Post-Sepsis State Induces Tumor-Associated Macrophage Accumulation through CXCR4/CXCL12 and Favors Tumor Progression in Mice

José M. Mota1,2, Caio A. Leite2, Lucas E. Souza3, Paulo H. Melo2, Daniele C. Nascimento2, Virginia M. de-Deus-Wagatsuma1,4, Jessica Temporal5, Florêncio Figueiredo6, Houtan Noushmehr4, José C. Alves-Filho2, Fernando Q. Cunha2, and Eduardo M. Rego1

Abstract

Survivors from sepsis are in an immunosuppressed state that is associated with higher long-term mortality and risk of opportunistic infections. Whether these factors contribute to neoplastic proliferation, however, remains unclear. Tumor-associated macrophages (TAM) can support malignant cell proliferation, survival, and angiogenesis. We addressed the relationship between the post-sepsis state, tumor progression and TAM accumulation, and polyphenotypic and genetic profile, using a mouse model of sepsis resolution and then B16 melanoma progression. CXCR4/CXCL12 inhibition in this context. Mice that survived sepsis showed increased tumor progression both in the short and long term, and survival times were shorter. TAM accumulation, TAM local proliferation, and serum concentrations of TGFβ, CXCL12, and TNFα were increased. Naïve mice inoculated with B16 together with macrophages from post-sepsis mice also had faster tumor progression and shorter survival. Post-sepsis TAMs had less expression of MHC-II and leukocyte activation-related genes. Inhibition of CXCR4/ CXCL12 prevented the post-sepsis–induced tumor progression, TAM accumulation, and TAM in situ proliferation. Collectively, our data show that the post-sepsis state was associated with TAM accumulation through CXCR4/CXCL12, which contributed to B16 melanoma progression. Cancer Immunol Res; 4(4); 1–11. ©2016 AACR.

Introduction

Sepsis is a leading cause of mortality worldwide (1), and despite the fact that implementation of early intervention-based approaches has improved outcomes (2), patients who survive from a first sepsis episode remain at a higher relative risk of death (3, 4) and of opportunistic infections (5) for at least 5 to 8 years. Post-sepsis states have been associated with an immunosuppressive phenotype, such as increased regulatory T cells (Treg; ref. 6), macrophages, and dendritic cells (7, 8). Döcke and colleagues reported monocyte/macrophage dysfunction after sepsis, characterized by human leukocyte antigens (HLA) downregulation and low tumor necrosis factor (TNF) production (9). Accordingly, Takahashi and colleagues showed that post-sepsis induced alternatively activated macrophages (M2-polarized) with no antibacterial activity in mice (10).

One important issue is to determine if the post-sepsis immunosuppressive state increases the incidence, or affects the evolution of neoplasms, similarly to what has been observed in organ recipients under immunosuppressive treatments (11). Cavassani and colleagues reported that the post-sepsis state resulted in increased tumor expansion in a Lewis carcinoma model (12). Post-sepsis shares several mechanisms implicated in the development of the protumor microenvironments described in cancer (13). This supports the idea of a link between increased tumor progression in post-sepsis and micro-environmental changes.

The tumor inflammatory stroma, which contains tumor-associated macrophages (TAM), neutrophils, dendritic cells, lymphocytes, pericytes, and fibroblasts, provides a number of distinct and complex mediators and mechanisms that promote tumor growth, adjacent tissue invasion, and metastasis (14–16). In this context, TAMs act as major players of tumor progression, stimulating cell proliferation, survival, angiogenesis, and immune escape (17). Clinically, TAM accumulation is a predictor of mortality in several cancer types (18).
Among the mechanisms associated with TAM accumulation, CCL2 is classically implicated in early monocyte recruitment to the tumor microenvironment (19). In addition, Tymoszuk and colleagues report the contribution of in situ TAM proliferation in a mouse model of spontaneous breast cancer (20). On the other hand, the CXCR4/CXCL12 axis keeps TAMs in a tumor’s hypoxic areas, thus contributing to neoangiogenesis and oxygen delivery (21, 22).

To evaluate the relationship between the post-sepsis state, tumor progression, and TAMs, we analyzed intratumoral macrophage accumulation, and phenotype and genetic profiles using a model of B16 melanoma expansion after the resolution of cecal and ligation-induced sepsis in mice.

Materials and Methods

Cells

B16-F10-luc (B16, Caliper Lifesciences) was cultured in RPMI containing 10% heat-inactivated FCS (Gibco), 100 U/mL penicillin (Gibco), 0.1 mg/mL streptomycin (Gibco), and amphotericin B 2 μg/mL (Gibco). Prior to use, cells were detached with trypsin-EDTA 0.25% (Gibco) with 70% to 80% of confluence and washed in PBS twice. The B16-F10 cell line was obtained in 2013 from ATCC and used at the third passage. Authentication was not made.

