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Markus Cicka Jr April 5, 2022

Adenosine Regulates the Formation of Macrophage Extracellular Traps Through the A2a Signaling Pathway

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Abstract

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By Markus Cicka Jr

Heart disease is one of the leading causes of death in developed countries across the world. During a myocardial infarction, immune cells, such as neutrophils and macrophages enter the site of ischemia and undergo various processes leading to increased inflammation in the heart and worse patient outcomes. Neutrophil extracellular traps or NETs, which are a mechanism where neutrophils eject their DNA into the extracellular space ensnaring pathogens, are currently recognized to play an important role in myocardial infarctions. More recently, macrophages have been discovered to undergo a process of DNA extrusion similar to NETosis. Here, I characterize murine bone marrow derived macrophages and their ability to produce extracellular traps (METs), stimulated by LPS treatment. I also investigate if the anti-inflammatory compound, adenosine, can inhibit the formation of these extracellular traps through the adenosine receptor signaling pathways. Determining how MET formation occurs will allow a greater understanding of the pathogenesis of aberrant inflammation and to assist in developing new therapies increase the survivability of myocardial infarctions in patients.

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Introduction

Immune Cell Recruitment in Cardiac Inflammation and Repair

Cardiovascular disease remains one of the leading causes of death in the United States[1]. One possible consequence of cardiovascular disease is the risk for myocardial infarctions. During a myocardial infarction, coronary arteries are blocked via a thrombus after atherosclerotic plaque rupture. This causes rapid myocardium cell death, which if not treated, can lead to death. The primary treatment option for myocardial infarction is thrombus removal to restore blood flow and oxygen to the myocardium[2]. The restoration of blood flow to the myocardium may also cause secondary injury through IRI (ischemia-reperfusion-injury)[3].

The immune system plays a key role in modulating both resolving and inflammatory aspects of myocardial damage[4]. Innate immune cells, such as neutrophils, blood monocytes, and macrophages are recruited to the infarcted myocardium via release of DAMPs (danger-associated molecular patterns) and ROS-production (reactive oxygen species) from the injured cardiomyocytes[5]. The neutrophils recruited to the site of injured myocardium release ROS molecules and proteases that are involved in the removal of cellular debris from the extracellular environment, playing into their role as positive mediators of myocardial repair[6]. However, these neutrophils may also cause further injury of the myocardium if ROS release is uncontrolled or the neutrophils undergo extracellular trap formation in this sterile environment, which may further increase inflammation in the heart[7,8].

Monocytes and macrophages also play a major role in cardiac remodeling, causing a second hit on the myocardium when recruited. During the myocardial infarction, CC chemokines, such as CCL2 and CCL7, are released, causing monocyte recruitment from the

blood[9]. This recruitment process is CCR2-dependent, resulting in pro-inflammatory, Ly6c^{high} macrophages to arrive at the area of infarct[10]. These macrophages express TNF-a, IL-1b, and other pro-inflammatory proteins and play a key role in phagocytosis of cellular debris from the heart[11,12]. The expression of these proteins sustain the inflammatory response in the heart. Approximately four days after the event, Ly6c^{low} macrophages predominate and become resolving mediators of inflammation due to the release of factors such as IL-10 and TGFb, which promote cardiac repair[11,13]. Therefore, there are multiple subtypes of macrophages that are responsible for inflammation and repair during a myocardial infarction[14,15]. In the context of both neutrophil and macrophages, targeting strength and duration of these cells' pro-inflammatory responses may reduce cardiac inflammation and preserve functionality of the myocardium[16].

Neutrophil Extracellular Traps: A Newly Recognized Form of Neutrophil Activation

NETs (neutrophil extracellular traps) were first described in a 2004 study by Brinkmann et. Al. They showed that neutrophils ejected their DNA into the extracellular space in response a bacterial challenge *in vitro*, but were also discovered *in vivo* experiments[17]. This process of extracellular DNA extrusion is call NETosis. The expelled DNA captures circulating bacteria, ensnaring them, and antimicrobial enzymes and peptides from the neutrophil granules assist in neutralizing the pathogens[18]. NET formation has now been connected to many chronic disease states such as rheumatoid arthritis, cystic fibrosis, cancer and recently SARS-CoV-2 infection[19-22]. Interestingly, NETosis has been discovered in the context of ischemia, such as in myocardial infarctions[23]. During myocardial infarctions, NETs have been discovered to increase expression of MCP-1/CCL2, leading to increased monocyte and fibroblast activity and recruitment. This may worsen the damage associated with the myocardial injury[24-26].

Neutrophil extracellular trap formation is characterized by the citrullination of the H3 histone[27]. The presence of H3 citrullination has been used to identify if neutrophil extracellular traps have formed[28-30]. The conversion of arginine to citrulline converts the positively charged arginine, which attracts the negatively charged DNA backbone, to the non-canonical amino acid citrulline, which is a neutral amino acid, thereby causing the DNA to decondense from the condensed state[31]. The enzyme responsible for this conversion in neutrophils is PAD4 (peptidyl arginine deiminase 4)[32]. PAD4 is part of a family of five proteins known as PADs (peptidyl arginine deiminases), which are found throughout various tissues in the body. PAD4 can localize to the nucleus and induce chromosome decondensation due to the presence of a nuclear localization sequence[33]. MPO (myeloperoxidase) is another enzyme, which generates ROS, that assists in the neutrophil extracellular trap formation as individuals with MPO-deficiency have decreased ability to produce NETs from isolated neutrophils *in vitro*[34]. NE (neutrophil elastase) also play a key role in NET formation through modulating chromatin release[35]. Since NETs induce more inflammation, timely NET clearance is necessary. DNase I has been shown to drive NET clearance in blood serum [36]. Additionally, macrophages can be involved in the clearance of NET structures from the extracellular environment through endocytosis[37,38].

Macrophages Also Produce Extracellular Traps

It has been recently discovered that macrophages can also undergo extracellular trap formation, which appear to be similar to neutrophil extracellular traps[39]. These METs (macrophage extracellular traps) have been studied using similar methods as NETs to determine their existence through use of electron scanning microscopy and extracellular DNA intercalating fluorescent dyes[40,41]. Many studies have described METs forming from a variety of bacterial infection[42-44]. However, METs are present in disease states such as AKI (acute kidney injury),

indicating that MET formation may play a role in the pathogenesis of many non-transmittable diseases[45]. MPO, elastase, and citrullinated H3, which are markers of NET formation, have been used to detect MET formation[46]. The PAD4 protein also plays a role in METosis as knockdown of PAD4 reduces extracellular DNA production[47]. Due to these similarity between cell types, it indicates that METs are morphologically similar to NETs and may be regulated in similar mechanisms. However, more studies need to be conducted to better understand the mechanism behind MET formation and under what circumstances the process occurs *in vivo* models.

The Advantages of Bone Marrow Derived Macrophages in Experimental Models

In order to study METosis, studies have used a variety of macrophages such as murine J774A.1 macrophage cell line or the human monocyte leukemia cell line (THP-1)[42,47]. These immortalized cell lines may have phenotypic variability that can affect the ability to translate their findings to wildtype cells and other models[48]. Therefore, primary cell lines, such as bone marrow derived macrophages, alveolar macrophages, and peritoneal macrophages can be used to avoid these potential issues. Peritoneal and alveolar macrophage harvesting produces a low number of cells[49]. Conversely, BMDMs (bone marrow derived macrophages) produce a high quantity of cells and can be produced from any genotype, including transgenic mice, depending on the experimental requirements[50]. The macrophages begin in an undifferentiated immature state. In order to differentiate the bone marrow cells into mature macrophages, a treatment of M-CSF (macrophage colony stimulating factor) throughout the differentiation process is required[51]. Furthermore, these bone marrow derived macrophages can be phenotypically modified by their environment to produce polarized macrophages for study[52]. BMDMs provide a good model for macrophage extracellular trap formation due to ease of culturing and relative homogenous population.

Macrophages Exist in Different Polarized States

Macrophages are diverse cells and have many sub-populations found throughout the body. Classically, macrophages are classified into two main categories: M1 macrophages and M2 macrophages[53]. M1 macrophages are described as pro-inflammatory and phagocytic[54]. The M1 cells can produce NO (nitric oxide), further indicating their role in increasing inflammation and their pathogen-killing abilities[55]. M2 macrophages are anti-inflammatory and promote repair in the body[56]. M2 classification further divides the cells into M2a, M2b, M2c, and M2d macrophages, which are developed through different treatment stimuli[57]. The classification of macrophages exists on a spectrum *in vivo* and macrophages can switch between M1-like and M2-like depending on the environment the cell is in[58]. M1 macrophages express proinflammatory cytokines such as IL-1b, TNF-a, CXCL10, IFIT1, and IL-12[59-61]. In contrast, M2 macrophages express anti-inflammatory and pro-repair cytokines, such as TGFb and IL-10[62]. Even with their different roles in the body, both phenotypes are involved in many different disease states such as bacterial infection, viral infection, cancer, insulin resistance, and cardiovascular disease[63]. Predominately, the M1 macrophages appear first in the disease state while the M2 macrophages increase in numbers later in the time course of the disease[64]. Due to the unique function of each macrophage type, the polarization state of the macrophages could be modulated and examined to determine if one phenotype directly results in worse outcomes than other for a specific disease.

Limitations of Macrophage M1 and M2 Classification

However, this classification of macrophages may be a simplification of the *in vivo* behavior of the macrophages. Macrophages are known to “reset” their expression of tissue-related genes after *in vitro* culturing after harvesting from tissue samples[65]. This indicates that

the macrophages used to create these classifications may not be truly representative of their environments. The M1 and M2 states are commonly described are considered the polar extremes of macrophages polarization and that there are many more transcriptional polarization states of macrophages[66]. There are numerous tissue-specific subclassifications of macrophages which may not have been fully characterized yet. Therefore, the nomenclature regarding macrophage polarization may not be as simple as it is currently defined, resulting in the current model being inadequate to describe all types of macrophages[67].

