation between laboratory parameters, we performed Pearson correlation analysis (or Spearman if necessary) for each pairwise association, using Bonferroni's correction for multiple comparisons. Significant relationships were included in a multivariate linear regression model against Hb as a dependent variable. For *ex vivo* experiment data with peripheral blood CD14+macrophages, Student's t-test for paired data was used to test differences in sample means, with a sample of 20 experiments (90% power to detect a 50% reduction of CD expression at a 5% level of statistical significance). All testing was two-sided and a $P<0.05$ was considered statistically significant. Analyses were performed by using SPSS version 17.0 (SPSS, Chicago, IL, USA).

Supplementary Table 1. Correlation of Hb levels with circulating levels of markers of inflammation, oxidative stress and iron metabolism in primary ovarian cancer patients

| Parameters	Correlation index
Inflammation	
IL-6	-0.520*
CRP	-0.663*
Fibrinogen	-0.310
Oxidative stress	
ROS	-0.510*
Iron metabolism	
Hepcidin	-0.650*
Ferritin	-0.590*
Serum iron	0.410*
Transferrin	0.158

*p<0.05 calculated with Spearman correlation test. Abbreviations: IL, Interleukin; CRP, C-reactive protein;

ROS, reactive oxygen species

a) CD14+/CD80+ Glucose uptake

b) CD14+/CD163+ Glucose uptake
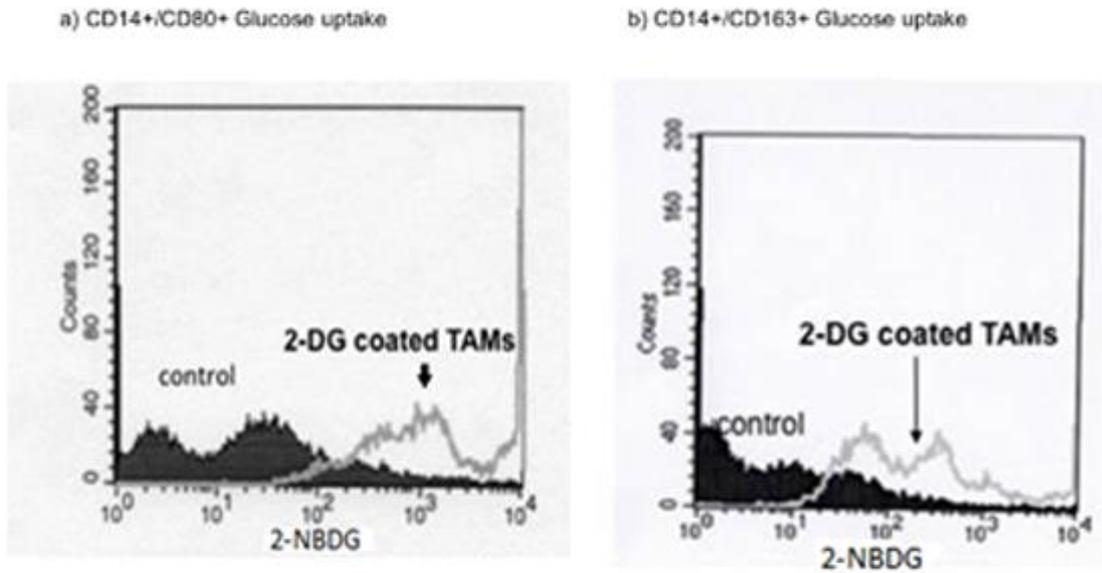
Supplementary Figure 1. Representative flow cytometry analysis of glucose uptake evaluated by using 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG), a fluorescently-labeled deoxyglucose analogue. Glucose uptake analysis in CD14+/CD80+ TAMs showed a higher mean fluorescence intensity (p<0.001) in comparison to CD14+/CD163+ TAMs isolated from ascites of patients with ovarian cancer
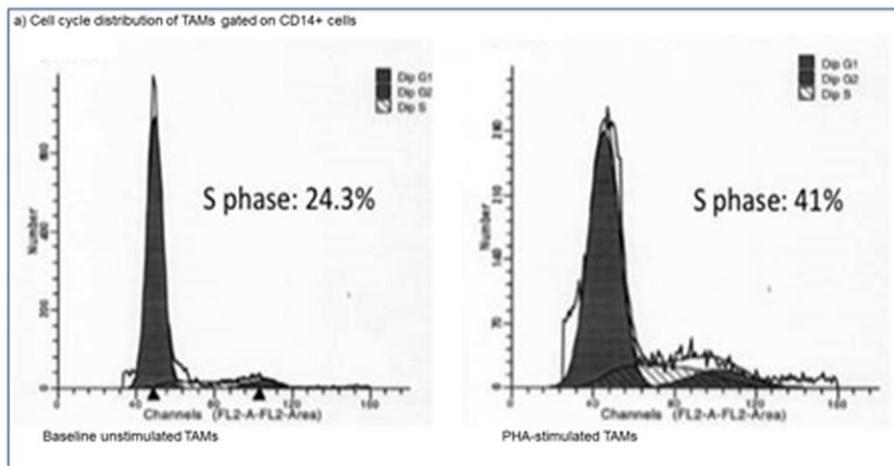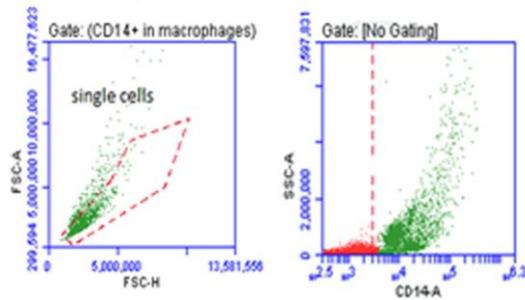
Supplementary Figure 2. Representative flow cytometry analysis of cell cycle distribution of