Sepsis and post-sepsis model

Severe sepsis was induced by cecal and ligation puncture (CLP), as described elsewhere (23). To induce survival and post-sepsis state, animals were treated with etrapenem (20 mg/kg, i.p., 6 hours after surgery, and every 12 hours for 3 days), as described (6). The controls received the same regimen of etrapenem.

Assays to evaluate tumor progression

Naïve or post-sepsis mice (15 days after CLP) were subcutaneously inoculated in the right flank with B16 melanoma. Different inocula were tested (100,000; 30,000, or 10,000 cells) to determine the most suitable one for our model. Tumor volumes were calculated as described (24). Tumor burden was measured through bioluminescence quantification (IVIS Lumina; Caliper Lifesciences) after α-luciferin (150 mg/kg, i.p.). Overall survival (OS) was monitored for 50 days.

Assays to evaluate metastasis in post-sepsis mice

Mice received 30,000 B16 melanoma cells subcutaneously (s.c.) 15 days after CLP induction. Animals were euthanized after 21 days and organs were harvested and scrutinized using magnification of ×100 with an optical microscope (Zeiss) to detect spontaneous metastasis. We evaluated the rate of metastasis per organ and per mouse. Also, we counted the number of metastatic sites in the lungs. In another set of experiments, post-sepsis (15 days after CLP) naïve mice were inoculated with 30,000 cells (s.c., right flank). Fourteen days after inoculation, primary tumors were surgically removed. Animals were then followed to assess mortality due to metastasis, as described (25). Additionally, post-sepsis, or naïve mice were submitted to a lung colonization assay (30,000 cells, injected i.v. through retro-orbital plexus). Eighteen days after inoculation with B16 cells (D+18), mice were euthanized and ex vivo pulmonary tumor burden was measured through bioluminescent signal.

Tissue digestion and flow cytometry

Tumor-bearing post-sepsis or naïve mice were euthanized at D+14 and tumor was harvested. Tumors were digested using collagenase type II 1 mg/mL (Sigma) and DNase type 10.1 mg/mL (Sigma) to prepare single-cell suspensions. Immunostaining was made with antibodies to CD45 (eBiosciences), F4/80 (eBiosciences), and CD206 (Abd Serotec) for assessment of TAM accumulation through flow cytometry (FACS CANTO, BD Biosciences).CXCR4 expression in TAMs was evaluated using anti-CXCR4 (BD Biosciences). For measurement of TAM proliferation an antibody to Ki67 (BioLegend) was used. The data were analyzed using FCS Express 3.0 (De Novo software).

Immunofluorescence

TAM accumulation was also measured by immunofluorescence assay (Leica DMi6000 B, Leica microsystems) with antibodies to F4/80 (rat anti-mouse, 1:50, Caltag Laboratories), followed by anti-rat Alexa Fluor 488 (1:100, Life Technologies). Nuclei were revealed with Hoechst 33342 (1 μg/mL, Life Technologies). Total F4/80+ cells per field (magnification of ×100) were quantified using ImageJ 1.44 (National Institutes of Health, Bethesda, MD).

ELISA

Serum and tumor and inguinal lymph nodes were harvested to detect IFNγ, IL10, TNFα, TGFβ, CXCL12, and CCL2 by ELISA (Duo Set, R&D Systems kits). Results were expressed in pg/mL (serum or lymph node supernatant) or pg/protein (tumor).

TAM isolation

Single-cell suspensions from tumors were prepared as described above. Next, tumor extracts were carefully layered onto a Percoll (Sigma) gradient (70%/30%) and centrifuged (1800 RPM, 23 minutes, 4°C). The lymphomononuclear correspondent layer was isolated, washed in PBS, diluted in 1% FBS supplemented RPMI, and cultured for 40 minutes at 37°C. Three vigorous washes ensured only the adherent cells remained in the plate. Prior to use, adherent cells were removed using a cell scraper. TAMs (CD45–F4/80+) were 70% to 80% pure, as confirmed through flow cytometry.

RNA isolation from TAM

RNA was isolated using a specific kit (Quick-RNA MicroPrep, Zymo Research), following the manufacturer’s instructions. RNA concentrations and absorbance ratios (A260/A230 and A260/ A280) were determined using NanoVue spectrophotometer (GE Healthcare Life Sciences). The four RNA samples with A260/280 ratio greater than 1.9 and closest to 2.0 from the 12 samples available of each studied group were selected for further processing in a microarray analysis.
RNA preparation for microarray

RNA preparation for microarray assay was made following the manufacturer's instructions (Agilent Technologies). To isolate Cy3–labeled cRNA, we used RNeasy Mini Kit (Qiagen). Quantifications were made using NanoVue (GE Healthcare Lifesciences). The concentrations of Cy3 (pmol/μL), cRNA (ng/μL), and 260:280 ratio were determined. cRNA were hybridized in microarray slides following two random sequential lotteries (SurePrint G3 Mouse Gene Expression 8 × 60k Microarray, G4852A, Agilent Technologies). Slides 1: 252800516673, Slide 2: 252800516674, Slide 3: 252800516788. Slides were scanned (G4900DA SureScan Microarray Scanner, Agilent Technologies), and data were extracted through Agilent Feature Extraction Software (Agilent Technologies).

