**SUPPLEMENTARY MATERIALS AND METHODS**

***Cell culture and Reagents***

MC38 and Luciferase-expressing MC38 cells (MC38/Luc) were obtained from Dr. Michael Lotze (University of Pittsburgh, Pittsburgh, PA). Hepa1-6 cell lines were purchased from ATCC (Manassas, VA). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100μg/ml streptomycin, 15mmol/L HEPES, 200mmol/L L-Glutamine, and incubated at 37oC in a humidified incubator containing 5% CO2. In some experiments, cancer cells were rendered either necrotic by incubation at 60°C for 60 minutes or hypoxic by placing in hypoxia chamber with 1% O2 for 12 hours. Supernatants from necrotic or hypoxic cancer cells were harvested and ere used as conditions media to stimulate neutrophils in subsequent assays. The reagents and antibodies used for Western Blots were as follows: anti-p38/p-p38, anti-p65/p-p65, anti-Stat3/p-Stat3 (Cell Signaling); anti-actin (Sigma); anti HIF-1-alpha (Abcam). TLR9 shRNA plasmids (Sigma-Aldrich) and TLR9 agonist (ODN 1668, 1μM) were purchased from InvivoGen. HMGB1 neutralizing antibody used at 10 μg/ml was gifted by Dr. Billiar (University of Pittsburgh, Pittsburgh, PA).

***Immunoblotting***

 Western blot assays were performed using whole cell lysates from either liver tissue or cell lines. Membranes were incubated overnight using the above mentioned antibodies.

***Cell Proliferation Assays***

Cell proliferation was assessed using an MTT assay. Cells (1 × 103 cells/well) were seeded on 96-well plates. Regular culture media, PMA-stimulated neutrophil media, PMA-stimulated neutrophil media + DNAse (250U/well), or HMGB1 neutralizing antibody were added to each well. At a series of time points, 10μl of MTT was added to each well and the cells were incubated at 37°C for 4 h. Then, 100μl dimethylsulfoxide was added to each well and mix thoroughly to dissolve the dark blue crystals overnight. The optical density (OD) was measured at 550 nm using a microplate reader (Bio-Rad Model 690, Richmond, CA, USA).

***Adhesion, migration and invasion assays***

For the adhesion studies, 5x105 neutrophils were plated in a 24-well tissue and allowed to adhere for 1 hour at 37oC 5% CO2. MC38 cells were stained with CFSE (Molecular Probes) for ten minutes and then 1x105 MC38 cells were added to the wells containing neutrophils with 100nM PMA with or without 1000U DNAse. MC38 cells added to the wells containing neutrophils without PMA served as control. Following incubation for 4 hours at 37oC 5% CO2, wells were washed with PBS and fives with 4% PFA. Using Olympus Floview 500 microscope, adhesion was quantified as the number of CFSE-labeled MC38 cells in 4 random hpf at x20.

Cell migration and invasion was studied using 6.5-mm Costar Transwell chambers with 8-μm pores (Corning). Invasion assays differed in that ECM gel (Sigma) was placed in the upper chamber. Cancer cells were trypsinized and resuspended in 0.1% serum containing DMEM media with 0.5% bovine serum albumin. A total of 2.5x105 cells were plated in the upper chambers in duplicate filters. DMEM medium with 10% serum was used as a chemoattractant in the lower chamber. Tumor cell suspension in the upper chamber were treated with neutrophil media collected from PMA-stimulated neutrophils, PMA-stimulated neutrophil media and 1000U of DNAse, PMA-stimulated neutrophil media and 10 μg/ml HMGB-1 neutralizing antibodies, or culture media alone. For PAD4 inhibition with YW4-03, neutrophils were preincubated with 8µm YW4-03 prior to PMA stimulation. After 24 h incubation at 37oC 5% CO2, the contents of the upper chambers were aspirated and washed with PBS and the non-migrating cells were removed by a cotton swab. Transwell membranes were then stained with crystal violet. Cells that migrated through the membrane to the lower surface were quantified in 4 random hpf by light microscopy and representative images were taken.