NETs and METs are Stimulated by Similar Compounds

Due to their similarity in morphology, NETs and METs share a similar number of activators that can induce extracellular trap formation. Macrophages are known to undergo METosis via live bacteria exposure, such as from *Klebsiella pneumoniae* and *Staphylococcus aureus*[43,68]. NETosis can also be modulated these same bacteria species indicating that NETosis and METosis may utilize similar mechanisms in the regulation of extracellular traps[69,70]. Specific targets in both NETs and METs are modulated by the same compounds. Pro-inflammatory cytokines, such as TNF-a are known to induce both NETosis and METosis, potentially indicating that heightened inflammatory states in the body result in aberrant extracellular trap formation[71,72]. PMA (Phorbol 12-myristate 13-acetate) has also been described to both stimulate extracellular trap formation in both cell types[68,73]. PMA is a PKC (protein kinase C) specific activator and has been show in neutrophils to activate PAD4, the protein required for ETosis in neutrophils[74]. LPS (lipopolysaccharide), a component of bacterial cell walls, has also been described to activate both METs and NETs[74,75]. Cell activation from LPS treatment is primary through the CD14/TLR4 signalizing pathway[76]. The TLR4 (toll-like receptor 4) pathway can interact with PKC, therefore, providing evidence that both PMA and LPS both may stimulate

extracellular trap formation using similar mechanisms[77,78]. A common target in neutrophils and macrophages may could be identified to inhibit extracellular trap formation in both cell types.

Extracellular Trap Inhibitors

NETs and METs further share a number of known inhibitors. The cytoskeletal polymerization inhibitor, Cytochalasin D, can inhibit both neutrophil and macrophage extracellular trap formation, indicating a key role of cytoskeletal movement required for these processes[79,80]. MPO is also a critical enzyme for ET (extracellular trap) formation as described above. Its inhibition through the use of 4-Aminobenzoic Acid hydrazide has further solidified its importance for ET formation[81,82]. Finally, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone, an elastase inhibitor, and Cl-Amidine, a PAD4 inhibitor, can inhibit ET formation in both cell types[83-86]. These inhibitors are key for determining the mechanism for ET formation, but may not be suitable for human use to inhibit aberrant ET formation. However, the biological compound adenosine has been shown to inhibit neutrophil extracellular traps and could be potent inhibitor of MET formation[87].

Adenosine's Role as an Anti-Inflammatory Nucleoside

Adenosine is a biologically derived compound that cells produce via degradation of ATP (adenosine triphosphate), which can modulate immune responses and inflammation[88-91]. Today, adenosine is already used in the cardiovascular field to stop supraventricular tachycardia[92,93]. Specific adenosine receptor agonists have been developed for more targeted receptor activation in the body[94]. In the body, adenosine can act through its 4 G-coupled protein receptors: A1, A2a, A2b, and A3[95]. The A2a and A2b adenosine receptors are Gs classes of G-proteins, while the A1 and A3 adenosine receptors are Gi classes of G-proteins[96]. This means that the A2a and A2b receptors increase production of cAMP (cyclic adenine monophosphate) intracellularly and

activate PKA (protein kinase A), while the A1 and A3 receptors reduce cAMP production and deactivate PKA[97]. In neutrophils, adenosine can inhibit neutrophil recruitment and adherence, while in macrophages, adenosine can inhibit pro-inflammatory cytokine production and cause the release of anti-inflammatory cytokines[98]. Specifically, activation of the A2a receptor, through adenosine or A2a agonists, has been proposed to play a key role in reducing inflammation in many studies[99-102]. In the context of a specific disease, adenosine has been discovered to reduce NET formation. Adenosine binds to adenosine A2a G-protein coupled receptors on the surface of neutrophils and increasing intracellular cAMP production, ultimately reducing NET formation[87]. Since NET and MET formation have been described to be similar, adenosine treatment is a promising inhibitor of macrophage extracellular trap formation due to its anti-inflammatory properties.

Experimental Aims

Aim 1: Determine the Optimal Concentration of M-CSF and Culture Conditions for Bone Marrow Derived Macrophage Differentiation into Mature Phenotype

Hypothesis: Macrophage differentiation will be the greatest with the highest concentration of M-CSF.

Rationale: In order to examine macrophage extracellular trap formation, an effective strategy needs to be devised to culture macrophages. Here, I will use murine bone marrow derived macrophages to create my model system for my *in vitro* work **[Figure 1]**.

Aim 2: Determine if Macrophages Undergo METosis in a Similar Mechanism as Neutrophils

Hypothesis: The molecular mechanisms governing macrophages extracellular trap formation will be homologous to neutrophil extracellular trap formation.

Rationale: Determining if macrophages possess the same proteins and have a similar mechanism for extracellular trap formation compared to neutrophils will provide insight into the potential modulation of both pathways. This may provide information on why macrophage extracellular trap formation initially developed, as well as develop an effective model of METosis, which can be used to test potential inhibitors that could be used to eventually inhibit excess inflammation **[Figure 2]**.

Aim 3: Determine if Macrophage Extracellular Trap Formation is Modulated by Adenosine Through Specific Receptor Subtypes

Hypothesis: Adenosine will signaling through the A2a receptor and will reduce extracellular macrophage ET production.

Rationale: Previous studies in the laboratory have describe that NETosis can be inhibited through the A2a adenosine receptor. Since both macrophages and neutrophils can undergo extracellular trap formation and play a role in inflammatory response in cardiac injury, adenosine provides a promising target for extracellular trap inhibition.

Materials and Methods

Animal Model

6-10 week old male and female C57BL/6 mice were obtained from Charles River Laboratory. All animal work was performed under an approved protocol of the Emory University Institutional Animal Care and Use Committee.

Macrophage Media

Sterile macrophage media was created by adding 89% RPMI (Roswell Park Memorial Institute Medium, x), 10% FBS (fetal bovine serum, #S11150H, R&D Systems, Minneapolis, MN), 1:100 streptomycin + penicillin (#B21110, R&D Systems, Minneapolis, MN), and 100 ng/mL M-CSF (#576404, Biolegend, San Diego, CA). This media was warmed to 37°C before being added to the cells. (Initially, the various concentrations were used, but after analysis, 100 ng/mL of M-CSF was used through all other experiments.)

Bone Marrow Macrophage Culturing

In order to harvest approximately $3 \times 10^6 – 4 \times 10^6$ macrophages per plate, a black six mouse was euthanized via CO2 and cervical dislocation. The abdomen and hind-legs were soaked in a 70% ethanol bath for one minute. Afterwards, the skin and muscles were peeled away from the tibia, fibula, and femur. The femur was dislocated from the pelvis and the entire leg was removed. The femur and tibia were separated, the autopodium was discarded, and the fibula was discarded. The remaining femurs and tibias were placed in a Petri dish filled with 10 mL of sterile PBS (phosphate-buffered saline, #21-040-CM, Corning, Corning, NY).

Using sterile scissors, the ends of the tibias and femurs were removed. The bone marrow of a single bone was flushed with 10mL of cold, sterile PBS in a 10 mL syringe with a 20 gauge needle and collected in a 50 mL conical tube. After all bones were flushed, the tube was

centrifuged at 200 G for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL sterile red cell lysis buffer (#11814389001, Sigma-Aldrich, St. Louis, MO) for 5 minutes. The tube was then centrifuged again at 200 G for 5 minutes and the supernatant was discarded. The cells were resuspended in macrophage media, counted on a hemocytometer and 4 million cells were plated in 10 mL of macrophage media in a 10 cm culture plate (#430167, Corning, Corning, NY). The cells were incubated at 37°C in a 5% CO2 incubator. On day three, the cells received 5 mL of additional macrophage media. The macrophages were then harvested on day 7.

For the initial determination of BMDM growth, the average number of macrophages were counted using phase-contrast microscopy on day 3, day 7, and day 10 at concentrations of 10 ng/mL, 50 ng/mL, or 100 ng/mL of M-CSF. Images were captured on an Olympus IX71 microscope.

Macrophage Harvesting

On day seven, the macrophage media on the plate was aspirated and discarded. The plate was then washed three times with 10 mL of sterile PBS. After the final wash, 2 mL of accutase (#A6964, Sigma-Aldrich, St. Louis, MO) was placed in the plate so that the entire plate was covered. The plate was placed in the incubator for 45 minutes to allow for the macrophages to detach. If the macrophages were still attached to plate after 45 minutes, a cell scraper was used to remove the remaining cells. 2 mL of complete macrophage media was added to the plate to quench the accutase. The media with the cells was moved to a conical tube and spun down at 100g for 10 minutes. The cells were then suspended in 1 mL of macrophage media and counted on a hemocytometer.

Immunofluorescent Staining

Approximately 200,000 macrophages were plated into each well in a 8 well slide (#125658, Thermo Fisher Scientific, Waltham, MA) in 500 µL of macrophage media. The slide was placed in an incubator and the cells were allowed to adhere to the plate overnight. The macrophage media was removed and the wells were washed three times with 200 µL of sterile PBS for 5 minutes.

If treatment of macrophages with activators and inhibitors of METosis (see chemical list under Macrophage SYTOX Assay Procedure) was required before staining, they were added to the wells for one hour to a total volume of 100 µL per well in RPMI. The treatments were washed with 200 µL PBS for 5 minutes before fixing with PFA.

Then, 200 µL of 4% PFA (paraformaldehyde, #15710, Electron Microscopy Sciences, Hatfield, PA) was added to each well for 15 minutes at room temperature. The 4% PFA was removed and the wells were washed three times with 200 µL of PBS. If the cells needed to be permeabilized for their antibody treatment, 100 µL of 3% Triton X-100 (#X100, Sigma-Aldrich, St. Louis, MO) was added to each well for 10 minutes. The 3% Triton X-100 was removed and each well was washed with 200 µL of PBS for 5 minutes.

The cells were blocked using 150 µL of 10% goat serum (#16210064, Thermo Fisher Scientific, Waltham, MA) in PBS for 1 hour at room temperature. The 10% goat serum was removed and 100 µL of 2% goat serum in PBS with antibodies were added to each well.

The following primary antibodies were used in various experiments:

- **CD11b** (1:100 anti-mouse, mouse, conjugated to Alexa Fluor 546, #sc-20050 Santa Cruz Biotechnology, Dallas, TX),
- **PAD4** (1:100 anti-mouse, rabbit, #17373-1-AP, Rosemont, IL),

- **F4/80** (1:100 anti-mouse, mouse, conjugated to Alexa Fluor 488, #sc-377009 Santa Cruz Biotechnology, Dallas, TX),
- **CITH3** (1:300 anti-mouse, rabbit, #ab5103, Abcam, Cambridge, UK),
- **A1** (1:50 anti-mouse, rabbit, #AAR-006, Alomone Labs, Jerusalem, Israel),
- **A2a** (1:50 anti-mouse, rabbit, #ab3461, Abcam, Cambridge, UK),
- **A2b** (1:100 anti-mouse, rabbit, #AAR-003, Abcam, Cambridge, UK),
- **A3** (1:100 anti-mouse, rabbit, #AAR-004, Abcam, Cambridge, UK).