Microarray data analysis

The microarray data were imported and analyzed using the open source software tool, Bioconductor 3.0 (http://www.bioconductor.org). All statistical tests were done using R (version 3.1.1). We used limma package (26) to perform quality, normalization, and differential analysis. Normalization was done applying quantile normalization, to ensure the distribution of probe intensities for each sample in a set of arrays are the same. One sample replicate from the TAM-naïve group (TAM naïve, rep2) was identified as an outlier due to low RNA concentrations and poor genetic material quality and therefore was removed from subsequent downstream analysis. Principal component analysis (PCA) methods were used to identify the relationship between groups of samples. A list of differentially expressed genes was defined with a log2 fold of 0.58 or greater (for upregulated genes) and −0.58 and less (for downregulated genes) with a false-discovery rate (FDR) < 0.01. Pathway analyses were analyzed using Nextbio database (http://www.nextbio.com). Hierarchical heatmaps were made using default parameters. All data were deposited for public access in Gene Expression Ombibus (GEO). The accession code is GSE64498.

Quantitative RT-PCR

qRT-PCR was conducted for specific genes related with macrophage polarization (M1, Nos2 and Tnf; M2, Arg1 and Mrc1). Briefly, 50 ng of total RNA was transcribed to cDNA by reverse transcriptase enzyme action Improm Pre-II (Promega). qRT-PCR reaction was done on ABI Prism 7500 Sequence Detection System (Applied Biosystems), using System SYBR green fluorescence (Applied Biosystems) for quantifying the amplification. The level of each gene was normalized to the levels of the housekeeping gene Gapdh. The results were analyzed by quantifying relative expression 2−ΔΔCt. Sequences of the primers used in this study are Gapdh (forward: CATCITCTGTGCACTGCCA, reverse: CGGC-CAAATTCGTTCAC), Nos2 (forward: CCCATGCGGACACGACAGA, reverse: TGCTCAAATCTGTGGTTC), Tnf (forward: GGCCTTGATCCGGATTTTGA, reverse: AGGGATGGAAGATGC- TCCAATATG), Arg1 (forward: GTTCCACCTGTACCTGACAG, reverse: GCAAAGCCATGTACGATT), and Mrc1 (forward: GTACTCCGGAGGTTCAGA, reverse: TTTTACGGCTCAATCCAC).

Bone marrow–derived macrophage co-inoculation with B16 cells

Animals were euthanized by anesthetic overdose 15 days after sepsis and bone marrow cells were collected using a 26G syringe and two washes of 3 mL of RPMI through lower leg bones. Red blood cells were lysed and the remaining cells were cultured in 10 mL 1929 medium (RPMI supplemented with 10% FBS and 20% of 1929 supernatant). On the third day, 10 mL of 1929 medium was added to the cultures. On the seventh day, the plates were washed with PBS to remove debris and dead cells, and adherent cells were removed using a cell scraper. Then, bone marrow–derived macrophages (BMDM) were s.c. co-inoculated with B16 cells in a proportion of 1:3 (10,000 and 30,000 cells, respectively) in naïve recipients. Tumor progression was assessed by tumor volumes and bioluminescent signal after d-luciferin (150 mg/kg, i.p.) injection at D+5. Survival was evaluated daily until D+50.

M1 and M2 macrophages polarization

M1- and M2-polarized macrophages were obtained as references for the microarray assessment. BMDMs were isolated from naïve mice as aforementioned. M1-polarization was induced by supplementing the medium at the third day of culture with IFNγ (50 ng/mL) and lipopolysaccharide (LPS, 10 ng/mL). For M2-polarization, BMDMs were supplemented with IL4 (20 ng/mL), IL13 (20 ng/mL), and IL10 (20 ng/mL) at the same time point. Macrophage differentiation and polarization were confirmed by flow cytometric analysis after staining with anti-F4/80, anti-M1/IC-II (M1 marker, eBioscience), and anti-CD206 (M2 marker).

Role of CXCR4/CXCL12 in TAM accumulation

In order to inhibit CXCR4/CXCL12 signaling, we designed an experiment in which 15-day CLP animals were inoculated with B16 (30,000 cells, s.c.). Then, the animals were administered with the specific inhibitor AMD3100 (5 mg/kg, i.p., at D+10 and D+15). Tumor progression, OS, TAM accumulation, and TAM extramedullar proliferation at D+14 were measured.