CD14+ TAMs. The mean percentage of proliferating (S phase) CD14+ TAMs at the time of

collection in primary ovarian cancer was 27±11. After PHA stimulation the percentage of TAMs in

the S phase of the cell cycle increased significantly (p=0.0008).
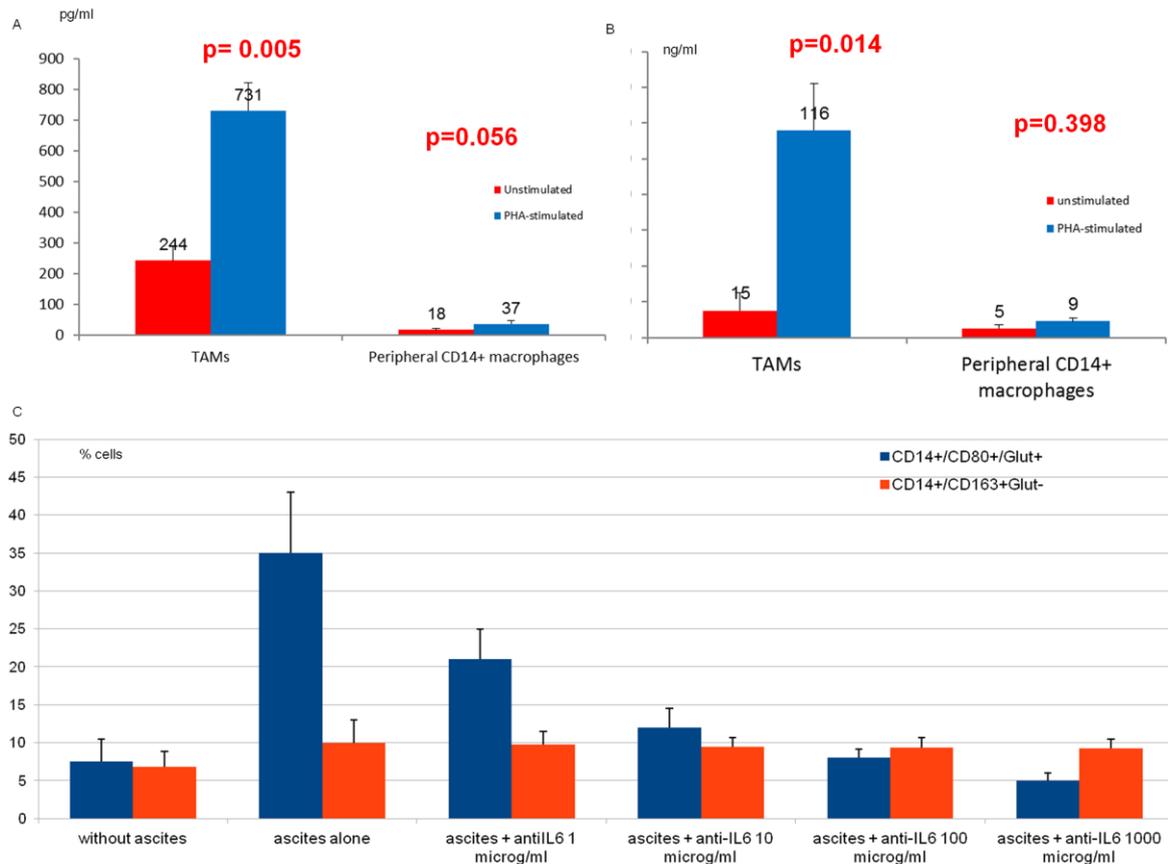
Supplementary Figure 3. Increased production of IL-6 and hepcidin by cultured TAMs and IL-6 mediated ability of malignant ascites to induce polarization of peripheral blood CD14+ macrophages. A) Mean levels of Interleukin-6 and B) hepcidin, assessed using a commercially available enzyme-linked immunosorbent assays (ELISA) kits (DRG, Marburg, Germany), in medium culture of unstimulated and PHA- stimulated TAMs isolated from ascites of ovarian cancer patients and CD14+ macrophages obtained by peripheral blood of the same patient. Cells were cultured at $1x10^6$ cells/mL in complete medium at 37°C in a humidified atmosphere with 5% CO2 without or with addition of phytohemoagglutinin (PHA) (5 µg/mL). After 24 hours non-stimulated and PHA-stimulated cultured cells were gently detached from the well and the cell suspension was

collected and centrifuged at 1500xg for 10 min to spin down the cells and supernatants were frozen at -80°C until assayed. C) Phenotype analysis of CD14+ macrophages isolated from peripheral blood of 20 patients with primary ovarian cancer at baseline and after 72 hours of culture without or with the addition of ascites +/- anti-IL-6 mAb tocilizumab at increasing doses (1-1000 mg/ml). Histograms represent the percentage of cells expressing the corresponding surface markers. All data are expressed as mean ± SD. $p < 0.05$ as calculated by Student's t-test for independent and paired data was considered significant. [a] $p < 0.05$ in comparison to baseline and unstimulated cells; [b] $p < 0.05$ in comparison to ascites-stimulated cells. Abbreviations: IL, Interleukin.