The 8-well slide was wrapped in tinfoil and placed in a refrigerator at 4°C overnight. The next day, the antibody solution was aspirated and the wells were washed three times for five minutes with 200 μL of PBS. The following secondary antibodies were applied when necessary:

- **Goat anti-rabbit Alexa Fluor 488** (1:1000 #AB_143165, Thermo Fisher Scientific, Waltham, MA)
- **Goat anti-rabbit Alexa Fluor 568** (1:1000 #AB_143157, Thermo Fisher Scientific, Waltham, MA)

The secondary antibodies were diluted in 2% goat serum in PBS. The slide was wrapped in tinfoil again and placed at room temperature for 2 hours. The secondary antibody solution was aspirated and the wells were washed three times with 200 μL of PBS for five minutes. The wells were then removed from the slide. One drop of ProLong Gold with DAPI (4′,6-diamidino-2-phenylindole, #P36931, Thermo Fisher Scientific, Waltham, MA) or DAPI dye (1:1000 in PBS, for 10 minutes, then ProLong Gold, #62248, #P10144, Thermo Fisher Scientific, Waltham, MA) was applied to each section of the slide and a glass coverslip was placed on the slide. The slide was placed in a dark drawer for overnight curing. The cells were then imaged using a ZEISS LSM 800 AIRYSCAN fluorescent microscope.

Macrophage SYTOX assay

After the macrophages were cultured for 7 days and harvested, the cells were plated at a density of 33,000 macrophages per well in a 96 black plastic bottom plate (#165305, Sigma-Aldrich, St. Louis, MO). The cells were allowed to adhere for 1 hour. The media in the wells was aspirated and phenol-free RPMI (#R8755, Sigma-Aldrich, St. Louis, MO) with the experiment groups were added to a total volume of 100 µL per well. The experimental concentrations of each chemical are listed below:

- **SYTOX Green** (1:100 #S7020, Invitrogen, Carlsbad, CA)
- **LPS** (50 µg/mL, #L2630, Sigma-Aldrich, St. Louis, MO)
- **Adenosine** (100 µM , #A9251, Sigma-Aldrich, St. Louis, MO)
- **CGS-21680** (5 µM, #ab120453, Abcam, Cambridge, UK)
- **ZM-241385** (10 µM, #1036, R&D Systems, Minneapolis, MN)
- **BAY60-6583** (50 µM, #4472, R&D Systems, Minneapolis, MN)
- **MRS1754** (100 nM, #2752, R&D Systems, Minneapolis, MN)
- **HEMADO** (1 µM, #1579, R&D Systems, Minneapolis, MN)
- **MRS3777** (100 nM, #2403 R&D Systems, Minneapolis, MN)
- **2'-MeCCPA** (10 µM, #2281 Tocris, Minneapolis, MN)
- **DPCPX** (50 µM, #0439 Tocris, Minneapolis, MN)
- **DMSO** (6 µL, #d8418, Sigma-Aldrich, St. Louis, MO)
- **Cl-Amidine** (1 µM, #10599, Cayman Chemical, Ann Arbor, Michigan)
- **KT5720** (1 µM, #1288, Tocris, Minneapolis, MN)

The fluorescence from the SYTOX green, which stains extracellular DNA, was read with a Synergy H1 Microplate Reader and Gene5 software (Biotek, Winooski, VT) at a setting of

485-nm excitation and 525-nm emission. The fluorescence was read every 5 min for 2 hours at 37°C.

Western Blotting

After harvesting, macrophages were suspended in 1 mL of RIPA buffer with protease inhibitor (1:100) (#89900, A32963, Thermo Fischer Scientific, Waltham, MA). The cells were then sonicated at 10 amps for 10 seconds three times, followed by centrifugation at 13,000 RPM at 4 degrees Celsius for 10 minutes. The pellet was discarded and the supernatant was kept.

Using the BCA Protein Assay (Pierce BCA Protein Assay Kit, #23225, Thermo Fisher Scientific, Waltham, MA), the protein concentration was calculated for each sample. 20 µg of protein at a ratio of 3:1 was added to 10:1 sample buffer to Mercaptoethanol (#M6250, Sigma-Aldrich, St. Louis, MO) for a total volume of 40 µL per well. The samples were boiled at 95 degrees Celsius for 5 minutes. The samples were added to a 8.5% SDS-page gel along with 3 µL of the protein ladder. The SDS-page was ran for 1.5 hours at 150 volts submerged in running buffer.

The blot membrane was prepared by soaking it in methanol (#A412, Thermo Fischer Scientific, Waltham, MA) for 30 seconds on each side and then briefly rinsed in water. The gel was transferred in placed in the holder with the following pieces: sponge, moistened pad, gel, blot, moistened pad, and 2 sponges. The holder was placed into the transferred apparatus with transfer buffer surrounded in an ice bath with an ice peak for 1.5 hours at 300 mA.

The blot was removed and placed in 5% milk in TBST for one hour to block at room temperature. Then, the primary antibody in blocking buffer was placed into the cold room for overnight incubation.

- **A1** (1:500 anti-mouse, rabbit, #AAR-006, Alomone Labs, Jerusalem, Israel)

- **A2a** (1:1000 anti-mouse, rabbit, #ab3461, Abcam, Cambridge, UK)
- **A2b** (1:500 anti-mouse, rabbit, #AAR-003 Alomone Labs, Jerusalem, Israel)
- **A3** (1:500 anti-mouse, rabbit, #AAR-004, Alomone Labs, Jerusalem, Israel)
- **GAPDH** (1:5000 anti-mouse, mouse, #ab8224, Abcam, Cambridge, UK)

After incubation, the blot was washed 3 times with TBST + 0.1% Tween 20 (#P9416, Sigma-Aldrich, St. Louis, MO). The secondary antibody was added in blocking buffer at room temperature for one hour.

- **Goat anti-mouse HRP** (1:5000 #ab205719, Abcam, Cambridge, UK)
- **Goat anti-rabbit HRP** (1:5000 #ab205718, Abcam, Cambridge, UK)

The blot was washed again 3 times with TBST + 0.1% Tween 20. The blot was developed by adding 1:1 of part A and part B of ECL Western Blotting Substrate (#PI32106, Fisher Scientific, Hampton, NH). The blot was developed in a dark room using a film reader for various exposure times (see figure legend for specific times).

Two Step Reverse Transcription Quantitative Polymerase Chain Reaction

Relative gene expression was measured using the two step reverse transcription quantitative polymerase chain reaction. A culture dish of macrophages received either no treatment or a treatment of 50 μg/mL LPS (#L2630, Sigma-Aldrich, St. Louis, MO) for one hour. Additionally, bone marrow cells were plated for one hour to allow for fibroblast adherence. The remaining cell suspension was collected for isolation. Finally, uncultured bone marrow cells were collected for RNA extraction. The RNA extraction was performed using Qiagen RNeasy MiniKit (#NC9677589, Thermo Fischer Scientific, Waltham, MA). After macrophage harvesting, approximately 3 x 10^6 – 4 x 10^6 macrophages were resuspended into 350 mL of RLT buffer (Qiagen RNeasy MiniKit) with 2-Mercaptoethanol (#M6250, Sigma-Aldrich, St. Louis,

MO). Then, 350 µL of 70% ethanol was added to the mixture. The solution was transferred to a spin column and centrifuged for 15 seconds at 8000 RPM. The flow-through was discarded and 700 µL of RW1 buffer (Qiagen RNeasy MiniKit) was added to the column and was centrifuged for 15 seconds at 8000 RPM. The flow-through was discarded and 500 µL of RPE buffer (Qiagen RNeasy MiniKit) was added, followed by centrifugation for 15 seconds at 8000 RPM and the flow-through was discarded. This step was repeated but was centrifuged for 2 minutes at 8000 RPM. A new collection tube was added and 30 µL RNase-free water (Qiagen RNeasy MiniKit) was added to the spin column and was centrifuged for 1 minutes at 8000 RPM. This step was repeated and the RNA was now dissolved in the flow-through.

The reverse transcription reaction master mix was created by the following specifications per sample:

- **10x RT Buffer** (2 µL, #4368813, Thermo Fisher Scientific, Waltham, MA)
- **10x Random Primers** (2 µL, #4368813, Thermo Fisher Scientific, Waltham, MA).
- **25x dNTPs (**0.8 µL, #4368813, Thermo Fisher Scientific, Waltham, MA)
- **Multiscribe Reverse Transcriptase** (1 µL, #4368813, Thermo Fisher Scientific, Waltham, MA)
- **RNase Inhibitor (**1 µL, #4368813, Thermo Fisher Scientific, Waltham, MA)

6.8 µL of the master mix and 13.2 of the RNA sample were combined in PCR reaction tubes (#AB-0451, Thermo Fischer Scientific, Waltham, MA). The reaction was ran in a DNA Engine Thermocycler (BioRad, Hercules, CA) and the following settings used for the reverse transcription reaction:

- 25 degrees Celsius – 10 minutes

- 37 degrees Celsius – 120 minutes
- 85 degrees Celsius – 5 minutes
- 4 degrees Celsius – hold

After cDNA was created, the qPCR step was performed in a Quantstudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). The following reaction mix was created per reaction well per gene:

- **TaqMan Fast Advanced Master Mix** (10 µL, #4444556, Thermo Fischer Scientific, Waltham, MA)
- **20x TaqMan Gene Expression Assays** (1 µL per primer mix)
    - **PPIA-FAM** (#Mm02342430_g1, Thermo Fischer Scientific, Waltham, MA)
    - **CXCL10-FAM** (#Mm00445235_m1, Thermo Fischer Scientific, Waltham, MA)
    - **IFIT1-FAM** (#Mm00515153_m1, Thermo Fischer Scientific, Waltham, MA)
    - **CD68-FAM** (#Mm03047343_m1, Thermo Fischer Scientific, Waltham, MA)
    - **TGFb-FAM** (#Mm01178820_m1, Thermo Fischer Scientific, Waltham, MA)
- H_2O (7 µL)

18 µL of the master mix was added to a MicroAmp™ Fast Optical 96-Well Reaction Plate (#4346906, Thermo Fischer Scientific, Waltham, MA) and 2 µL of cDNA sample was added per well. The reaction was completed with duplicates per gene and 2 wells per gene were used for a

negative control (no cDNA addition). The plate was spun down at 1200 RPM for 5 minutes. The following reaction protocol was used:

- 50 degrees Celsius – 2 minutes
- 95 degrees Celsius – 2 minutes
- (95 degrees Celsius – 1 second, 60 degrees Celsius – 20 seconds) x 40 cycle

CT values were measured and the delta-delta CT was calculated comparing the unstimulated macrophages and the LPS-treated macrophages. PPIA was used as the housekeeping gene.