Statistical analysis

All nonmicroarray data were analyzed with GraphPad Prism v.5.0 (GraphPad software). Parametric data were tested with ANOVA followed by the Bonferroni post-test or Student t test, when appropriate. Nonparametric data were analyzed using the Fisher exact test and Kaplan–Meier curves were analyzed by log-rank test. Statistical significance was set at P < 0.05.

Results

Increased tumor progression

In order to determine the number of cells needed to induce tumors, naïve and post-sepsis mice were inoculated with 10,000, 30,000, or 100,000 B16 melanoma cells 15 days after CLP (see Materials and Methods). We observed tumor development in all mice that received 30,000 or 100,000 cells. Only 40% of naïve mice developed macroscopic tumors by D+42 when inoculated with 10,000 B16 cells, in contrast with post-sepsis groups, in which 80% developed tumors (Fisher exact test: P = 0.02). Based on these results, we selected 30,000 B16 cells as the inoculum for further assays.

Post-sepsis mice showed increased tumor volumes and reduced OS (data not shown for 10,000 and 100,000 B16 cells). When the mice were inoculated with 30,000 B16 cells, significantly larger tumor volumes were detected in post-sepsis mice (Fig. 1A), which had shorter survival times than naïve tumor-bearing controls (Fig. 1B). The results were also confirmed by bioluminescence measurement (Fig. 1C and D).
Moreover, the long-lasting effects of sepsis were evaluated by s.c. inoculating mice with 30,000 B16 cells either 30 days (Supplementary Fig. S1A and S1B) or 60 days (Supplementary Fig. S1C and S1D) after CLP. In both situations, the mice in post-sepsis groups presented larger tumors and died sooner.

Higher metastatic burden

Supplementary Fig. S2 depicts the experimental protocols used to evaluate metastasis and lung colonization. An increased number of metastatic lesions in the lungs was observed at D +21 after inoculation in post-sepsis mice (Supplementary Fig. S2B). Mortality due to metastasis was evaluated by removing the primary tumors and following up the length of survival (see Materials and Methods). Mice did not present with local recurrences of melanoma lesions. Increased mortality due to metastasis in the post-sepsis group was detected (Supplementary Fig. S2C). In addition, lung colonization after 30,000 B16 cells intravenous injection was also increased in post-sepsis mice (Supplementary Fig. S2D), as evaluated by bioluminescence quantification at D +18.

Increased numbers of TAMs

First, we observed that the percentage of leukocytes (CD45+) was increased in tumor samples from sepsis-surviving animals. Post-sepsis mice had higher percentages of TAMs at D +14 (Fig. 2A) as well as increased absolute numbers of TAMs (Fig. 2B). Differences in TAM percentages were not detected at D +21 (Fig. 2C), in contrast to the absolute numbers (Fig. 2D). Representative plots are presented in Fig. 2E. Immunofluorescence staining for F4/80+ cells in frozen tumor sections further demonstrated the higher TAM accumulation in post-sepsis mice (Fig. 2F–I).

The spleen and draining lymph nodes of post-sepsis tumor-bearing mice also had increased percentages of Tregs, but no differences in intratumoral Tregs (Supplementary Fig. S3A–S3C). Differences in CD3+CD4+ and CD3+CD8+ T-cell intratumoral subpopulations were not detected between the groups (Supplementary Fig. S4A–S4D).

Increased concentrations of CXCL12, TNFα, and TGFβ

In an attempt to understand the mechanism underlying the post-sepsis increase of TAM accumulation, we screened chemokines and cytokines related to the inflammatory process and to macrophage recruitment (Fig. 3). The concentrations of CCL2 (Fig. 3B) and CXCL12 (Fig. 3C) were increased in the serum of post-sepsis mice injected with vehicle in comparison with naïve mice. The presence of the tumor by itself also increased CXCL12 (Fig. 3C). Post-sepsis tumor-bearing mice had higher TNFα (Fig. 3D), TGFβ (Fig. 3A), and CXCL12 compared with naïve mice injected with tumor cells (Fig. 3C). We also quantified the same chemo/cytokines within the tumor mass of naïve and post-sepsis mice. Only TNFα concentrations were increased in tumor masses from post-sepsis mice after 30 days after sepsis induction were also performed. TGFβ was increased in post-sepsis mice after 30 days. Differences in serum concentrations of CCL2, CXCL12, and TNFα were not detected (data not shown).