***Tumor Burden Analysis***

Mice were sacrificed at desired timepoints and the livers were harvested in toto for analysis. Liver and mice weights and gross metastatic surface nodules were assessed. Intrahepatic tumor burden was assessed by calculating the percentage of hepatic tissue replaced by tumor i.e. the hepatic replacement area (HRA) ([1](#_ENREF_1)). On H&E stained histological sections at 40x magnification, the tumor occupied area was quantitatively assessed by Image J (NIH). Results were presented as the mean of the percentage of tumor occupying area (mm2) with respect to the entire area of one capture (mm2).

***ELISA***

HMGB1 levels were measured in the media from stimulated neutrophils using an ELISA kit (IBL International).

***Establishment of TLR9 Stable Knockdown Cells***

 One day before transfection, 2×105 cells were seeded onto 6-well plates. TLR9 shRNA was transfected into cells with Lipofectin 2000. After 24 h, transfected cells were spread onto 100-mm culture dish at 1:100 dilution. To select for stable transfectants, cells were cultured in DMEM with 10 µg/ml puromycin (Sigma-Aldrich) for 4 weeks. Clones with puromycin resistance were selected and expanded ([2](#_ENREF_2)).

***Quantification of NETs***

To quantify NETs in cell culture supernatant and in mouse and human sera, a capture ELISA myeloperoxidase (MPO) associated with DNA was performed as described previously ([3](#_ENREF_3)). Briefly, to show that circulating nucleosomes in sera are derived from NETs, we tested myeloperoxidase, a prominent granular component of neutrophils, attached to nucleosomes. MPO-DNA complexes were identified using a capture ELISA. For the capture antibody, Mouse MPO ELISA kit (Hycult biotech, HK210-01) was used according to the manufacturer’s directions. 100μl of sample was added to the wells and incubated for 1 hour. After washing three times (300μl each), 100μl incubation buffer containing a peroxidase-labeled anti-DNA mAb (component No.2, Cell Death ELISAPLUS, Roche; Cat. No: 11774424001) was used. Values for soluble NET formation are expressed as percentage increase in absorbance above control. Serum nucleosome quantification was performed using Cell Death Kit (Roche).

***Quantitative real-time PCR***

Total RNA was extracted from the cell using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instruction. mRNA for the cytokines of interest and β-actin were quantified in duplicate by SYBR Green two-step, real-time reverse transcription polymerase chain reaction (RT-PCR). After removal of potentially contaminating DNA with DNAse I (Life Technologies), 2 μg of total RNA from each sample was used for Clontech cDNA synthesis kit (Clontech) to generate first-strand cDNA. PCR reaction mixture was prepared using SYBR Green PCR Master Mix (PE Applied Biosystems). Thermal cycling conditions were 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min on an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). Each gene expression was normalized with β-actin mRNA content.

***Confocal microscopy***

For immunofluorescence staining, liver sections were fixed, stained, and imaged using confocal microscopy as previously described ([4](#_ENREF_4)). Liver tissue or neutrophils were incubated with the specific primary antibodies for Ly6G (1:100, BD Bioscience), citrullinated-histone H3 (Cit-H3 1:50; Abcam), or histone H2A.X (1:800, Abcam). All slides were scanned under the same conditions for magnification, exposure time, lamp intensity and camera gain. Confocal images were acquired using Olympus Fluoview 1000 microscope with a PlanApo N (×40 with and without a 2.5 digital zoom). Sequential scanning was applied for acquiring individual emission channels when multiple fluorophores were involved. The thickness of the sections were imaged by focusing on the top of the section, setting the Z-axis to 0, and then refocusing to the bottom of the section, an average of 14 sections were acquired. Data images were acquired using FV10-ASW software and imported into Imaris (BITPLANE) for 3D volume reconstruction.

***Bioluminescent imaging***

Bioluminescent imaging was also used to monitor tumor burden at 7 day intervals in a nonlethal manner. Prior to imaging, mice were anesthetized using Isofluorane followed by i.p. injection of luciferin at a concentration of 300 mg/kg in 0.1ml PBS 8 min before imaging. The mice were then placed in the chamber for an IVIS 200 optical imaging system (Xenogen Corp., Hopkinton, MA, USA). Photon emission in the region of interest over the tumor sites was quantified using Living Image software v. 3.0 (Xenogen Corp.) and quantified as total photon counts.

**References**

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