Cell Fluorescence Calculations

Cellular fluorescence was calculated using Fiji, an ImageJ add-on. The background integrated intensity and mean grey were determined from Fiji from the background of the immunofluorescence image. Then, the outline of 20 macrophages in each image were selected and the integrated intensity and mean grey were captured. The corrected total cell fluorescence was calculated using the following equation: Integrated Density – (Area of selected cell X Mean fluorescence of background). Imaged used were a representative sample.

Image Processing and Statistical Analysis

Image processing was performed on ZEN Blue 3.1 Imaging Software and Fiji Imaging Software[103]. Statistical analysis was performed on GraphPad Prism 9.3.1. All values plotted are mean with standard deviation. Two-way ANOVA with Tukey multiple comparisons test was used for comparing total number of cells per day and concentration of M-CSF. One-way ANOVA with Tukey multiple comparisons test was used for comparison of adenosine on adenosine receptors. Brown-Forsythe and Welch ANOVA with Dunnett multiple comparisons test was used for of CITH3 expression with treatments. The statistical tests and the sample sizes

used in each experiment are stated in the figure legends. $P < 0.05$ was considered statistically significant.

Results

Characterization of Bone Marrow Derived Macrophages

Before examining if macrophages could produce extracellular traps, a method for consistently and easily culturing macrophage needed to be developed. Bone marrow cells were treated with various concentrations of M-CSF for 10 days. It was determined that the highest number of macrophages was produced on day 7 after isolation compared to day 3 and day 10, likely due to the cells dying on day 10 and not fully differentiating by day 3. These day 7 cells were treated with 100 ng/mL of M-CSF, indicating that the highest concentration of M-CSF produces the greatest number of cells **[Figure 3]**.

Immunofluorescent staining of the bone marrow derived macrophages showed that the macrophages did express F4/80 and CD11b. The expression of these macrophage cell surface markers indicate that the 100 ng/mL M-CSF treatment for 7 days resulted in the differentiation of the bone marrow cells into macrophages. Since these cells were confirmed to be macrophages, PAD4 staining was also performed. The macrophages expressed PAD4 indicating that the macrophages produce the key protein required for extracellular trap formation. Therefore, they also may be capable of creating extracellular traps in a similar mechanism to neutrophils **[Figure 4]**.

The expression of the 4 adenosine receptor subtypes was also detected in the murine BMDMs through immunofluorescent staining and western blotting. The murine macrophages expressed the A1, A2a, A2b, and A3 adenosine receptors providing a mechanism for which adenosine could act to reduce extracellular trap formation. However, this assay does not indicate which receptor adenosine may act through in the macrophages, but rather provided evidence that adenosine could act through any of its receptors. **[Figure 5, Figure 6]**.

Adenosine Inhibits Macrophages Produce Extracellular Traps Induced by LPS

In order to test if macrophages formed extracellular traps, a treatment of LPS (50 μg/mL) was added for 120 minutes as well as SYTOX DNA intercalating dye to examine if there was an increase in extracellular DNA production. The results show that the LPS-treated macrophages produce more extracellular DNA compared to the macrophage only control over the examined time period. By comparing to the macrophage only control, the expulsion of DNA can be contributed solely to the LPS treatment, rather than nonspecific cell death. Adenosine (100 μM) was added to LPS-macrophages to determine if adenosine was able to inhibit the extracellular trap formation over 120 minutes of treatment. It was determined that adenosine treatment significantly reduced extracellular trap formation by 41.6% at 60 minutes. This shows that adenosine has anti-ETosis effects in both macrophages and neutrophils. Next, the assay was repeated with an A2a agonist, CGS-21680 (5 μM), to elucidate the specific receptor pathway that adenosine may act through. At 60 minutes, A2a agonism significantly reduced extracellular trap formation by 37.7%. Treatment with the A2a antagonist, ZM-241385 (10 μM) and adenosine (100 μM) inhibited adenosine's anti-ETosis action **[Figure 7]**. These results showed that the A2a signaling pathway may play a significant role in modulating macrophage extracellular trap formation.

Though adenosine's action appeared to act through the A2a receptor pathway similar to neutrophils, the remaining other three adenosine receptor subtypes were investigated for their anti-ET action in macrophages. Macrophages treated with LPS (50 μg/mL) and A1 receptor agonist, 2'-MeCCPA (10 μM), did not have significantly reduced MET formation compared to LPS only positive control. The A2b receptor agonist BAY60-6583 (50 μM) and the A3 receptor agonist HEMADO (1 μM) also did not significantly reduce macrophage extracellular trap

formation. These results further indicated that A2a receptor activation is likely responsible for the inhibition of MET formation rather than any of the other adenosine receptor subtypes. The other adenosine receptors were also subjected to antagonism with their specific receptor antagonists with adenosine and LPS treatment: A1 DPCPX (50 μM), A2b MRS1754 (100 nM), and A3 MRS3777 (100 nM). In all experimental groups, antagonism of the adenosine receptors did not significantly differ from their respective agonist treatment further showing that the A1, A2b, and A3 receptors do not play a major role in regulating MET formation, unlike the A2a receptor **[Figure 8]**.

Expression of CitH3 in Response to Various Treatments

After discovering that adenosine can inhibit MET formation, macrophages were examined to determine if adenosine and other potential MET-modulating compounds could directly affect levels of citrullinated H3 histones. Macrophages were treated with various compounds for 60 minutes and immunostained for citrullinated H3 and the macrophage marker, F4/80. Corrected total cell fluorescence was calculated in each treatment group and plotted. 60 minutes of LPS (50 μg/mL) treatment marked a significant increase in CITH3 fluorescence compared to macrophages only, showing that macrophage extracellular trap formation does result in the citrullination of the H3 histone. Interestingly, adenosine (100 μM) treatment does not reduce the amount of CITH3 in the macrophages. This suggests the action of adenosine as an anti-MET compound acts downstream of H3 modification/PAD4 activation. Though, further testing needs to be completed to discern this conclusion fully. Moreover, blocking adenosine's action through addition of a PKA inhibitor, KT5720 (1 μM) also does not result in change of CITH3 expression showing again that adenosine may act downstream of the citrullination of the H3 histone. Treatment with the irreversible PAD4 inhibitor, Cl-Amidine (1 μM), does

significantly reduce CITH3 fluorescence. This supports evidence that H3 citrullination is PAD4-dependent in macrophages. Finally, the addition of DMSO causes a significantly large increase in CITH3 expression compared to the macrophages only group. These results indicate that the citrullination of the H3 histone is accomplished in MET formation, but adenosine's action may be downstream of PAD4 modulation.

Increased M1-Like Gene Expression of LPS-Treated Macrophages

It was examined if M-CSF derived macrophages displayed more M1 or M2-like characteristics as this may have influenced their ability to produce METs. RNA was extracted and cDNA reverse transcribed from bone marrow cells, nonadherent bone marrow cells after 1 hour (to prevent fibroblasts from influencing qPCR results), macrophages only, and macrophages treated with LPS (50 μg/mL) for 1 hour. The CD68 results show that two bone marrow cells are not macrophages since they do not express CD68. The two macrophage groups do express CD68, showing that indeed these are macrophages. One M1 pro-inflammatory gene, IFIT1, was expressed in both macrophage groups. This possibly indicates that M-CSF treatment may slightly polarize the macrophages to be more M1-like. However, the other M1 pro-inflammatory gene, CXCL10, was only expressed in the LPS-treated macrophages. Interestingly, the macrophage only group expressed higher levels of TGFb than the LPS-treated macrophage group. TGFb is classically an M2 expressed gene, meaning that the LPS-treated macrophages became more inflammatory with LPS treatment and subsequent METosis. Both of the bone marrow cell groups did not express any of the tested genes **[Figure 10]**.

Discussion

Ischemic heart disease affects thousands of American each year. Hypoxia-independent damage can be driven through aberrant immune cell recruitment and activation. These cells, such as neutrophils and macrophages, can be activated and form extracellular traps in response to the local environment in the heart, further increasing the inflammatory response and potentially worsening the outcome in patients. Therefore, the targeting of macrophage extracellular trap formation may improve survivability in patients and the severity of the disease.

Extracellular traps form when a cell expels its DNA into the extracellular environment dependent on the citrullination of the H3 histone. Adenosine has been shown to reduce NET formation, however, MET inhibition remained untested. Therefore, I sought to examine whether adenosine treatment could inhibit MET formation.

I first showed that murine bone marrow cells differentiate into macrophages at the highest confluence on day 7 with 100 ng/mL of M-CSF. The bone marrow derived macrophages were positive for both CD11b and F4/80, which are both macrophage-specific markers. I also confirmed that these macrophages have the protein necessary for extracellular trap formation, PAD4. These results supported the role of BMDMs as a model for macrophage physiology. Further analysis through immunofluorescent staining and western blots revealed that the macrophages express all 4 adenosine receptor subtypes, providing a potential mechanism for which adenosine could bind and inhibit METosis.

I also demonstrated that the LPS greatly increased the fluorescence of extracellular DNA compared to the macrophage only control. In parallel to neutrophils, there was a significant decrease in the fluorescence of the adenosine-treated macrophages over a time period of 120 minutes. Specific A2a agonism and antagonism revealed that MET inhibition was modulated

through the A2a signaling pathway. In contrast, the remaining adenosine receptors, A1, A2b, and A3 played no significant role in adenosine-driven MET inhibition. This shows that METs are similar to NETs as both cell types' extracellular traps can be inhibited by adenosine and primarily through the same A2a receptor pathway. However, these results do not fully exclude the possibility that the other adenosine receptors a less significant role in MET formation. Future experiments could involve using several transgenic knockout mice for each adenosine receptor to ensure adenosine cannot bind to these receptors and examine if MET inhibition is decreased in result.