Differences in gene expression

Because we have demonstrated a quantitative early increase of TAM accumulation in post-sepsis mice, we decided to compare the global gene expression of TAMs from naïve and post-sepsis mice using Agilent microarrays with almost 60,000 probes (39,430 mRNA and 16,251 long noncoding RNAs). M1- and M2-polarized macrophages were used for comparison.
Figure 4A depicts the PCA for the aforementioned groups, using an unsupervised approach. The differences between TAMs from naïve and TAMs from post-sepsis mice were only mild, and their gene expression profiles were distinct from M1 and M2 macrophages.

We then focused our analysis on the differences between TAMs from post-sepsis and TAMs from naïve mice. We identified 61 genes to be upregulated and 98 genes to be downregulated (Table 1) using log2 fold cutoffs at |0.58| and adjusted P value (FDR) < 1% (Fig. 4B). Among the downregulated genes we detected genes associated with leukocyte activation (e.g., Cd83, which is a marker of dendritic cell maturation; Cd86, which is a marker of macrophage classic or M1 activation). Also, genes related to major histocompatibility complex type II (MHC-II; e.g., H2-Eb1 and H2-Ab1) were downregulated. Among chemokine-related genes, Ccl5 and Cxcr4 were downregulated in TAMs from post-sepsis mice.

A qRT-PCR analysis of specific genes related to macrophage polarization was carried out. Post-sepsis–derived TAMs exhibited reduced gene expression of Nos2, and a trend toward higher gene expression of Tnf, Arg1, and Mrc1 was detected (Supplementary Fig. S5A–S5D).

In order to evaluate whether macrophages derived from post-sepsis mice could contribute to tumor progression, we co-inoculated BMDMs from naïve or post-sepsis mice with B16 cells in naïve recipients (Fig. 5A). BMDMs from post-sepsis or naïve mice were primarily in an M0, as indicated by the low flow cytometric expression of CD206 and TNFa. BMDMs from post-sepsis mice led to larger tumor volumes (Fig. 5B) and shorter OS (Fig. 5C) of the recipients. All animals co-inoculated with BMDMs from post-sepsis mice died within 50 days, whereas approximately 60% of animals co-inoculated with BMDMs from naïve mice survived in the same time span. Tumor burden increase was confirmed by bioluminescence quantification at D+21 (Fig. 5D and E).
CXCR4/CXCL12 inhibition

Based on the increased CXCL12 detected in the serum of post-sepsis mice, we investigated if the pharmacologic inhibition of this pathway could reverse the effect of post-sepsis on tumor progression. AMD3100, a specific antagonist of the CXCR4/CXCL12 pathway, was administered at D+10 and D+15 after B16 cell inoculation. As shown in Fig. 6A, AMD3100 reverted the increase of post-sepsis–induced tumor volumes but resulted in nonsignificant changes in tumor volumes in the naive group. Post-sepsis and naive tumor-bearing mice that received AMD3100 survived longer than vehicle-treated controls (Fig. 6B). The percentage of TAMs expressing CXCR4 was similar between naive and post-sepsis groups (Fig. 6C). CXCR4/CXCL12 blockade through AMD3100 inhibited the TAM accumulation associated with post-sepsis state (Fig. 6D and E). Additionally, AMD3100 administration inhibited the ability of post-sepsis BMDMs to increase tumor growth (Supplementary Fig. S6).

The percentage of TAMs expressing Ki67 was increased in tumors from post-sepsis mice, and the inhibition of CXCR4/CXCL12 by AMD3100 reverted this finding. Of note, Ki67 levels were not increased in CD45− cells (Supplementary Fig. S7A–S7C).

Discussion

Here, we describe the increase of melanoma B16 progression in sepsis-surviving mice, which was associated with tumor microenvironmental TAM accumulation through CXCR4/CXCL12 signaling. In addition, the post-sepsis state was associated with an increased pulmonary metastatic burden and increased lung colonization. The effect of sepsis on B16 melanoma tumor progression proved to be long-lasting, because it was observed even when the neoplastic cells were inoculated 30 or 60 days after CLP-induced sepsis. Accordingly, Weycker and colleagues have shown that patients with sepsis present an
increased long-term mortality (3). Also, Otto and colleagues proved that sepsis survivors are at higher risk of opportunistic infections after a period between 16 and 150 days following sepsis resolution (5). If the clinical post-sepsis state is associated with higher tumor incidence, tumor progression or metastasis still remains elusive.

TAM accumulation is a relevant marker of unfavorable prognosis in cancer (18). It has been associated with increased tumor progression, neoangiogenesis, and immune escape in several tumor types (17, 27). We assessed TAMs (F4/80⁺CD206⁺) through flow cytometry and immunofluorescence and found that they accumulated heavily in post-sepsis mouse tumors at an early stage of tumor progression (D+14) when the tumor sizes were not different between the groups. In a later phase (D+21), the relative amounts of TAMs showed no difference between the groups.