The results from the quantification of the citrullinated H3 histone assay pose questions regarding how adenosine specifically inhibits MET formation. CITH3 expression increases in response to LPS treatment, which matches the current understanding of histone modification via PAD4 activation leading to ETosis. However, the adenosine treatment did not significantly reduce CITH3, which was an interesting finding. In addition, treatment with the PKA inhibitor, KT5720, also had no significant effect in decreasing CITH3 expression. The combination of these results suggest that adenosine's action as an anti-MET compound must act downstream of the citrullination of the H3 histone. One possible mechanism is that adenosine, through binding to the A2a receptor, could activate PKA, which then phosphorylates and inhibits enzymes such as MPO and specific elastases. Further analysis of this pathway should be accomplish to best understand how adenosine functions in macrophages. Importantly, the DMSO control showed high CITH3 expression. Both the KT5270 and the Cl-Amidine were dissolved from the stock into DMSO. Therefore, these expression levels are likely slightly lower than the ones measured due to the DMSO activating the macrophages.

The qPCR results provide some insights into how macrophage differentiation operates. Both macrophage groups express IFIT1, one pro-inflammatory gene, while LPS-treated macrophages express both IFIT1 and CXCL10. The macrophage only group both expressed IFIT1 and TGFb but LPS treatment lead to decreased TGFb expression and increased IFIT1 and CXCL10 expression. These results indicate that M-CSF differentiation leads to an intermediate polarization of the macrophages, while 60 minute of LPS treatment leads to increased M1-like macrophage development. Therefore, the macrophages used to produce METs in my assays were M1-like macrophages. An interesting question that still needs to be addressed is if M2-like macrophages can produce METs or do all macrophages switch phenotype towards M1 before undergoing METosis. A different activator of METosis could be found that does not result in macrophage polarization to M1 or development using GM-CSF could be attempted to produce more M2-like macrophages.

In conclusion, these data suggest that BMDMs can produce METs in response to LPS treatment and treatment with adenosine can inhibit this process *in vitro*. These findings, along with previous knowledge about NETs, show that these processes have mechanisms which can be inhibited. The next step in this field would be to test whether adenosine works *in vivo* to reduce METosis and improve cardiac recovery post myocardial infarction. Only then, can adenosine be further explored therapeutically to be given to patients who are suffering from an myocardial infarction to reduce aberrant immune activity and improve their outcomes.

Figures

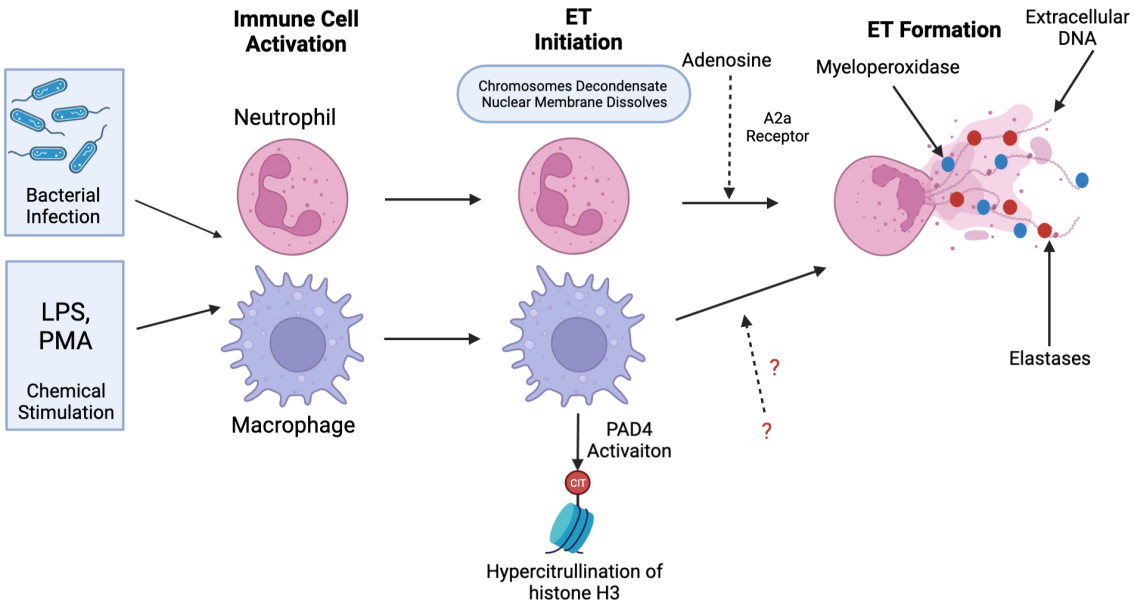


Figure 1: Extracellular Trap Formation Schematic

Diagram illustrating the mechanism of extracellular trap formation. Adenosine is known to reduce neutrophil extracellular trap formation through the A2a receptor. It is unknown how adenosine acts on macrophage extracellular trap formation, indicated by the question marks. Dotted arrows indicate inhibition. Schematic created using BioRender.

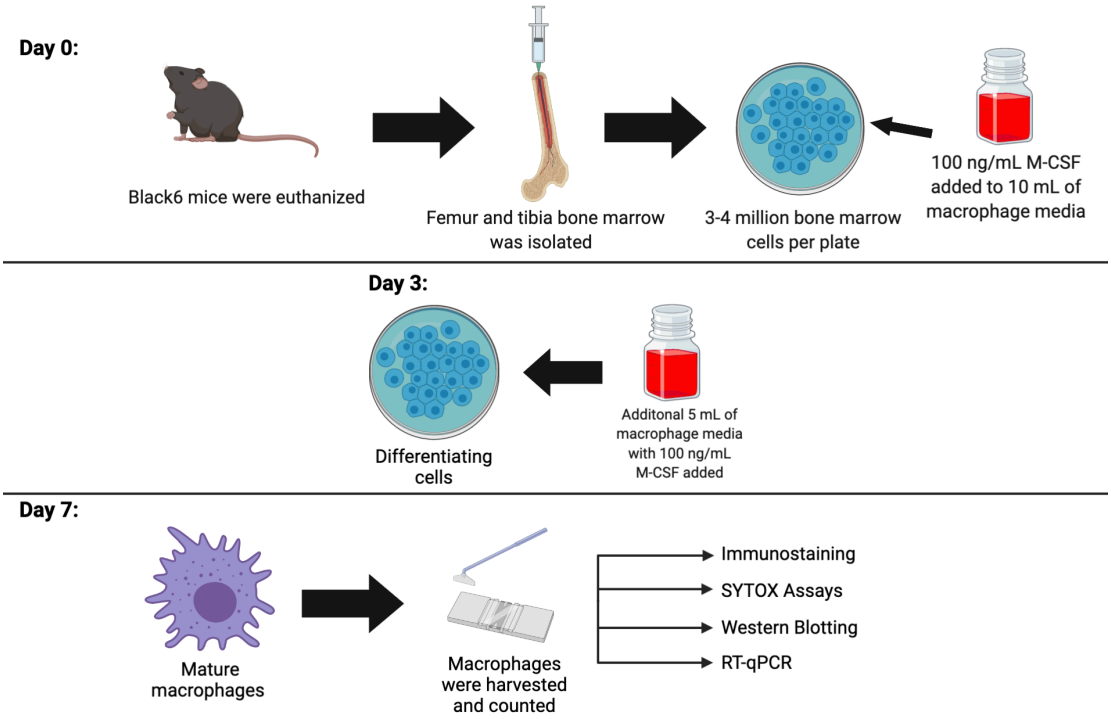


Figure 2: Macrophage Differentiation and Culturing Schematic

Diagram illustrating the procedure of murine bone marrow harvesting and differentiation into macrophages. Protocol was created after experiments to determine days of differentiation and concentration of M-CSF. Schematic created using BioRender.

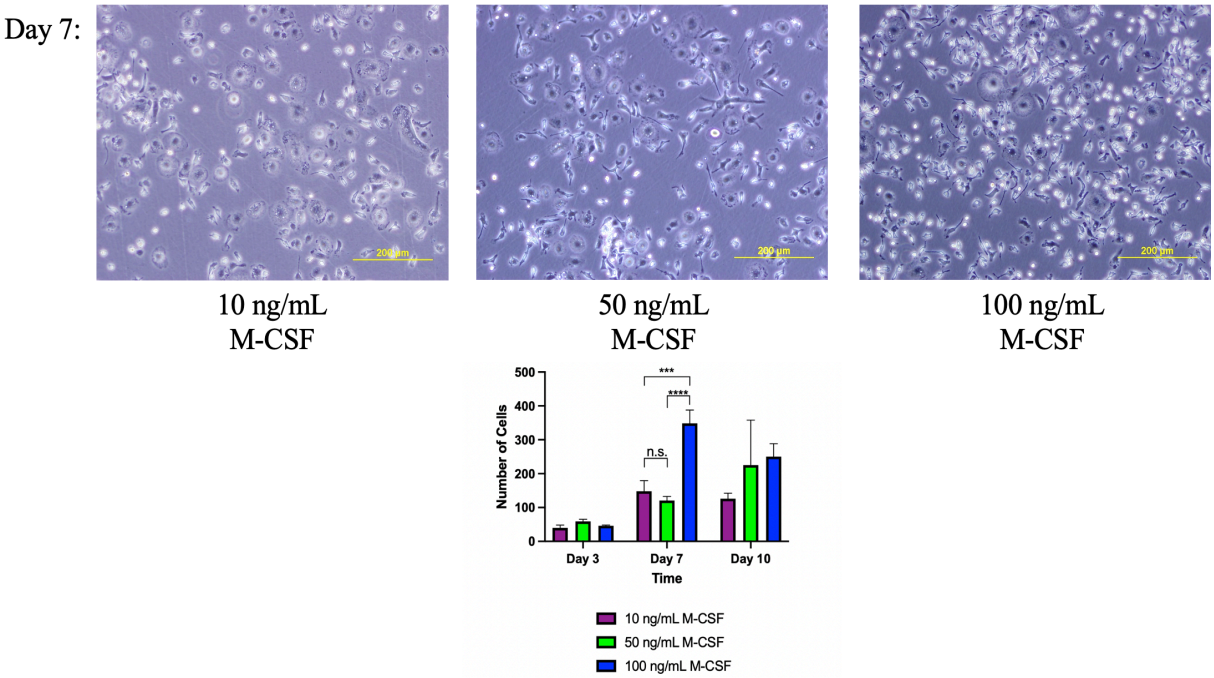


Figure 3: 100 ng/mL M-CSF Produces Greatest Macrophage Confluence on Day 7

Bone marrow cells were isolated and cultured for 10 days. The cells were cultured with macrophage media with 10 ng/mL, 50 ng/mL, or 100 ng/mL M-CSF. Phase-contrast images were taken on days 3, 7, and 10. Images shown are on day 7. The number of cells on each day per image under microscope were counted. Representative images of macrophage plates are shown. Two-way ANOVA with Tukey's multiple comparison was used. Overall ANOVA $p < 0.0001$. Error bars indicate standard deviation. $n = 3$. $p > 0.05$ = n.s, $p \leq 0.05$ = *, $p < 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****.

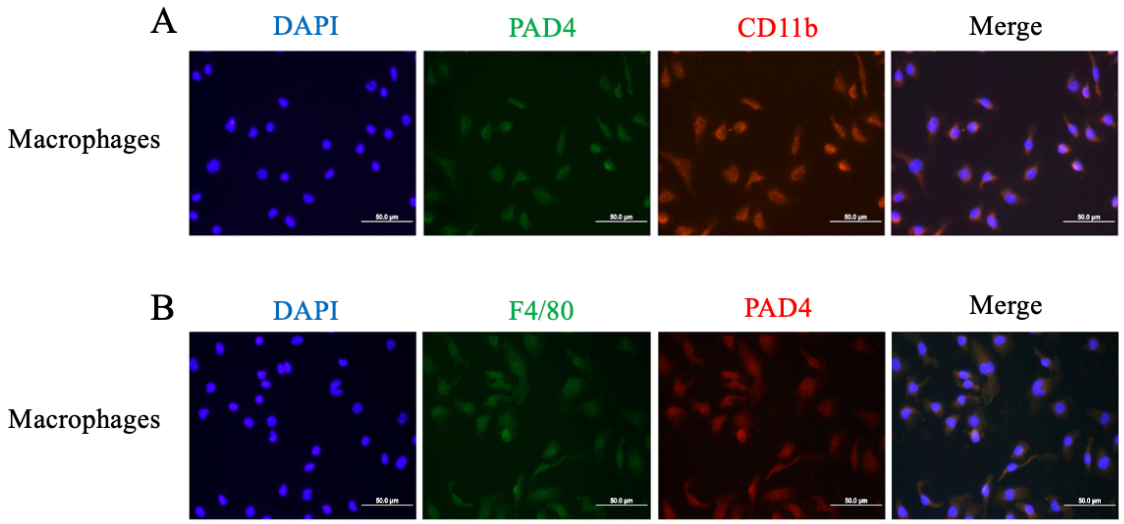


Figure 4: Murine Bone Marrow Derived Macrophages Express Macrophage Markers CD11b and F4/80 and Extracellular Trap Enzyme PAD4.

Murine bone marrow cells were differentiated with 100 ng/mL of M-CSF for 7 days. Cells were fixed and stained with the macrophage marker CD11b and the extracellular trap modulator, PAD4 (A), as well as another macrophage marker F4/80 and PAD4 (B). All primary antibodies were used at a concentration of 1:100. Secondary antibodies for PAD4 were used at a concentration of 1:1000. Representative images of macrophages are shown.

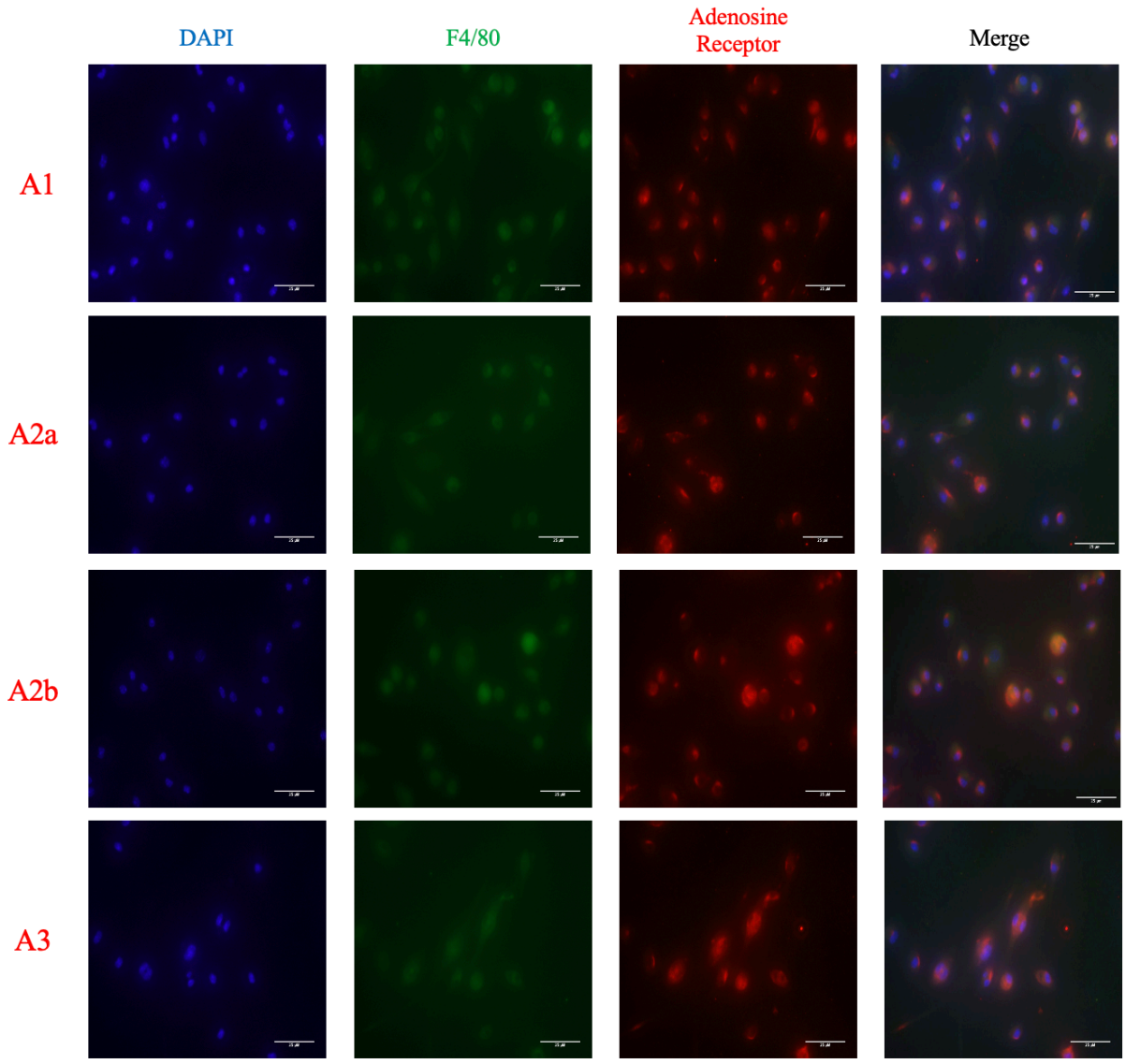


Figure 5: Murine Bone Marrow Derived Macrophages Express A1, A2a, A2b, and A3 Adenosine Receptors Through IF Staining

Murine bone marrow cells were differentiated with 100 ng/mL of M-CSF for 7 days. Cells were fixed and stained with adenosine receptors A1, A2a, A2b, and A3 and macrophage marker F4/80. Primary antibody concentrations were: A1: 1:50, A2a: 1:50, A2b: 1:100, A3: 1:100, F4/80: 1:100. Secondary antibody concentration for all adenosine receptors was 1:1000. Representative images of macrophages are shown.

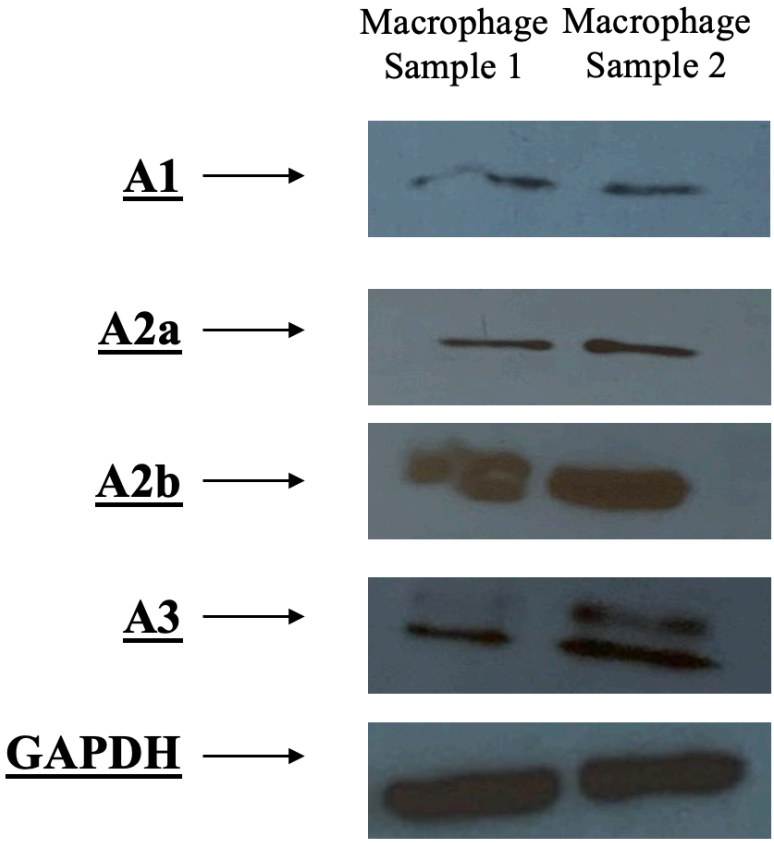


Figure 6: Western Blots of Murine Macrophage A1, A2a, A2b, and A3 Adenosine Receptors

Murine bone marrow cells were differentiated with 100 ng/mL of M-CSF for 7 days. Cells were harvested and western blot analysis was performed to test for the presence of all adenosine receptor subtypes. Primary antibody concentrations were: A1: 1:500, A2a: 1:1000, A2b: 1:500, A3: 1:500, GAPDH: 1:5000. Secondary antibody concentration for adenosine receptors was 1:5000. Exposure time for each protein were: A1: 10 minutes, A2a: 10 minutes, A2b: 10 minutes, A3: 10 minutes, GAPDH: 1 minute. Representative images of macrophages are shown.