Classically, TAM accumulation depends on bloodstream-derived monocyte infiltration mediated by CCL2, CCL7, VEGF, and other cytokines (27–29). However, Tymoszuk and colleagues demonstrated in situ proliferation of CD11b⁺F4/80⁺ cells through BrdUrd labeling and Ki67 staining in a model of spontaneous breast cancer (20). This suggests an important contribution of in situ TAM proliferation. Corroborating these findings, we observed that Ki67 positivity in TAM cells was increased in post-sepsis mice. This finding could represent a contribution of local macrophage proliferation for TAM accumulation in the post-sepsis state.

Figure 4.
Comparison between the gene expression profiles of TAMs from post-sepsis and naive mice. A, first two principal components are plotted elucidating the group relationship between TAMs from naive and from post-sepsis mice. B, volcano plot comparing TAM from post-sepsis with TAMs from naive mice. Fold change was set at 0.58 for upregulated and 0.58 for downregulated gene expression. Significance was set at FDR < 0.01. 98 genes were downregulated (green) and 61 were upregulated (red); mRNA, messenger RNA; lncRNA, long noncoding RNAs; UNK, unknown RNA; FDR, false discovery rate; M1, M1-polarized macrophage; M2, M2-polarized macrophage.
We confirmed the importance of post-sepsis macrophages to tumor progression by co-inoculating BMDMs together with B16 cells in naïve mice. Post-sepsis–derived BMDMs co-inoculation resulted in higher tumor progression and lower OS. Cho and colleagues found that the inoculation of M2-polarized macrophages provoked increased progression and metastasis in a breast cancer model, possibly due to increased angiogenesis (30).

In order to discern whether TAMs from post-sepsis mice have acquired an M2-like phenotype, the global gene expression profile

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>P</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd2</td>
<td>−2.91</td>
<td>0.0028</td>
<td>Adhesive properties; costimulatory molecule</td>
<td>Bimal et al., 2012 (31)</td>
</tr>
<tr>
<td>Ccr7</td>
<td>−2.76</td>
<td>0.0017</td>
<td>CCL19 and CCL21 receptor; related to classic macrophage activation</td>
<td>Oh et al., 2012 (32)</td>
</tr>
<tr>
<td>H2-DMb1</td>
<td>−2.14</td>
<td>0.0017</td>
<td>MHC class II–related molecule</td>
<td>Cho et al., 1991 (33)</td>
</tr>
<tr>
<td>Cd24a</td>
<td>−2.10</td>
<td>0.0067</td>
<td>Cell adhesion molecule; DC marker</td>
<td>Qu et al., 2014 (34)</td>
</tr>
<tr>
<td>Cd74</td>
<td>−1.82</td>
<td>0.0026</td>
<td>MIF receptor; related to classic macrophage activation</td>
<td>Leng et al., 2003 (35)</td>
</tr>
<tr>
<td>H2-Ed1</td>
<td>−1.82</td>
<td>0.0017</td>
<td>MHC class II–related</td>
<td>Widera and Flavell, 1984 (36)</td>
</tr>
<tr>
<td>Cd86</td>
<td>−1.80</td>
<td>0.0002</td>
<td>Costimulatory molecule; related to classic macrophage activation</td>
<td>Cavanaugh et al., 2013 (37)</td>
</tr>
<tr>
<td>Selpg</td>
<td>−1.70</td>
<td>0.0017</td>
<td>P-selectin glycoprotein ligand; cell adhesion molecule</td>
<td>Tchernychev et al., 2003 (38)</td>
</tr>
<tr>
<td>H2-Ab1</td>
<td>−1.68</td>
<td>0.0007</td>
<td>MHC class II–related</td>
<td>Lacaze et al., 2009 (39)</td>
</tr>
<tr>
<td>Cxcr4</td>
<td>−1.62</td>
<td>0.0064</td>
<td>CXCL12 receptor; TAM accumulation in hypoxic areas</td>
<td>Solinas et al., 2009 (27); Schioppa et al., 2003 (21)</td>
</tr>
<tr>
<td>Marco</td>
<td>−1.62</td>
<td>0.0067</td>
<td>Scavenger receptor; related to alternative macrophage activation</td>
<td>Tomioka et al., 2012 (40)</td>
</tr>
<tr>
<td>H2-Ob</td>
<td>−1.60</td>
<td>0.0059</td>
<td>MHC class II–related</td>
<td>Karlsson and Peterson, 1992 (41)</td>
</tr>
<tr>
<td>Il2rB</td>
<td>−1.51</td>
<td>0.0017</td>
<td>IL2 receptor β chain; IL2 is a cofactor for macrophage classic activation</td>
<td>Han et al., 1999 (42)</td>
</tr>
<tr>
<td>Ccl5</td>
<td>−1.44</td>
<td>0.0074</td>
<td>Proinflammatory chemokine; M1 macrophage marker</td>
<td>Sica and Mantovani, 2012 (43)</td>
</tr>
<tr>
<td>Cd209a</td>
<td>−1.42</td>
<td>0.0063</td>
<td>C-type lectin; mediates recognition and phagocytosis</td>
<td>Lu et al., 2013 (44)</td>
</tr>
<tr>
<td>Il6ra</td>
<td>−1.41</td>
<td>0.0072</td>
<td>IL6 receptor; modulates macrophage phenotype</td>
<td>Mauer et al., 2014 (45)</td>
</tr>
</tbody>
</table>

NOTE: Differences in gene expression comparison between TAMs from post-sepsis and TAMs from naïve mice were only mild. It was considered a fold change of 0.58 to upregulated genes and −0.58 to downregulated genes. Statistical significance was set at $P < 0.01$. Among all, 61 genes were found to be upregulated and 98 downregulated. This table shows only the selected genes that could be in such a way related to TAM functions. Genes associated with leukocyte activation, macrophage polarization, and MHC II are shown here.

Abbreviations: DC, dendritic cell; MIF, macrophage migration inhibitory factor.

Figure 5.

Bone marrow (BM)–derived macrophages (Mφs) from post-sepsis mice increased tumor progression. A, experimental protocol. B, recipients of post-sepsis Mφs presented increased tumor progression in comparison with controls. $n = 10$ (naïve Mφs) and $n = 9$ (post-sepsis Mφs). C, mice injected with post-sepsis Mφs + B16 cells presented increased mortality. $n = 10$ (naïve Mφs) and $n = 9$ (post-sepsis Mφs). D, increased tumor burden in the post-sepsis Mφs group was confirmed at D+21 through bioluminescent signal quantification after D-luciferin (150 mg/kg, i.p.) administration. $n = 7$ (naïve Mφs) and $n = 5$ (post-sepsis Mφs). E, representative mice from each group. Graphs represent two independent experiments. Horizontal red lines, mean; individual data, scattered dot plots. $^* P < 0.05$. 

Published OnlineFirst January 27, 2016; DOI: 10.1158/2326-6066.CIR-15-0170
of isolated TAMs was analyzed. The PCA showed a distinct clustering of TAMs from naïve and TAMs from post-sepsis mice. The microarray analysis revealed few similarities between TAMs and M2-macrophages in contrast with those reported in the literature (46). One could speculate that this could have occurred due to suboptimal purity of TAM isolation (70%–80%), when compared with bone marrow–derived macrophages (almost 100%).

Sixty-one genes were upregulated, and 98 genes were downregulated in post-sepsis–derived TAMs compared with naïve-derived TAMs. Genes related to the M2 phenotype, such as Il10, Arg1, and Fizz1, were not differentially expressed in the microarray analysis. However, we detected a reduced expression of Nos2, a classic M1 marker, in TAMs from post-sepsis mice in additional qRT-PCR assays. Of note, Marco, a gene upregulated in IL10-induced M2 macrophages (47), showed higher expression in TAMs from post-sepsis mice. In spite of these findings, it is unclear whether Marco is an M2 marker or a marker of innate macrophage activation (48). On the other hand, reduced expression of leukocyte activation and MHC-II–related genes were detected in these cells. In addition, Cd86, which is a costimulatory molecule-related gene, was downregulated. The latter two molecules are more likely associated with M1 rather than M2 polarization (47).

Because TGFβ has been implicated in monocyte recruitment, monocyte-to-macrophage differentiation and acquisition of protumoral functions by TAMs (49), we assessed its serum concentrations, which were increased in post-sepsis tumor-bearing mice. In addition, CXCL12 was increased in the serum of these mice. Along this line, Wang and colleagues reported that TGFβ increases the response of CXCL12 chemotactic signaling (50). Based on these results, we tested whether CXCR4/CXCL12 inhibition through AMD3100 administration would affect tumor progression and TAM accumulation. When signaling through CXCR4 was blocked in vivo, this reversed the effect of the post-sepsis state on tumor size and also improved OS.