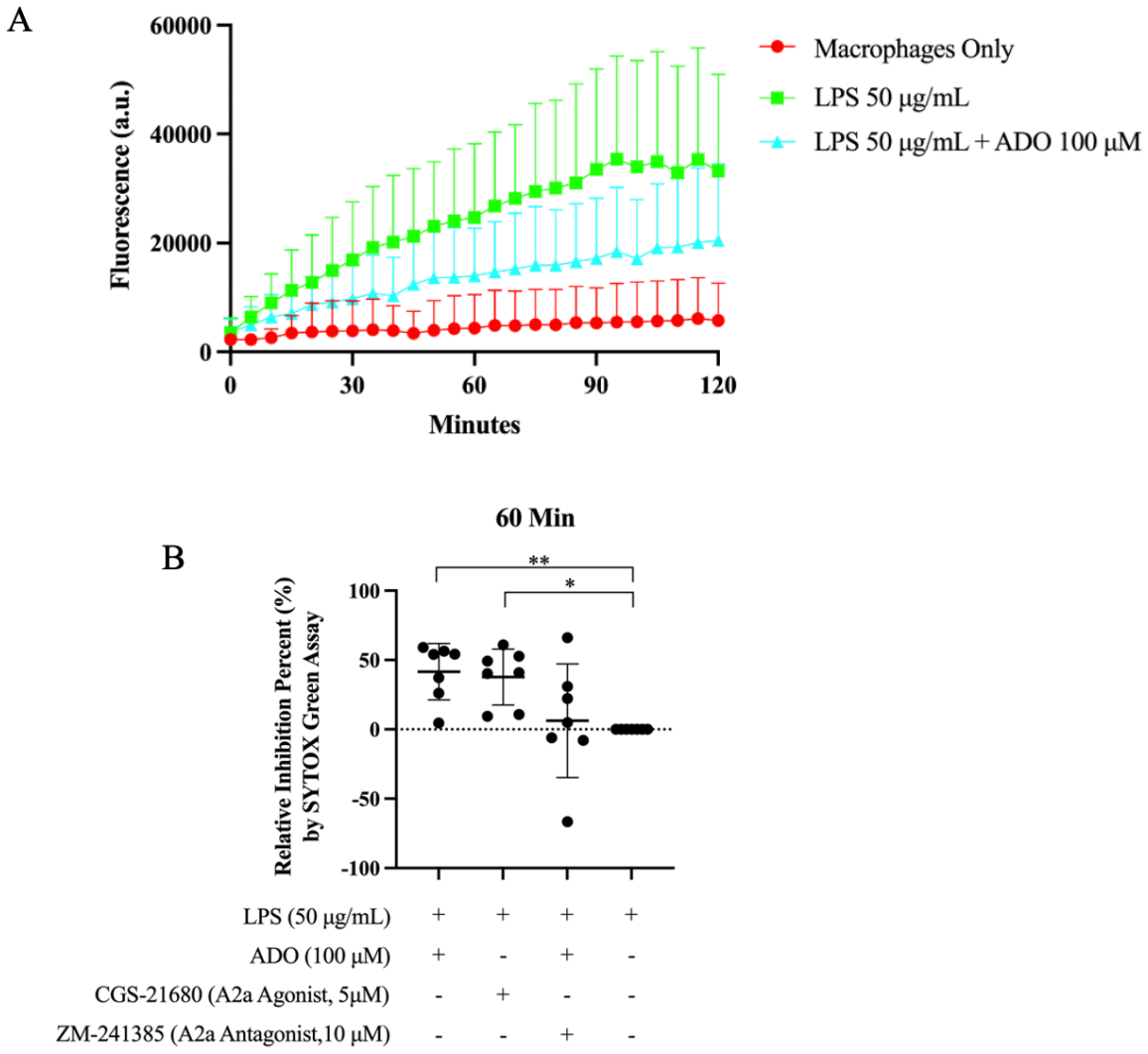


Figure 7: Adenosine Inhibit LPS-Induced Murine Macrophage Extracellular Trap Formation Through the A2a Adenosine Receptor

Murine macrophages produce extracellular traps in response to 50 µg/mL LPS over a time period of 120 minutes. Treatment with 100 µM of adenosine reduces extracellular trap formation over the same period of time. Average fluorescence was plotted. n = 7 (A). Adenosine reduces extracellular DNA production by 41.6% at 60 minutes while the A2a agonist, CG2-21680, reduces extracellular by 37.7% at 60 minutes. Treatment with an A2a antagonist, ZM-241385 blocks adenosine's action. One-way ANOVA with Tukey's multiple comparison was used. Overall ANOVA $p = 0.0170$. Error bars indicate standard deviation. n = 7. $p > 0.05$ = n.s, $p \leq 0.05$ = *, $p < 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****.

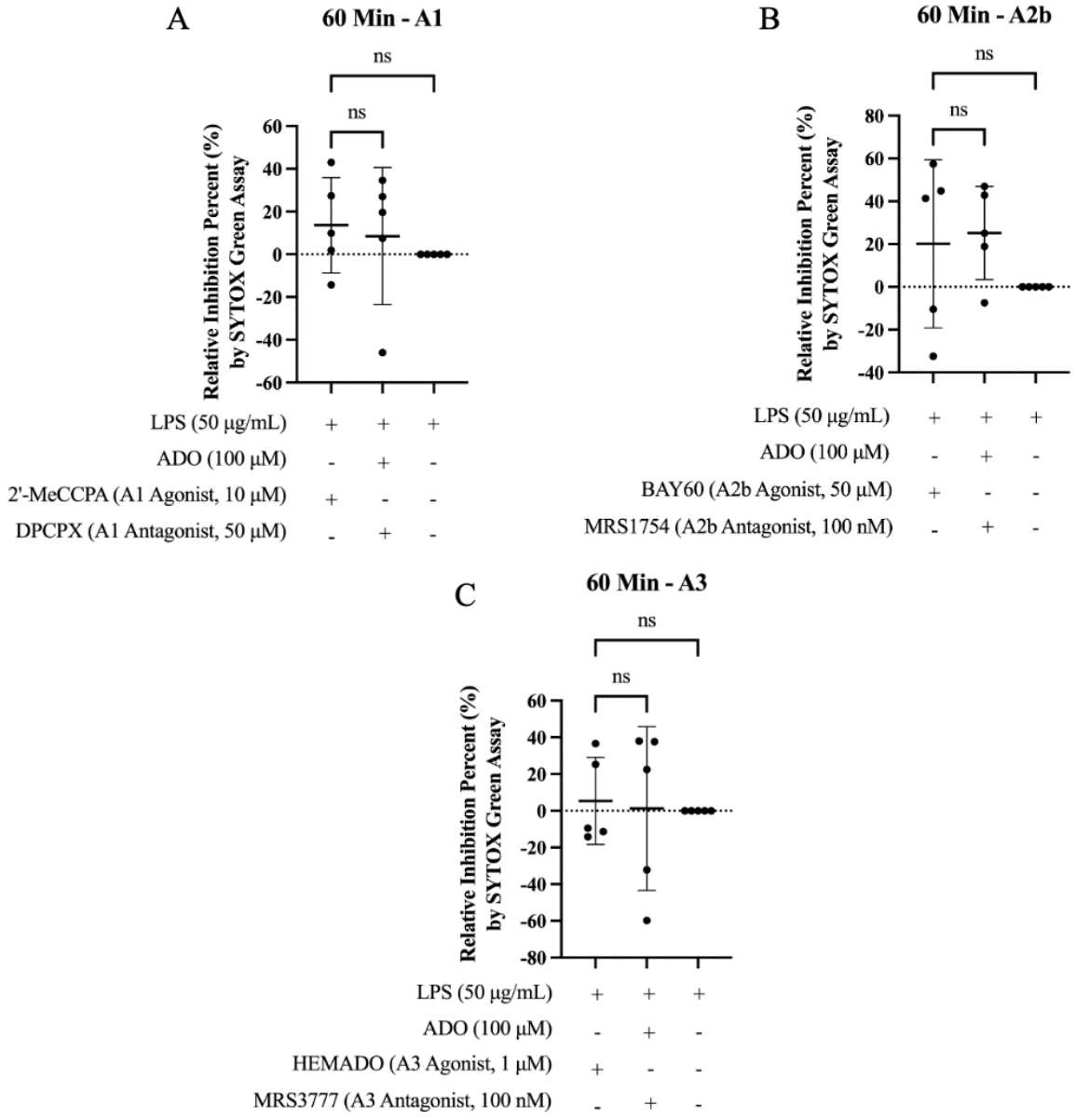
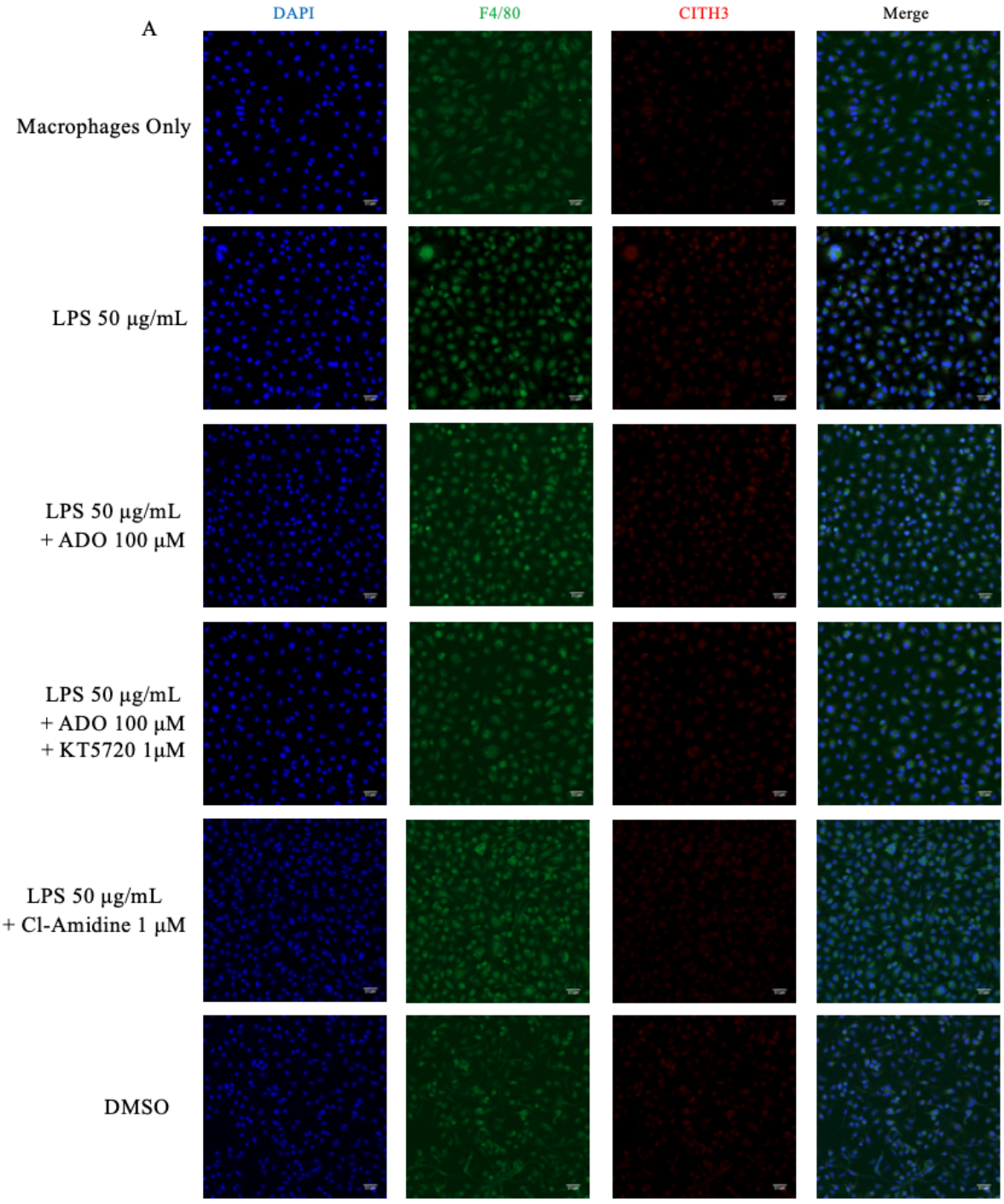