**Figure 6.**
CXCR4/CXCL12 blockade reverted the post-sepsis effect on tumor progression and TAM accumulation. A, the tumor volumes of post-sepsis treated with AMD3100 were not different from untreated naïve mice. n = 6 (naïve plus vehicle), n = 10 (naïve plus AMD3100), n = 5 (post-sepsis plus vehicle), and n = 12 (post-sepsis plus AMD3100). The arrows indicate the days in which AMD3100 was administered. B, OS was improved after CXCR4/CXCL12 blockade. Both naïve and post-sepsis mice that received AMD3100 showed survival improvement compared with their untreated counterparts. n = 5 (post-sepsis without tumor), n = 6 (naïve plus vehicle), n = 11 (naïve plus AMD3100), n = 7 (post-sepsis plus vehicle), and n = 13 (post-sepsis plus AMD3100). C, TAMs (CD45+ F4/80+) from naïve and post-sepsis mice expressed the same levels of CXCR4. n = 5 (naïve) and n = 6 (post-sepsis). D, AMD3100 reverted the TAM accumulation in tumors of post-sepsis mice at D+14. n = 5 (naïve plus vehicle), n = 5 (naïve plus AMD3100), n = 5 (post-sepsis plus vehicle), and n = 5 (post-sepsis plus AMD3100). E, representative dot plots of the post-sepsis plus vehicle group and the post-sepsis plus AMD3100 group. All graphs represent two independent experiments. Horizontal red lines, mean; individual data, scattered dot plots. *P < 0.05; ***, P < 0.001.
These findings were also associated with the reduction of TAM accumulation. CXCR4/CXCL12 is a known pathway implicated in TAM accretion in hypoxic areas of tumor microenvironments (22). Our results are in agreement with Beider and colleagues, who described that multiple myeloma cells recruit monocytes to their microenvironmental niche, differentiate them to macrophages that then polarize into M2-like phenotype through CXCR4/CXCL12 (51). The impediment of TAM accumulation was, at least in part, dependent on the reduction of extra-medullary proliferation, because we observed reduced numbers of Ki67– F4/80+ cells.

Other cells may take part in the post-sepsis–associated tumor progression. In this regard, Cavassani and colleagues had previously assessed post-sepsis neoplastic expansion and demonstrated the Treg-dependent increase in tumor burden using a heterotopic Lewis lung carcinoma model (12). Zhou and colleagues have demonstrated the frequent association of Tregs and TAMs within the tumor microenvironment and reported that Treg/TAM colocalization is associated with worse outcome in patients with hepatocellular cancer (52). This could be relevant in the context of our findings, because Tregs have a role in the post-sepsis immunosuppressive state (6). However, we could not detect significant differences in Treg numbers in the tumor microenvironment of post-sepsis mice.

In summary, sepsis has a long-lasting effect, which may favor neoplastic expansion. In our model, TAMs accumulated in the tumor microenvironment of post-sepsis mice in an early phase, which is, at least in part, dependent on CXCR4/CXCL12 signaling. Overall, our data indicate at least three potential mechanisms of post-sepsis–induced tumor progression in mice: a possible direct effect of CXCL12 in tumor progression, increased TAM accumulation and proliferation (perhaps in response to increased CXCL12 in serum), and intrinsically altered bone marrow–derived macrophages (and thus potentially TAMs). The evaluation of such phenomena in the clinical setting should be a matter of future concern. Specifically, it is important to determine if sepsis-surviving patients have a higher risk of cancer development, progression, or mortality, and if CXCR4/CXCL12 blockade could reverse this effect.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Mota, C.A. Leite, P.H. Melo, F. Figueiredo, F.Q. Cunha, E.M. Rego

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Mota, C.A. Leite, P.H. Melo, V.M. de-Deus-Wagatsuma, J. Temporal, F. Figueiredo, H. Noushmehr, F.Q. Cunha

Writing, review, and/or revision of the manuscript: J.M. Mota, P.H. Melo, V.M. de-Deus-Wagatsuma, H. Noushmehr, F.Q. Cunha, E.M. Rego

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Mota, V.M. de-Deus-Wagatsuma, J.C. Alves-Filho, F.Q. Cunha

Study supervision: J.M. Mota, J.C. Alves-Filho, F.Q. Cunha, E.M. Rego

Acknowledgments

The authors gratefully acknowledge Giuliana Bertozzi, Priscila Scheucher and Amélia Goes de Araújo for their technical assistance. They dedicate this work to the memory of Professor Ronaldo de Albuquerque Ribeiro, MD, PhD.

Grant Support

E.M. Rego has been awarded a grant from São Paulo Research Foundation (FAPESP) under grant agreements 2013/14228-3 and 2013/08135-2 and University of São Paulo–Nucleo Apoio Pesquisa (NAP).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 12, 2015; revised December 10, 2015; accepted December 11, 2015; published OnlineFirst January 27, 2016.

References


Role of TAMs in Post-Sepsis Tumor Progression


Cancer Immunology Research

Post-Sepsis State Induces Tumor-Associated Macrophage Accumulation through CXCR4/CXCL12 and Favors Tumor Progression in Mice

José M. Mota, Caio A. Leite, Lucas E. Souza, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-15-0170

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2016/01/27/2326-6066.CIR-15-0170.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerimmunolres.aacrjournals.org/content/early/2016/02/29/2326-6066.CIR-15-0170. Click on “Request Permissions” which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.