Figure 8: A1, A2b, and A3 Adenosine Receptors Do Not Play a Key Role of Modulating Macrophage Extracellular Trap Formation

Murine macrophages produce extracellular traps in response to 50 µg/mL LPS over a time period of 120 minutes. A1 agonist 2'-MeCCPA, A2b agonist BAY60-6583, and A3 agonist HEMADO do not reduce extracellular trap formation (A, B, C). A1 antagonist DPCPX, A2b antagonist MRS1754, and A3 antagonist MRS3777 do not differ from their respective agonist inhibition. One-way ANOVA with Tukey's multiple comparison was used. Overall ANOVA for A1 $p = 0.4566$, A2b $p = 0.2573$, and A3 $p = 0.8731$ Error bars indicate standard deviation. $n = 5$ for each individual receptor. $p > 0.05$ = n.s, $p \leq 0.05$ = *, $p < 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****.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DAPI
F4/80
CITH3
Merge
Macrophages Only
LPS 50 µg/mL
LPS 50 µg/mL
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LPS 50 µg/mL
+ ADO 100 µM
+ KT5720 1µM
LPS 50 µg/mL
+ Cl-Amidine 1 µM
DMSO

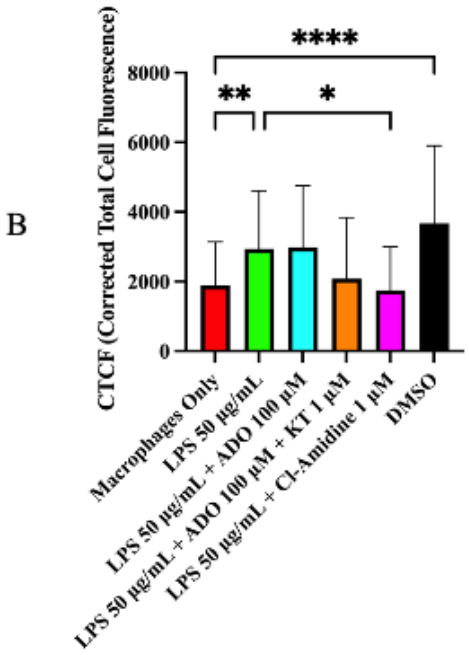

Figure 9: Citrullination of the H3 Histone Increases in Response to 60 Minutes LPS Treatment

Murine bone marrow cells were treated with various compounds for 60 minutes. Cells were fixed and stained for citrullinated H3 histone and the macrophage marker F4/80. Primary antibody concentrations were: CITH3 1:300, F4/80 1:100. Secondary antibody concentration for CITH3 was 1:1000. Representative images of macrophage plates are shown (A). Quantification of immunofluorescence was calculated using corrected total cell fluorescence formula in Fiji/ImageJ. LPS treatment increased CITH3 fluorescence compared to macrophages only and treatment with Cl-Amidine, a PAD4 inhibitor, reduced CITH3 fluorescence. One-way ANOVA with Tukey's multiple comparison was used. Overall ANOVA $p < 0.0001$. Error bars indicate standard deviation. $n = 3$. $p > 0.05$ = n.s, $p \leq 0.05$ = *, $p < 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****.

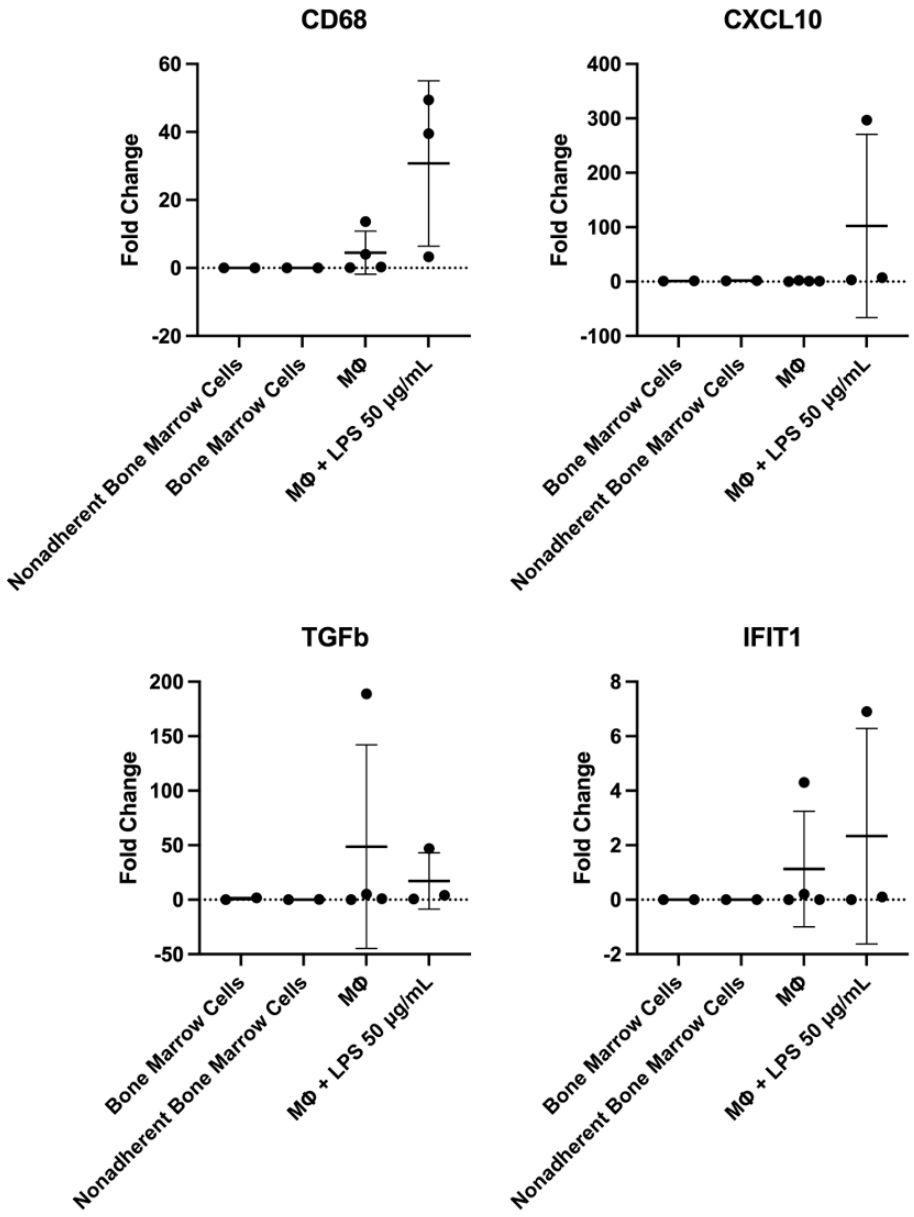

Figure 10: Macrophages Treated With LPS Express M1-Like Genes

RNA from various groups of bone marrow cells and macrophages were isolated and cDNA was transcribed. qPCR was performed on each RNA sample and the delta-delta CT was calculated using the macrophages only as the baseline CT. Macrophages treated with LPS trended towards increased expression of pro-inflammatory genes (IFIT1 and CXCL10), while the macrophages only trended towards increased expression of the anti-inflammatory gene TGFb. Both group of undifferentiated bone marrow cells did not express CD68, a macrophage specific marker. Error bars indicate standard deviation. n = 2 for bone marrow cells, n = 2 for nonadherent bone marrow cells, n = 4 for macrophages only, and n = 3 for macrophages treated with LPS.

Non-Standard Abbreviations

- AKI – Acute kidney injury
- ATP – Adenosine triphosphate
- BMDMs – Bone marrow derived macrophages
- cAMP – Cyclic adenosine monophosphate
- CCL2 – Chemokine (C-C motif) ligand 2
- CCL7 – Chemokine (C-C motif) ligand 2
- CD14 – Cluster of differentiation 14
- CD68 – Cluster of differentiation 68
- DAMPs – Damage-associated molecular patterns
- GAPDH – Glyceraldehyde 3 phosphate dehydrogenase
- GM-CSF – Granulocyte-macrophage colony stimulating factor
- H3 – Histone 3
- IFIT1 – Interferon induced protein with tetratricopeptide repeats 1
- IL-10 – Interleukin 10
- IL-12 – Interleukin 10
- IL-1b – Interleukin 1 beta
- IRI – Ischemia reperfusion injury
- LPS – Lipopolysaccharide
- Ly6c – Lymphocyte antigen 6 complex, locus C1
- M-CSF – Macrophage colony stimulating factor
- MCP-1 – Monocyte chemoattractant Protein 1
- METs – Macrophage extracellular traps

- MPO – Myeloperoxidase
- NETs – Neutrophil extracellular traps
- NO – Nitric oxide
- PAD4 – Protein-arginine deiminase type-4
- PADs – Protein-arginine deiminases
- PKA – Protein kinase A
- PKC – Protein kinase C
- PMA – Phorbol 12-myristate 13-acetate
- ROS – Reactive oxygen species
- TGFb – Transforming growth factor beta
- TLR4 – Toll like receptor 4
- TNFa – Tumor necrosis factor alpha

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